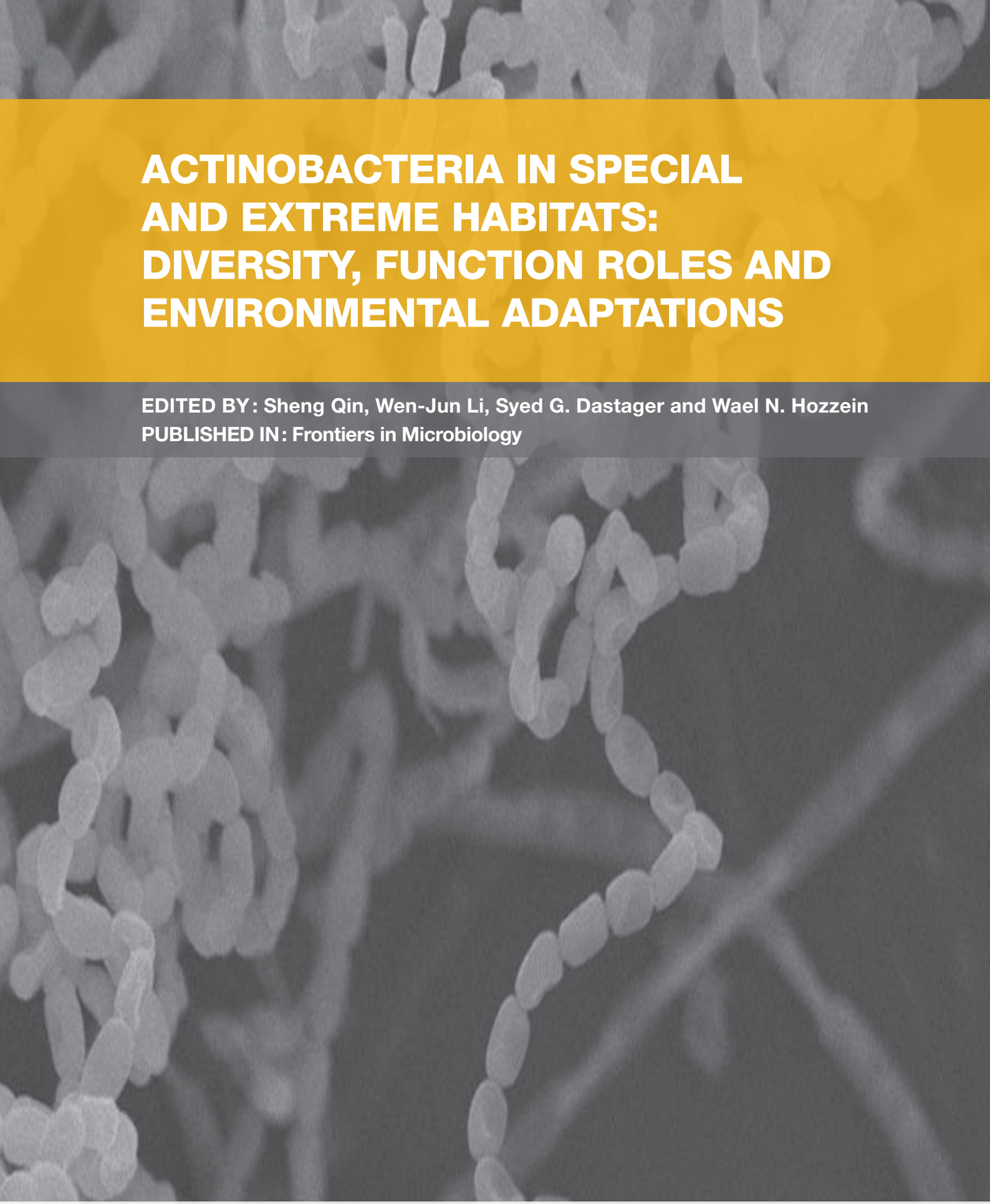

ACTINOBACTERIA IN SPECIAL AND EXTREME HABITATS: DIVERSITY, FUNCTION ROLES AND ENVIRONMENTAL ADAPTATIONS

EDITED BY: Sheng Qin, Wen-Jun Li, Syed G. Dastager and Wael N. Hozzein

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ACTINOBACTERIA IN SPECIAL AND EXTREME HABITATS: DIVERSITY, FUNCTION ROLES AND ENVIRONMENTAL ADAPTATIONS

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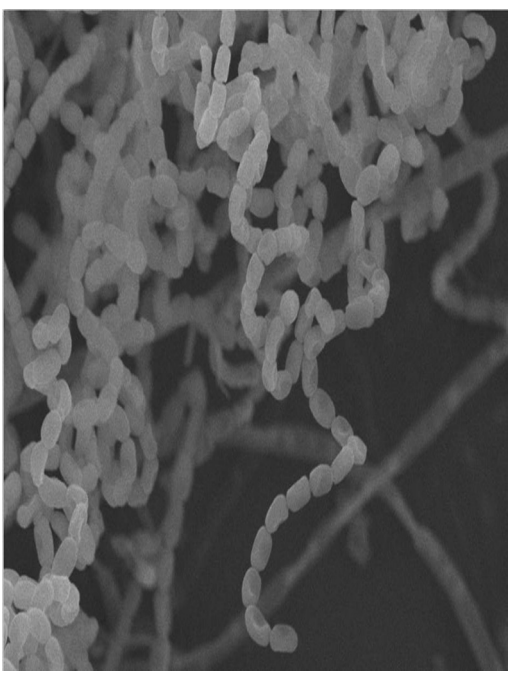

Scanning electron micrograph of an endophytic Streptomyces strain grown on ISP 2 medium for 21 days at 28 °C.
Image by Sheng Qin.

Actinobacteria are highly diverse prokaryotes that are ubiquitous in soil, freshwater and marine ecosystems. Although various studies have focused on the ecology of this phylum, data are still scant on the diversity, abundance and ecology of actinobacteria endemic to special and extreme environments, such as gut, plant, alkaline saline soil, deep sea sediments, hot springs and other habitats. Actinobacteria are well-known producers of a vast array of secondary metabolites, many of which have useful applications in medicine and agriculture. Furthermore, actinobacteria also have diverse functions in different environments apart from antibiotic production. For example,

actinobacteria are reported to contribute to the break-down and recycling of organic compounds. They play a significant role in fixation of nitrogen, improvement plant growth, biodegradation, bioremediation and environmental protection. Therefore, understanding the actinobacterial diversity and distribution in such special environments is important in deciphering the ecological roles of these microorganisms and for biotechnological bioprospecting. Recent advances in cultivation, DNA sequencing technologies and -omics (metagenomics, metaproteomics etc) methods have greatly contributed to the rapid advancement of our understanding of microbial diversity, function and they interactions with environment. Furthermore, comparative genomic studies can provide overall information about actinobacterial speciation, evolution, metabolism and environment adaptation mechanisms. This research topic comprising reviews and original articles highlights the recent advances regarding the unexpectedly diverse/rare group of actinobacteria with special selective isolation methods or culture-independent methods, as well as their biological activities, ecophysiologica function and mechanisms from diverse special and extreme environments.

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EDITORIAL
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Editorial: Actinobacteria in Special and Extreme Habitats: Diversity, Function Roles, and Environmental Adaptations

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Keywords: actinobacteria, special and extreme environments, diversity, omics technologies, activities, environmental adaptation

The Editorial on the Research Topic

Actinobacteria in Special and Extreme Habitats: Diversity, Function Roles, and Environmental Adaptations

The phylum *Actinobacteria* composes a diverse group of Gram-positive bacteria with high G + C content, which are abundant in soils and present in various special and extreme habitats. Actinobacteria have made a significant contribution to the health and well-being of people throughout the world (Demain and Sanchez, 2009). However, the increasing emergence of new diseases and pathogens, and the antibiotic resistance question in recent years have caused a resurgence of interest in finding new biologically active compounds for drug discovery. Thus, previously unexplored ecological niches and regions in the world have been pursued as sources of novel actinobacteria and antibiotics and other useful biologically active agents (Tiwari and Gupta, 2012). With Prof. William C. Campbell and Satoshi Omura winning the Nobel Prize in Physiology or Medicine in 2015 for their discovery of Avermectin, the discovery of new antibiotics from actinobacteria is expected to enter a new golden age.

Actinobacteria have been isolated from diverse ecosystems, including alkaline saline soil, marine sponges, and deep sea sediments, hot springs, guts, and medicinal plants. They have broad applications potential in agriculture and environmental protection apart from antibiotic production due to their diverse ecological functions. During the last few decades, actinobacterial resource research has focused on special habitats and extreme environments; however, due to the limitations of isolation and cultivation methods, our knowledge of the diversity and ecology of extremophilic actinobacteria is at best fragmentary (Bull, 2011). Recent advances in microbial cultivation, next generation sequencing (NGS) technologies and -omics (metagenomics, metaproteomics etc) methods have greatly contributed to the rapid advancement of our understanding of actinobacterial diversity from special and extreme habitats (Qin et al., 2012; Hamedi et al., 2013; Orsi et al., 2016). Still, the physiological functions of actinobacteria and their environmental interactions await further investigation.

We proposed this research topic to highlight the current advances and knowledge related to actinobacteria from extreme environments. In this Research Topic e-book "Actinobacteria in special and extreme habitats: diversity, function roles and environmental adaptations" we collected 17 articles, including 4 reviews and 13 original articles that focus on actinobacterial species diversity from different special and extreme habitats, as well as the bioactive secondary metabolites,

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functional genes and potential ecological functions of actinobacteria. We are grateful to all authors who have submitted contributions to this research topic.

Actinobacteria in extreme habitats represent not only extensive taxonomic diversity, but also high genetic diversity of their biosynthetic pathways for synthesizing novel biological compounds. Mohammadipanah and Wink review the diversity and biotechnological potential of actinobacteria from arid and desert habitats. The article by Shivlata and Satyanarayana also reviews the taxonomic diversity of thermophilic and alkaliphilic actinobacteria, and discusses their potential applications in industry, agriculture and biotechnology. Sun H. M. et al. provide an example of physiological characteristics of a predominant actinobacterial group, found in their survey of highly diverse culturable but rare actinobacteria in desert soil crusts. Interestingly, the article by Riquelme et al. explores the actinobacteria in volcanic caves using culture-dependent and culture-independent methods; the results help fill in the gaps in our knowledge of actinobacterial diversity and their potential ecological roles in the volcanic cave ecosystems. Two articles by Yang et al. and Tang et al. use 16S rRNA gene clone library construction to describe the diversity of actinobacteria in the ecologically sensitive Yanshan Mountains zone and in cold springs sediments in China; they found that biogeographical isolation and biogeochemical factors might be major factors influencing actinobacterial distribution. Many articles focusing on marine actinobacteria are also present. Ser et al. and Tan et al. report bioactive *Streptomyces* species from coastal mangrove soil in Malaysia and their antioxidative metabolites. Marine actinobacteria, particularly coral and sponge-associated actinobacteria, have attracted increasing attention in recent years. Sun W. et al. explore the culturable actinobacterial diversity from sponges in the South China Sea that produce aromatic polyketides. The report by Mahmoud and Kalendar focuses on the diversity of coral-associated actinobacteria; the results may be helpful to understand how corals thrive under harsh environmental conditions. The inner tissue of higher plants is a special habitat. The article by Khieu et al. provides evidence that actinobacteria associated with medicinal plants have the potential to produce novel biological compounds. Finally, Trujillo et al. review the endophytic actinobacteria, in particular the interaction and environmental adaptations of *Micromonospora* co-occurring with plants.

We are delighted to present this research topic in Frontiers in Microbiology. We hope that this e-book will be interesting and useful to the readers of the journal and broaden the knowledge of actinobacteria in harsh environments. The information available above is promising but still limited. In the future, the application of innovative isolation and cultivation techniques, and –omics methods will undoubtedly unveil more unexpected and exciting aspects of actinobacteria in special and extreme habitats, and illuminate especially their ecophysiological function in nature.

AUTHOR CONTRIBUTIONS

SQ organized this topic and wrote the editorial article. WL also wrote the editorial article. SD and WH are the co-editors of the topic and discussed the writing.

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REVIEW
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Thermophilic and alkaliphilic *Actinobacteria*: biology and potential applications

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Microbes belonging to the phylum *Actinobacteria* are prolific sources of antibiotics, clinically useful bioactive compounds and industrially important enzymes. The focus of the current review is on the diversity and potential applications of thermophilic and alkaliphilic actinobacteria, which are highly diverse in their taxonomy and morphology with a variety of adaptations for surviving and thriving in hostile environments. The specific metabolic pathways in these actinobacteria are activated for elaborating pharmaceutically, agriculturally, and biotechnologically relevant biomolecules/bioactive compounds, which find multifarious applications.

Keywords: ***Actinobacteria*, thermophiles, alkaliphiles, polyextremophiles, bioactive compounds, enzymes**

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Introduction

The phylum *Actinobacteria* is one of the most dominant phyla in the bacteria domain (Ventura et al., 2007), that comprises a heterogeneous Gram-positive and Gram-variable genera. The phylum also includes a few Gram-negative species such as *Thermoleophilum* sp. (Zarilla and Perry, 1986), *Gardenerella vaginalis* (Gardner and Dukes, 1955), *Saccharomonospora viridis* P101^T (Pati et al., 2009), *Ferrimicrobium acidiphilum,* and *Ferrithrix thermotolerans* (Johnson et al., 2009). Actinobacteria are either aerobes or anaerobes, motile or non-motile, and spore-/non-spore forming bacteria with a high G+C content (>55 mol%; Ensign, 1992). The genome size of actinobacteria ranges from 0.93 Mb (*Tropheryma whipplei*; Bentley et al., 2003) to 12.7 Mb (*Streptomyces rapamycinicus*; Baranasic et al., 2013), that exists either as a circular or linear form. Actinobacteria occur in diverse ecological niches such as terrestrial and aquatic ecosystems (fresh and marine waters), characterized by a complex life cycle that includes their existence either as dormant spores or actively growing hyphae. They are highly diverse in their morphology ranging from coccoid (e.g., *Micrococcus*) and rod-coccoid (e.g., *Arthrobacter*), fragmenting hyphal forms (e.g., *Nocardia*) to branched mycelium (e.g., *Streptomyces*; Barakate et al., 2002). Reproduction in actinobacteria occurs either by vegetative mode via fragmentation of mycelia or by asexual mode (spore or conidia formation). They produce either a single spore (monosporic) or a pair of spores (bisporic), or many spores (oligosporic) on aerial or substrate mycelium. The oligosporic actinobacteria show distinct patterns of spore arrangement (hooked, straight, or wavy) on the mycelium, depending on the taxa.

Actinobacteria represent one of the most primitive lineages among prokaryotes (Koch, 2003) which are believed to have evolved about 2.7 billion years ago (Battistuzzi and Hedges, 2009). Antibiotic production by actinobacteria is considered to be a key driving factor in the evolution of prokaryotes that led to the diversification of archaea and Gram-negative bacteria (diderm) from Gram-positive bacteria (monoderm; Gupta, 2011). Actinobacteria form a distinct branch on the 16S rRNA gene tree (Zhi et al., 2009), and are distinguished from other bacterial taxa on the basis

of their distinct gene arrangement patterns (Kunisawa, 2007) and conserved indels present in both the 23S rRNA and proteins (e.g., cytochrome C oxidase subunit I, CTP synthetase, and glutamyl-tRNA synthetase; Gao and Gupta, 2005). Their classification has been revised many times in the past. According to the recent system of classification, these are placed under Phylum XXVI, *Actinobacteria* in the Domain II (Bacteria) in Bergey's Manual of Systematic Bacteriology, volume 5. This phylum contains a large array of chemotaxonomically, morphologically and physiologically distinct genera, grouped into six major classes (*Actinobacteria, Acidimicrobiia, Coriobacteria, Nitriliruptoria, Rubrobacteria,* and *Thermoleophilia*; Goodfellow et al., 2012).

Actinobacteria are an ecologically significant group, which play a vital role in several biological processes such as biogeochemical cycles, bioremediation (Chen et al., 2015), bioweathering (Cockell et al., 2013), and plant growth promotion (Palaniyandi et al., 2013). They not only produce a large array of pharmaceutically important bioactive compounds (antibiotics, antitumor agents, anti-inflammatory compounds, and enzyme inhibitors) but also an enormous number of industrially and clinically important enzymes. Since the discovery of streptomycin (first discovered antituberculosis drug from actinobacteria), the drug discovery and development programmes have inclined toward the antimicrobial agents than chemical compounds. Subsequently, a large number of actinobacterial species have been searched for the discovery of clinically valuable compounds. The phylum *Actinobacteria* contains several genera encompassing antibiotic producing species. The genus *Streptomyces* is a prominent source of secondary metabolites, especially antibiotics. *Streptomyces* species are known to produce more than 50% of the total known microbial antibiotics ($\geq$10,000). Despite the availability of enormous number of clinical drugs, many pharmaceutical companies and research laboratories are engaged in the search for new therapeutic drugs in order to combat the microbial pathogens. Multidrug resistant pathogenic strains are constantly emerging, which cause severe disease outbreaks in several countries. In order to find novel bioactive compounds of pharmacological and industrial relevance, actinobacteria have been isolated from exotic and unexplored locations such as desert (Kurapova et al., 2012), marine (Manivasagan et al., 2013), and wetland (Yu et al., 2015) areas. On the premise that the extremophilic actinobacteria could be a source of new valuable metabolites (Bull, 2010) with gene clusters for the synthesis of novel biomolecules, attempts are being made to isolate actinobacteria from extreme environments.

Extremophilic/Extremotolerant Actinobacteria

Actionobacteria are known to occur not only in normal environments, but also in extreme environments, which are characterized by acidic/alkaline pH, low or high temperatures, salinity, high radiation, low levels of available moisture, and nutrients (Zenova et al., 2011). The diverse physiology and metabolic flexibility of extremophilic/extremotolerant actinobacteria enable them to survive under hostile and unfavorable conditions. The high abundance of actinobacterial species was recorded in all extreme environments (Bull, 2010) which had broken the traditional paradigm of restricted predominance of actinobacteria in soil and fresh water habitats. Enormous data has been reported on actinobacteria isolated from normal environments (neutral pH and temperature ranging 20–40°C). Only a few investigations have been carried out to understand the diversity of actinobacteria in the extreme environments, their ecological role and adaptation. Polyextremophiles and polyextremotolerant actinobacterial species also exist in environments with two or more extreme conditions. Polyextremophiles can adapt to environments with multiple stresses (Gupta et al., 2014), which include alkalithermophilic, thermoacidophilic, thermophilic radiotolerant, haloalkaliphilic, and thermoalkalitolerant actinobacteria. Their incidence has been documented in distinct extremes of geographical locations such as the Arctic (Augustine et al., 2012) and Antarctic (Gousterova et al., 2014) regions, oceans (Raut et al., 2013), hot springs (Chitte and Dey, 2002), and deserts (Kurapova et al., 2012).

The extremophilic actinobacteria exhibit several adaptive strategies such as antibiosis, switching between different metabolic modes (i.e., autotrophy, heterotrophy, and saprobes) and production of specific enzymes to survive under unfavorable environmental conditions (high temperature, alkaline, and saline). The thermotolerance is attributed to the presence of high electrostatic and hydrophobic interactions and disulfide bonds in the proteins of thermophiles (Ladenstein and Ren, 2006). They have certain special proteins known as chaperones which aid in refolding the partially denatured proteins (Singh et al., 2010). Several other proteins are also synthesized that bind to DNA and prevent their denaturation at elevated temperatures. Some actinobacteria have acquired multiple adaptive mechanisms to survive in environments with two or more stresses. A thermophilic *Streptomyces* sp., isolated from desolated place, produced enzymes of the autotrophic metabolic pathway such as carbon monoxide dehydrogenase (CODH; Gadkari et al., 1990). The enzyme CODH facilitates the microbial growth in nutrient deprived condition by oxidizing the available inorganic compound such as carbon monoxide into CO_2 which is further fixed by RuBisCO enzyme into microbial biomass through Calvin–Benson cycle (King and Weber, 2007). The thermophilic chemolithoautotroph, *Acidithiomicrobium* sp., isolated from geothermal environment, utilizes sulfur as an energy source (Norris et al., 2011). The antibiosis is another principal strategy through which actinobacteria sustain by killing other microbial flora under nutrient limited conditions. Acidophiles and alkaliphiles have acquired proton pumps to regulate H^+ concentrations inside and outside the cell for maintaining physiological pH inside (Kumar et al., 2011). Alkaliphiles contain the negatively charged cell wall polymers which stabilize the cell membrane by reducing the charge density at the cell surface (Wiegel and Kevbrin, 2004). The adaptive strategy of haloalkaliphiles includes additional tolerances to the salt environment by synthesizing and accumulating high amount of compatible solutes (Roberts, 2005) that prevent desiccation through osmoregulation. They also have Na^+/H^+ antiporter to exclude excessive salt content from inside of the cell.

Actinobacteria are also known to show tolerance to extremely harmful radiations such as gamma and UV rays, and have been isolated from various radioactive sites. The three thermophilic *Rubrobacter* species such as *R. radiotolerans, R. xylanophilus* (Ferreira et al., 1999), and *R. taiwanensis* (Chen et al., 2004) have been reported to be radiotolerant. The resistance mechanism has not been adequately understood, but the complete whole genome analysis of *R. radiotolerans* RSPS-4 revealed the presence of genes encoding proteins involved in DNA repair system, oxidative stress response, and biosynthetic pathways of compatible sugars (trehalose and mannosylglycerate) which might be playing a role in mitigating the damage caused by radiations (Egas et al., 2014). In recent years, a few more alkalitolerant and radiotolerant actinobacterial species such as *Microbacterium maritypicum* (Williams et al., 2007), *Microbacterium radiodurans* GIMN 1.002T (Zhang et al., 2010), *Cellulosimicrobium cellulans* UVP1 (Gabani et al., 2012), *Kocuria* sp. ASB 107 (Asgarani et al., 2012), and *Kocuria rosea* strain MG2 (Gholami et al., 2015) have been documented. These two alkalitolerant *Kocuria* strains were isolated from Ab-e-Siah radioactive spring of Iran. The *Kocuria* sp. ASB 107 is a psychrotrophic strain which shows tolerance to ionizing radiation (upto 90% lethal doses) such as ultraviolet (256 nm-UV) and corona discharge. The *Kocuria rosea* strain MG2 was shown to endure the high dosage of harmful UV-C radiation. This actinobacterium can grow in a wide pH range (5–11 with optimum growth at pH 9.2) and salt concentration (0–15%). Gholami et al. (2015) performed the cell viability analysis on *Kocuria rosea* strain MG2 under multiple stresses. After 28 days of incubation under desiccation condition, the cells of *Kocuria* strain were found to be viable and showed high tolerance to the radiation and strong oxidant such as H_2O_2 (1–4%). The hydrogen peroxide is a well-known antimicrobial agent which damages biological membranes by generating hydroxyl radicals. They seem to exhibit both enzymatic (catalase and peroxidase) and non-enzymatic antioxidant defense systems (carotenoids) to diminish the effect of radiation or strong oxidants or other stresses (Gholami et al., 2015).

The resilience and adaptability of extremophilic/extremotolerant actinobacteria confer them a competitive advantage over other microbes. Besides helping them to survive under extreme conditions, the physiology and metabolic flexibility also trigger them to produce industrially valuable compounds (Singh et al., 2013). The production of biomolecules by extremophiles mitigates the risks of other microbial contaminations, besides providing thermostable, alkalistable, and halotolerant compounds. Enzymes produced by the extremophilic/extremotolerant actinobacteria are functional under extreme conditions, thus, making them suitable candidates for application in industrial processes, where harsh conditions/treatment methods are used. This review focuses on the physiology, phylogeny, ecological roles, and potential applications of thermophilic and alkaliphilic actinobacteria.

Thermophilic and Thermotolerant Actinobacteria

Thermophilic actinobacteria thrive at relatively high temperatures ranging from 40 to 80°C (Tortora et al., 2007). They are widespread, commonly found in moldy hay (Corbaz et al., 1963), self-heating plant residues, cereal grains, sugar cane bagasse (Suihko et al., 2006), decaying vegetable materials, and compost heaps (Henssen and Schnepf, 1967). These are of two types: strictly thermophilic and moderately thermophilic actinobacteria. The former can grow in the temperature range between 37 and 65°C, but optimum proliferation takes place at 55–60°C. While moderately thermophilic actinobacteria thrive at 28–60°C and require 45–55°C for optimum growth (Jiang and Xu, 1993). Another group known as thermotolerant actinobacteria can survive at temperatures up to 50°C (Lengeler et al., 1999).

Physiology

Thermophilic actinobacteria are strictly aerobes and obligate chemoorganotrophs in nature and thrive on decaying organic matter (dead animal and plant materials). There are certain thermophilic actinobacteria such as *Streptomyces thermoautotrophicus* (Gadkari et al., 1990) and *Acidithiomicrobium* sp. (Norris et al., 2011) which are obligate chemoautotrophs, growing solely on CO_2+H_2 and sulfur, respectively. Other nutritive modes such as facultative chemoautotrophy (e.g., *Strepyomyces* strain G26; Bell et al., 1988) and facultative methylotrophy (e.g., *Amycolatopsis methanolica*; Boer et al., 1990) have been observed among thermophilic actinobacteria. The diverse metabolic physiology facilitates the colonization of thermophilic actinobacteria in distinct topographical zones. Prevalence of thermophilic actinobacteria has been documented in sites ranging from the Desert Steppe Zone of Mongolia (Kurapova et al., 2012) to the subtropical area of Argentina (Carrillo et al., 2009) and hydrothermal vents to residential heating systems (Fink et al., 1971). Actinobacteria found in these environments are primarily fast growing and spore forming. The spores produced are of thermoduric type and are stable at higher temperatures for longer duration, even for days in some cases. This appears to provide an additional ecological advantage over other bacteria, making them easier to adapt back to their vegetative forms with the advent of favorable conditions.

Systematics, Taxonomy, and Phylogeny

Thermophilic and thermotolerant species exist in the diverse genera of phylum Actinobacteria (**Table 1**). Among them, the genera such as *Thermopolyspora*, *Thermomonospora*, *Thermotunica, Thermocatellispora, Thermobispora*, *Acidothermus, Acidimicrobium*, and *Thermoleophilum* include only thermophilic species, while other genera include both thermophilic and mesophilic species. All these genera belong to four classes such as *Actinobacteria, Acidimicrobiia, Rubrobacteria,* and *Thermoleophilia* of the phylum *Actinobacteria* (shown in **Figure 1**).

Monospore producing thermophilic actinobacteria belong to three major genera *Saccharomonopora*, *Thermomonospora,* and *Micromonopsora*. The genus *Saccharomonospora* was first described by Nonomura and Ohara (1971) for monosporic actinobacteria with cell wall type IV (meso-DAP, arabinose, and galactose), which includes mostly mesophilic actinobacteria except *Saccharomonospora xinjiangensis* (Jin et al., 1998) and

TABLE 1 | Thermophilic and thermotolerant actinobacterial species.

Actinobacteria	Growth conditions		Location of isolation	References
	Temperature (°C)	pH		
Microbispora siamensis DMKUA 245^T	25–50	–	Soil sample, Thailand	Boondaeng et al., 2009
Georgenia sediminis SCSIO 15020^T	24–60	6–10	Sea sediment, Austria	You et al., 2013
Actinokineospora soli YIM 75948^T	25–55	7–9	Soil sample, China	Tang et al., 2012
Marinactinospora thermotolerans SCSIO 00652^T	10–55	6–9	Sea sediment, Northern South China	Tian et al., 2009
Saccharomonospora viridis SJ-21	35–60	7–10	Hot water spring, India	Jani et al., 2012
Actinomadura miaoliensis BC 44T-5^T	22–55	7.0	Soil sample, Taiwan	Tseng et al., 2009
Streptosporangium sp.	–	–	Soil of Mongolia Desert Steppe Zone	Kurapova et al., 2012
Streptomyces Calidiresistens YIM 7808^T	40–65	7.0	Hot spring sediment, South-west China	Duan et al., 2014
Nocardiopsis yanglingensis A18	25–55	6.5–8.5	Compost of button mushrooms	Yan et al., 2011
Amycolatopsis ruanii NMG112^T	20–50	4–10	Soil sample	Zucchi et al., 2012
A. thermalba SF45^T				
A. granulosa GY307^T				
Pseudonocardia thermophila JCM3095	–	–	–	Yamaki et al., 1997
Thermomonospora curvata B9T	40–65	7.5–11	Composted stable manure	Chertkov et al., 2011
Thermobifida fusca (formerly named as *Thermomonospora fusca*)	35–53	10–11	–	McCarthy and Cross, 1984
Thermotunica guangxiensis	37–65	6–9	Mushroom residue compost, China	Wu et al., 2014b
Thermopolyspora flexuosa DSM 41386^T	40–60	6–9	Soil from the Pamir Mountains	Krasilnikov and Agre, 1964
Thermocatellispora tengchongensis	28–58	6–8	Soil sample, South-west China	Zhou et al., 2012
Saccharopolyspora thermophila 216^T	45–55	–	Soil sample, China	Lu et al., 2001
Thermobispora bispora R51^T	50–65	–	Decaying manure, Berlin	Henssen, 1957
Thermoleophilum album ATCC 35263	45–70	6.5–7.5	Mud samples	Zarilla and Perry, 1984
Acidothermus cellulolyticus 11B	37–70	4–6	Acidic hot springs, Yellowstone National Park	Barabote et al., 2009
Acidimicrobium ferrooxidans TH3	45–50	2	Icelandic geothermal site	Clark and Norris, 1996
Aciditerrimonas ferrireducens IC-180^T	35–58	2.0–4.5	Solfataric field, Japan	Itoh et al., 2011
Acidithiomicrobium sp.	50	3	Geothermal environments	Norris et al., 2011
Ferrithrix thermotolerans Y005T	43	1.3	Mine site, UK	Johnson et al., 2009
Rubrobacter taiwanensis LS-28	30–70 (optimum 60)	6–11	Lu-shan hot springs, Taiwan	Chen et al., 2004
Rubrobacter radiotolerans	46–48	7.0–7.4	Hot springs, Central Portugal	Ferreira et al., 1999
R. xylanophilus	60	7.5–8.0		

S. viridis. The genus *Thermomonospora* was originally proposed only for thermophilic actinobacteria (Henssen, 1957), which comprised three thermophilic species *T. curvata, T. lineata,* and *T. fusca*. Only *T. curvata* could be maintained as pure culture among the three. Afterwards, one mesophilic actinobacterium (*T. mesophila*) was transferred from the genus *Actinobifida* to the genus *Thermomonospora* (Nonomura and Ohara, 1971). Consequently, some other *Thermomonospora* species such as *T. mesouviformis* (Nonomura and Ohara, 1974) and *T. curvata, T. alba, T. chromogena, T. fusca,* and *T. mesophila* (McCarthy and Cross, 1984) were identified. Later on, the *T. mesouviformis* was reassigned as a synonym of *T. alba* (McCarthy and Cross, 1984). One more species, *T. formosensis* (Hasegawa et al., 1986), was isolated and introduced into this genus. McCarthy (1989) described a total of six species (*T. curvata, T. alba, T. chromogena, T. fusca, T. mesophila,* and *T. formosensis*) in the ninth edition of Bergey's Manual of Determinative Bacteriology. Zhang et al. (1998) proposed a polyphasic taxonomy based classification system for the six *Thermomonospora* species. *T. formosensis* and *T. mesophila* were reclassified as *Actinomadura formosensis* and *Microbispora mesophila*, respectively. *T. alba* and *T. fusca* were transferred to the genus *Thermobifida* and named as *Thermobifida alba* and *Thermobifida fusca*, respectively (Zhang et al., 1998). The genus *Themomonospora* is now left with only two species (*T. curvata* and *T. chromogena*). However, *T. chromogena* (shown in red square in **Figure 1**) appears distantly from *T. curvata* on 16S rRNA tree. It shows close ribosomal gene sequence similarity with *Thermobispora bispora*. The detailed study of *T. chromogena* revealed the presence of total six rRNA operons (rrn) in the genome, among which, one operon (rrnB) shows sequence similarity with rRNA of *Thermobispora bispora*. The thermophilic actinobacterium *T. chromonogena* might have acquired this operon from *Thermobispora bispora* or other related microorganism through horizontal gene transfer (Yap et al., 1999). The species of *Thermobifida* genus produces single spore on dichotomously branched hyphae. This genus includes only four species (shown in **Figure 1**). Among them, *Thermobifida fusca* is well-studied, which produces a

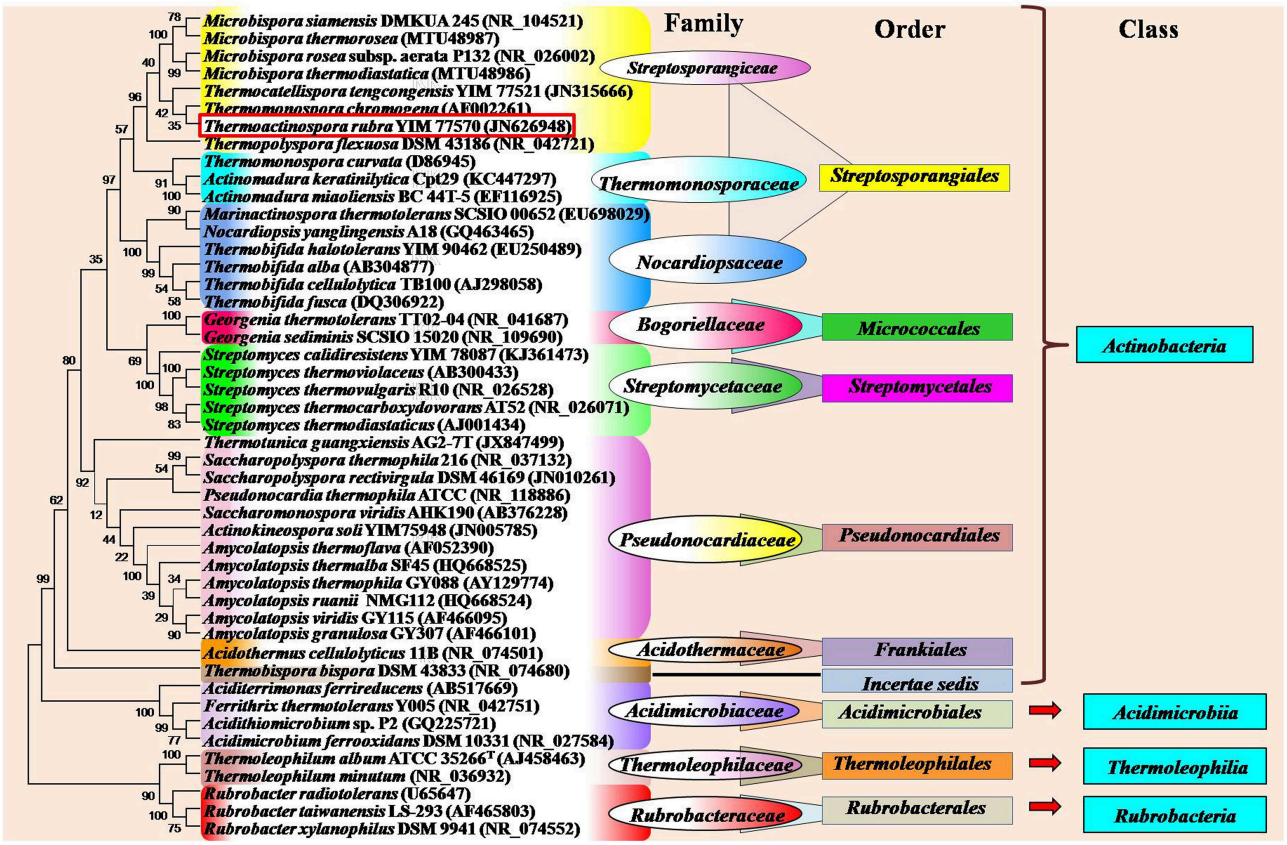

FIGURE 1 | Phylogram indicating the placement and relatedness of some thermophilic and thermotolerant actinobacterial strains belonging to four classes (*Actinobacteria*, *Acidimicrobiia*, *Rubrobacteria*, and *Thermoleophilia*) of the phylum *Actinobacteria*. The numbers given at branch nodes indicate (%) bootstrap value. Phylogenetic tree was generated using Mega5.2 software with 1000 bootstrap replications. Bar 0.02 substitutions per 100 nucleotide positions.

number of industrially important enzymes and other bioactive compounds.

Bisporic thermophilic actinobacteria are included into two genera (*Thermobispora* and *Microbispora*). A thermophilic actinobacterium, *Thermobispora bispora* [earlier known as *Microbispora bispora* (Lechevalier, 1965) and *Thermopolyspora bispora* (Henssen, 1957)] has been isolated from decaying manure in Berlin, Germany (Henssen, 1957), and described as a type species of the genus *Thermobispora* based on thermal preference, chemotaxonomic features, and ribotyping (Wang et al., 1996). The genus contains only single species *T. bispora* that belongs to the class *Actinobacteria* (Goodfellow et al., 2012). In recent years, a few thermotolerant species were identified belonging to the genus *Microbispora* (shown in **Figure 1**).

Oligospore forming thermophilic actinobacteria are majorly included in the genera *Thermopolyspora, Saccharopolyspora,* and *Streptomyces*. A thermophilic actinobacterium, *Thermopolyspora flexuosa,* is the only species of the genus *Thermopolyspora* (Krasilnikov and Agre, 1964), which forms a short chain of spores on sporophore. This species had been subjected to several reclassifications and subsequently assigned into different genera such as *Nocardia* (Becker et al., 1964; Lechevalier et al., 1966), *Micropolyspora* (Krasil'nikov et al., 1968), *Actinomadura* (Cross and Goodfellow, 1973; Lacey et al., 1978), *Microtetraspora* (Kroppenstedt et al., 1990), and later into the genus *Nonomuraea* (Zhang et al., 1998). Once again the taxonomic position of this actinobacterium has been reconsidered and transferred from the genus *Nonomuraea* to the genus *Thermopolyspora* and rechristened as *Thermopolyspora flexuosa* on the basis of 16S rRNA sequence, chemotaxonomy, morphological, and physiological properties (Goodfellow et al., 2005).

The genus *Saccharopolyspora* includes both mesophilic and thermophilic species. The thermophilic species such as *S. rectivirgula* [formerly named as *Micropolyspora faeni, Thermopolyspora polyspora* (Henssen, 1957), and *Thermopolyspora rectivirgula* (Krasilnikov and Agre, 1964)] has been isolated from moldy hay. It causes severe farmer's lung disease. Another species of thermophilic *Saccharopolyspora*, *S. thermophila* was isolated from a garden soil collected from the Xishan Mountain, Beijing (Lu et al., 2001). Goodfellow et al. (1987) isolated a number of thermophilic *Streptomyces* species from diverse habitats. *Streptomyces thermovulgaris* had been reported as the causative agent of bacteremia (Ekkelenkamp et al., 2004), which has been further designated

as a synonym of *S. thermonitrificans* (Kim et al., 1999). Some other thermophilic *Streptomyces* such as *Streptomyces* sp. G26 (Bell et al., 1988), *S. thermoautotrophicus* (Gadkari et al., 1990), *S. thermocarboxydovorans,* and *S. thermocarboxydus* (Kim et al., 1998) have been reported to be carboxydotroph, which are capable of oxidizing the toxic carbon monoxide gas into innocuous CO_2, thus, lowering its atmospheric concentration to safer levels.

Non-sporulating thermophilic actinobacteria belong to the genus *Rubrobacter* (Suzuki et al., 1988) which includes many thermophiles or radiotolerant thermophiles and mesophiles. A thermophilic and radiotolerant actinobacterium, *R. radiotolerans* was formerly described as *Arthrobacter radiotolerans* (Yoshinaka et al., 1973), which tolerates both gamma and UV radiations (Suzuki et al., 1988). The complete genome sequence of *R. radiotolerans* RSPS-4 has been recently annotated to elucidate the radiation resistant mechanism (Egas et al., 2014). Other thermophilic actinobacteria belonging to this genus are *R. xylanophilus* (Carreto et al., 1996), *R. taiwanensis* (Chen et al., 2004), *R. calidifluminis,* and *R. naiadicus* (Albuquerque et al., 2014). The non-sporulating genus, *Amycolatopsis* also includes a few thermophilic actinobacteria (shown in **Figure 1**). *Aciditerrimonas ferrireducens* (Itoh et al., 2011), *Acidithiomicrobium* sp. (Norris et al., 2011), *Ferrithrix thermotolerans* (Johnson et al., 2009) and *Acidimicrobium ferrooxidans* (Clark and Norris, 1996) are non-spore forming thermoacidophilic actinobacteria belonging to the class *Acidimicrobiia*. *Aciditerrimonas ferrireducens* exhibits both heterotrophic and autotrophic mode of nutrition. It is capable of reducing ferric ions to facilitate the autotrophic growth under anaerobic conditions, while the last two catalyze both the processes (dissimilatory oxidation of ferrous iron and reduction of ferric iron). *Acidimicrobium ferrooxidans* displays facultative autotrophic growth, which is capable of fixing atmospheric CO_2 in the absence of organic matter, while *Ferrithrix thermotolerans* exhibits only heterotrophic mode of nutrition. Another thermoacidophilic actinobacterium, *Acidothermus cellulolyticus* 11B was isolated from hot-springs (Mohagheghi et al., 1986), which belongs to the order *Frankiales*. It produces a number of thermostable cellulases, among which, a cellulase (endoglucanases E1) shows higher thermostability and substrate specificity as compared to other actinobacterial cellulases (Thomas et al., 1995).

Adaptation of Thermophilic and Thermotolerant Actinobacteria

Thermotolerant/thermophilic actinobacteria have acquired diverse strategies for homeostasis such as comparatively higher GC content in their genomes, substitution of amino acids in proteins and contain specific components in the cell wall. Mostly thermophiles are known to incorporate comparatively higher quantity of charged amino acids (Asp, Glu, Arg, and Lys) than polar amino acids (Asn, Gln, Ser, and Thr) in their proteins (Suhre and Claverie, 2003). Same trend of increased content of charged amino acids except lysine was observed in the proteins of *Thermobifida fusca* (Lykidis et al., 2007). The genus *Corynebacterium* includes mostly mesophilic actinobacteria with the exception of *C. efficiens* which is capable to grow up to 45°C (Fudou et al., 2002). The comparatively high GC content may provide the thermotolerance to the *C. efficiens*. Amino acid substitution has also been noticed in the enzymes involved in the biosynthetic pathway of industrial valuable amino acids (glutamic acid and lysine) which enhances the production yield of amino acids, thereby adding an industrial importance to this actinobacterium (Nishio et al., 2003). Another thermotolerant actinobacterium, *Saccharomonospora xinjiangensis* contains specific phospholipid [unknown glucosamine-containing phospholipids (GluNU)] in the cell wall, which is considered to be involved in favoring the growth at high temperatures (45–50°C; Jin et al., 1998). *Acidothermus cellulolyticus* belongs to the family *Acidothermaceae* and the order *Frankiales*, can grow optimally at 55°C and pH 5.5. It comes close to the genus *Frankia* on the phylogenetic tree constructed on the basis of the 16S rRNA (Normand et al., 1996), recA (Maréchal et al., 2000), and shc nucleotide sequences (Alloisio et al., 2005). The thermal adaptation in *A. cellulolyticus* may be attributed to the presence of higher GC content compared to the *Frankia* species. The inverse nucleotide preference for G and A at the first and third codon positions has also been observed. Moreover, the proteins contain repetitive patch of the amino acids (IVYWREL) as compared to proteins of *Frankia* species. The amino acid patch might provide thermostability to proteins of *Acidothermus cellulyticus* (Barabote et al., 2009).

Characteristic Features of Thermophilic and Thermotolerant Actinobacteria

All thermophilic and thermotolerant actinobacteria except the genera (*Amycolatopsis, Rubrobacter, Ferrithrix, Acidothermus, Aciditerrimonas, Acidimicrobium,* and *Thermoleophilum*) are spore formers. Mostly they are non-acid fast, non motile, and aerobes except the genus *Amycolatopsis* which includes both aerobes and facultative anaerobes. All are Gram-positive with the exception of *Thermoleophilum* sp., *Ferrithrix* sp., and a species (*S. viridis*) of the genus *Saccharomonospora*. The accurate status of thermophilic actinobacteria has been validated only after the advent of polyphasic taxonomy. Cell wall (peptidoglycan) composition is one of the major feature of the genus specific classification. On the basis of amino acid and sugar contents, actinobacterial cell wall is grouped into four major types i.e., type-I [LL-DAP (diaminopimelic acid) and glycine], type-II [amino acids (meso-DAP and glycine) and sugars (arabinose and xylose)], type-III (meso-DAP with or without madurose), type-IV (meso-DAP, arabinose and galactose; Lechevalier et al., 1966), and other cell wall type V–X. The majority of the thermophilic actinobacteria have a cell wall type-III, while a few genera (*Saccharomonospora, Saccharopolyspora,* and *Amycolatopsis*) are known to contain cell wall type IV. Only one species of the genus *Streptomyces* has cell wall type-I. Other cellular components considered for chemotaxonomic classification include phospholipids, fatty acids, mycolic acid, menaquinones type, and GC content (% mol). The major respiratory menaquinones of thermophilic and thermotolerant actinobacteria are MK-9 variants. The

presence of other menaquinones MK-8 (*Rubrobacter*) and MK-10 (*Thermobifida*) have also been reported (Goodfellow et al., 2012) in thermophilic actinobacteria.

Ecological Importance

Thermophilic and thermotolerant actinobacteria are known to possess unique metabolic rates and physical properties that prove to be beneficial in a variety of ecological roles.

Composting

Composting is a self-heating, aerobic, and biodegradation process that supplies humus and nutrients to the soil (Rawat and Johri, 2013). The composting involves the synergistic action of bacteria, actinobacteria, and fungi, wherein the actinobacteria proliferate in the later stages of composting. The predominance of thermotolerant actinobacteria is generally observed in thermobiotic condition generated by the preceding bacteria. During the initial stage of thermobiotic condition, the compost is colonized by thermotolerant actinobacteria (*Streptomyces albus* and *Streptomyces griseus*) and subsequently by the thermophilic actinobacteria (Goodfellow and Simpson, 1987). Actinobacteria genera such as *Streptomyces*, *Amycolatopsis*, *Microbispora*, *Cellulosimicrobium*, *Micrococcus*, *Saccharopolyspora*, *Micromonospora*, *Thermobispora*, *Thermomonospora*, *Thermobifida,* and *Planomonospora* were reported to be involved in the composting process. The composition of actinobacterial communities varies during various stages of composting (Xiao et al., 2011). They also suppress the growth of plant pathogens by secreting antibiotics along with the breakdown of organic matter which provides an additional advantage of using compost in order to enhance soil nutrients and also suppressing the development of plant diseases. Moreover, the addition of compost to contaminated soil enhances the bioremediation rates of pollutants such as polycyclic aromatic hydrocarbons, petroleum, pesticides, and heavy metals (Chen et al., 2015).

Antimicrobial Activity

Thermotolerant actinobacteria such as *Streptomyces tauricus*, *S. toxytricini*, *S. coeruleorubidis*, *S. lanatus,* and *Streptosporangium* sp. have been found to inhabit the rhizosphere of many plants in the desert of Kuwait during the hot season (Diab and Al-Gounaim, 1985). The rhizosphere inhabiting actinobacteria exhibit antimicrobial activity, thus protect the plant from the attack of phytopathogens (Xue et al., 2013). Some thermotolerant actinobacteria isolated from the Himalayan Mountains, have also been shown to exhibit antagonistic activity against pathogenic bacteria and fungi. They include mostly *Streptomyces* species such as *S. phaeoviridis* and *S. griseoloalbus*, *S. viridogens,* and *S. viridogens.* The *S. phaeoviridis* and *S. griseoloalbus* exhibit antibacterial activity against both Gram-positive and Gram-negative bacteria, including methicillin resistant and vancomycin resistant strains of *Staphylococcus aureus.* The other two *Streptomyces* species (*S. viridogens* and *S. rimosus*) are capable of suppressing the growth of pathogenic fungi (*Fusarium solani*, *Rhizoctonia solani*, *Colletotricum falcatum,* and *Helminthosporium oryzae*), therefore, these *Streptomyces* species could be used as the bio-pesticides for agricultural production (Radhakrishnan et al., 2007).

Plant Growth Promotion

Actinobacteria secrete many volatile secondary metabolites which play significant roles in the suppression of plant diseases and the alleviation of biotic or abiotic stresses. Moreover, many actinobacteria species are known to secrete the iron chelating organic molecules such as siderophores which sequester the solubilized form of iron (Fe^{+3}) and immobilize it in the rhizosphere of plants growing in the iron deficient soil. The siderophores modulate either the plant growth, directly or indirectly, by enriching the other plant beneficial microbes in the rhizosphere zone (Palaniyandi et al., 2013). Dimise et al. (2008) showed that a soil dwelling cellulolytic actinobacterium, *Thermobifida fusca* partakes in plant growth promotion by synthesizing the siderophore (fuscachelins) through non-ribosomal peptide biosynthetic pathways.

Nitrogen Fixation

The *Frankia* and some non-*Frankia* actinobacteria have been shown to fix the atmospheric nitrogen (Gtari et al., 2012). A thermophilic actinobacterium, *Streptomyces thermoautotrophicus* which is an autotrophic carboxydotroph, has an unusual characteristic of nitrogen fixation (Ribbe et al., 1997). In this actinobacterium, the process of nitrogen fixation is coupled to the oxidation of carbon monoxide. The electrons generated during the oxidation process of CO reduce molecular oxygen into oxygen free radicals. The manganese-containing superoxide oxidoreductase oxidizes the formed free radicals into O_2 and release electrons. The released electrons are further utilized by the enzyme nitrogenase in order to reduce N_2 into ammonia. The notable feature of nitrogenase of *S. thermoautotrophicus* is its insensitivity to O_2 and O_2^- radicals. Furthermore, it also differs from other known nitrogenases in terms of protein structure and requirement of Mg^{2+} and ATP. Valdes et al. (2005) reported that some *Thermomonospora* species are also capable of fixing atmospheric nitrogen.

Hypersensitivity Pneumonitis

Besides their beneficial activities, thermophilic actinobacteria such as *Saccharomonospora viridis* (Pati et al., 2009) and *Saccharopolyspora rectivirgula* (Pettersson et al., 2014) have been reported to cause severe respiratory diseases such as Farmer's lung and bagassosis. The Farmer's lung and bagassosis are a type of hypersensitivity pneumonitis (HP). The major cause of these allergic reactions is attributed to the exposure to moldy molasses, when densely colonized by spore-forming thermophilic actinobacteria.

Alkaliphilic and Alkalitolerant Actinobacteria

The actinobacteria have long been known to thrive in soda lakes, salt alkaline lake, and alkaline soil. Their occurrence has also been observed in neutral environments. The alkalitolerant actinobacteria are capable of growing in the comparatively

broader range of environments from neutral to alkaline pH. Alkaliphilic actinobacteria are, therefore, categorized into three major groups: alkaliphilic (grow optimally at pH 10–11), moderately alkaliphilic (grow in a pH range of 7–10) but show poor growth at pH 7.0, and alkalitolerant actinobacteria (grow in the pH range between 6 and 11; Jiang and Xu, 1993). Baldacci (1944) presented the first report on alkaliphilic actinobacteria. Thereafter, Taber (1960) isolated alkaliphilic actinobacteria from the soil. The occurrence of alkaliphilic and alkalitolerant actinobacteria has been reported from various habitats including deep sea sediment (Yu et al., 2013), alkaline desert soil (Li et al., 2006), and soda lakes (Groth et al., 1997). Mikami et al. (1982) studied the distinct chemotaxonomic patterns of cell wall of a total six alkaliphilic *Streptomyces* species [*Streptomyces caeruleus* ISP 5103 (reclassified as *Actinoalloteichus cyanogriseus*, Tamura et al., 2008), *S. alborubidus* ISP 5465 (reclassified as *Nocardiopsis alborubida*), and *S. autotrophicus* ISP 5011, *S. canescens* ISP 5001, *S. cavourensis* ISP 5300, and *S. hydrogenans* ISP 5586] which show optimum growth at pH 11.5. Among them, the first three contained meso-diaminopimelic acid. Subsequently, the taxonomic positions and applications of alkaliphilic actinobacteria in various fields have been described by Groth et al. (1997) and Duckworth et al. (1998).

Physiology, Characteristic, and Taxonomic Features of Alkaliphilic and Alkalitolerant Actinobacteria

The alkaliphilic and alkalitolerant actinobacteria are known to occur in environments of high salinity (known as haloalkaliphiles or haloalkalitolerants) or in thermobiotic conditions (termed as alkalithermophile or alkalithermotolerants). Alkalithermophiles and alkalithermotolerant actinobacteria have also been isolated from saline habitats with their halophilic and halotolerance characteristic (Zenova et al., 2011). One such polyextremotolerant actinobacterium, *Microbacterium sediminis* has been isolated from deep sea that possesses the psychrotolerance, thermotolerance, halotolerance, and alkalitolerance attributes (Yu et al., 2013). Other reported polyextremophilic actinobacteria include alkaliphilic and thermotolerant actinobacteria [*Streptomyces alkalithermotolerans* (Sultanpuram et al., 2014) and *Georgenia satyanarayanai* (Srinivas et al., 2012)], thermophilic and alkalitolerant (*Streptomyces thermoalcalitolerans*; Kim et al., 1999), and haloalkaliphilic actinobacteria [*Nitriliruptor alkaliphilus* (Sorokin et al., 2009)]. They are either aerobes or microaerobes or facultative anaerobes. All alkaliphiles and alkalitolerants are Gram-positive. These exist as either halophiles or non-halophiles. Most alkaliphilic and alkalitolerant actinobacteria are non-motile and spore- or non-spore formers.

Some alkaliphilic actinobacterial species belonging to the genus *Streptomyces* (Mikami et al., 1982), *Micromonospora* (Jiang and Xu, 1993), *Nocardioides* (Yoon et al., 2005), *Microcella* (Tiago et al., 2005), *Cellulomonas* (Jones et al., 2005), *Nesterenkonia* (Luo et al., 2009), *Streptosporangium* (Gurielidze et al., 2010), *Corynebacterium* (Wu et al., 2011b), *Georgenia* (Srinivas et al., 2012), *Nocardiopsis, Isoptericola, Nesterenkonia* (Ara et al., 2013), *Saccharomonospora* (Raut et al., 2013), *Saccharothrix* (Jani et al., 2014), and *Arthrobacter* (Kiran et al., 2015) have been isolated and well-characterized. Among them, the genus *Nocardiopsis* has been found to be prominent in alkaline environments (Ara et al., 2013). All the genera belong to the class *Actinobacteria* except the genus *Nitriliruptor* that belongs to the class *Nitriliruptoria* (shown in **Figure 2**). There are a few well-characterized alkalitolerant species such as *Citricoccus alkalitolerans* (Li et al., 2005), *Spinactinospora alkalitolerans* (Chang et al., 2011), and *Haloactinopolyspora alkaliphila* (Zhang et al., 2014) which proliferate in sites ranging from neutral to alkaline pH.

Ecological Significance

Microbial Decomposition in Hypersaline or Haloalkaline Sites

The microbial degradation of recalcitrant molecules takes place rapidly in the environment with acidic or neutral pH. However, the hypersaline and extreme haloalkaline conditions of lakes and mangroves limit most of the microbial hydrolytic activity on complex biomolecules such as cellulose, lignin, and chitin. Only haloalkaliphilic or haloalkalitolerant bacteria and actinobacteria are capable to proliferate and contribute in the decomposition of recalcitrant biopolymers in haloalkaline zones. A number of alkalitolerant or alkaliphilic actinobacteria have been isolated from mangrove, soda lakes and marine sediment. The two *Isoptericola* species i.e., *Isoptericola chiayiensis* (Tseng et al., 2011) and *Isoptericola rhizophila* (Kaur et al., 2014) were isolated from mangrove soil sample, Taiwan and rhizosphere of *Ficus benghalensis* (banyan tree) in Bhitarkanika mangrove forest, India, respectively. These two species are capable of hydrolyzing organic matter into simpler forms which are further assimilated by plants. The second most abundant biopolymer, chitin is produced by brine shrimp in bulk quantities in hypersaline soda lakes. Sorokin et al. (2012) showed the high prevalence of haloalkaliphilic chitinolytic bacteria and actinobacteria in hypersaline sediments and soda soils. The other chitinolytic actinobacteria species include *Isoptericola halotolerans*, *Nocardiopsis* sp., *Glycomyces harbinensis*, and *Streptomyces sodiiphilus* which are capable of degrading chitin completely and more rapidly than the bacterial population (Sorokin et al., 2012). Other alkaliphiles, *Nocardiopsis prasina* OPC-131 (Tsujibo et al., 2003), *Streptomyces* and *Nocardia* sp. (Bansode and Bajekal, 2006) are reported to display chitinolytic activity.

Chitin Amendment

Chitin amendment is a soil management approach to suppress or inhibit the growth of plant pathogens or parasites. The addition of chitin enhances the pathogenic suppressiveness of soil (Kielak et al., 2013). This strategy not only involves the chitinolytic action of the soil or rhizosphere microflora but also induces desired changes in the metabolism of the endophytic microflora of plants (Hallmann et al., 1999). The *Arthrobacter* sp., *Corynebacterium aquaticum*, *Micrococcus luteus*, *Mycobacterium parafortuitum*, and other bacterial species were found during the chitin facilitated amendment of the soil and rhizophere

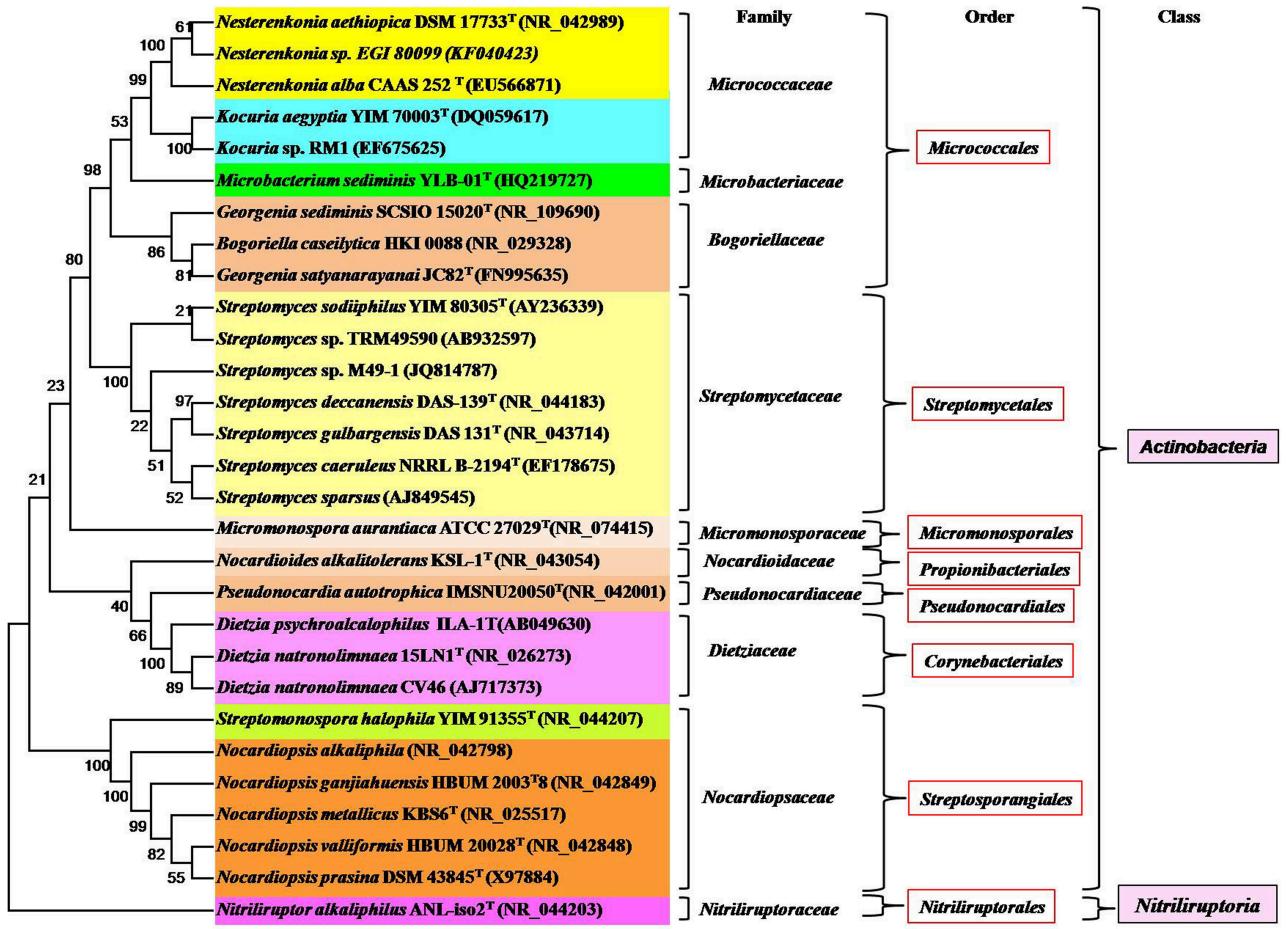

FIGURE 2 | Phylogram indicating the placement and relatedness of some alkaliphilic, alkalitolerant, alkalithermophilic and alkalithermotolerants actinobacterial strains belonging to two classes (*Actinobacteria* and *Nitriliruptoria*) of the phylum *Actinobacteria*. The numbers given at branch nodes indicate (%) bootstrap value. Bar 0.02 substitutions per 100 nucleotide positions.

zone of cotton plants (Hallmann et al., 1999). The microbial community has been found to change with the alteration of physical properties (pH and temperature) of soil. The enzyme chitinase produces short oligosaccharide chains and chitin derivatives which have various industrial applications. Besides biotechnological applications, the chitinases that are particularly active at high pH find application in plant pathogen suppression by hydrolyzing the cell wall component (chitin) of fungi, thereby inhibiting the fungal growth and spread of infection. The alkalistable chitinase producing *Isoptericola jiangsuensis* (Wu et al., 2011a) and *Nocardioides* sp. (Okajima et al., 1995) can be applicable for such soil amendment practices. The amendment of chitin with apatite has also been found to sequester the metals in marine sediments (Kan et al., 2013).

Biotransformation

The nitriles (RC≡N) are organic compounds, synthesized by chemical methods (ammoxidation, hydrocyanation, and dehydration of amides and oximes) or biologically produced by anaerobic degradation of amino acids (Harper and Gibbs, 1979). The cyanogenic plants also release nitrile compounds in the environment (Vetter, 2000). The nitriles are commonly used in the synthesis of other useful organic compounds or manufacturing of rubber (gloves) and super glue. Moreover, the selective hydrolysis or reduction of nitriles yields valuable compounds such as amides, acids, and amines. Despite their various uses, nitriles cannot be easily degraded and are known to persist for longer periods in the environment, causing toxic or hazardous effects on biological systems, therefore, nitriles have to be metabolized into non-toxic forms. The two enzymatic pathways [nitrile hydrolase/amidase (two steps) and nitrilases (single step)] are reported to be involved in the conversion of nitriles into carboxylic acid and ammonia. Some nitrile degrading bacteria, actinobacteria, and fungi have been isolated and characterized. Most of the well-known nitrile degraders are neutrophiles. Sorokin et al. (2007) showed that a microbial consortium could degrade nitriles completely. This consortium consists of an actinobacterium (*Nitriliruptor alkaliphilus* ANL-iso2^T) and a bacterium (*Marinospirillum* sp.

strain ANL-isoa). *Nitriliruptor alkaliphilus* ANL-iso2^T is an obligate alkaliphile and moderately salt-tolerant which plays a major role in the hydrolysis of isobutyronitrile (iBN; Sorokin et al., 2009). This actionobacterium has a nitrile hydratase/amidase pathway to metabolize isobutyronitrile (iBN) into isobutyroamide, isobutyrate and ammonia which are further scavenged by *Marinospirillum* sp. strain ANL-isoa. *Nitriliruptor alkaliphilus* ANL-iso2^T is also capable of utilizing propionitrile (C3), butyronitrile (C4), valeronitrile (C5), and capronitrile (C6) as carbon and nitrogen source, thus, indirectly cleaning the environment. This strain can, therefore, be applied as a potential candidate for bioremediation or other environmental biotechnological purposes.

Bioweathering

Weathering is a disintegration process of rock constituents into smaller fragments. These components are further broken down into mobilized forms of essential nutrients (e.g., P and S) and elements (e.g., Na, K, Mg, Ca, Mn, Fe, Cu, Zn, Co, and Ni). The essential nutrients and elements are brought into crop lands or fields through wind or water. Microbial populations (bacteria and actinobacteria) occupying the rock zones show high resistance to radiations, desiccation and limited nutrient conditions. The filamentous microbes are capable of enhancing the weathering process as they penetrate through the rocks by the growing mycelia. The *Streptomyces* species are most commonly observed in rock weathering sites, since they have filamentous structure and are capable of growing as oligotroph (Cockell et al., 2013). They have a great efficiency to utilize the recalcitrant organic matter and form anthrospore under water stress. Cockell et al. (2013) reported that the indigenous microbial population of Icelandic volcanic rocks includes *Arthrobacter, Knoellia, Brevibacterium, Rhodococcus*, and *Kribbella* species. The investigation of the altered stones and monuments in the Mediterranean basin also revealed the presence of actinobacterial species which involved in the weathering of stones and monuments. These species belong to the three genera *Geodermatophilus*, *Blastococcus*, and *Modestobacter* of the family *Geodermatophilaceae* (Urzì et al., 2001). Similarly, other actinobacterial species such as *Nocardioides, Kibdelosporangium* (Abdulla, 2009), *Arthrobacter*, and *Leifsonia* (Frey et al., 2010) are known to accelerate the weathering process. Furthermore, some other actinobacteria capable of carrying out withering of rocks are also alkalitolerant such as [*Isoptericola nanjingensis* H17T (Huang et al., 2012) and *Arthrobacter nanjingensis* A33T (Huang et al., 2015)] and have been isolated from soil samples of Nanjing, China.

Plant Growth Promotion

Actinobacteria are well-known to exhibit antimicrobial and insecticidal properties and help in suppression of plant pathogenesis, thereby indirectly promoting plant growth. They also make iron available to plants for their growth (Francis et al., 2010). The plants and microbes can take up iron only in its reduced form (Fe^{+2}), while the iron exists as oxidized form (Fe^{+3}) in alkaline soils. Alkaliphilic actinobacteria reduce the iron (from Fe^{+3} to Fe^{+2} forms) and make it into soluble form which can be assimilated by plants and microbes for their growth (Valencia-Cantero et al., 2007). These actinobacteria are also capable of solubilizing phosphorus in alkaline conditions as solubility of phosphorus decreases in acidic or alkaline soils (Palaniyandi et al., 2013). An alkaliphilic strain, *Kocuria rosea* HN01 reduces Fe^{+3} into the soluble form (Fe^{+2}), thus, making the iron available to plants growing in the alkaline soil (Wu et al., 2014a).

Humic Acid Reduction

The oxidation and reduction of humic acid have a significant importance during the anaerobic biotransformation of organic and inorganic pollutants. The quinone moieties of humic acid act as center for oxido-reductive reactions (Lovley et al., 1996). The oxidized form of humic acid accepts electrons released from mineralization of organic pollutants. In addition, the reduced form of humic acid is also involved in biotransformation by reducing insoluble pollutants (oxidized) to soluble form (reduced). An alkaliphilic actinobacterium, *Corynebacterium humireducens* is capable of carrying out such biotransformation and catalyzes the reduction of the humic acids (Wu et al., 2011b) as well as the reduction of a quinone into hydroquinone. The hydroquinone speeds up the process of mineralization of pollutants such as 2,4-dichlorophenoxy acetic acid (Wang et al., 2009). The reduced humic acid could further be used to reduce the insoluble Fe^{+3} into soluble Fe^{+2} ions making them available for plant assimilation.

Applications of Thermophilic and Alkaliphilic Actinobacteria

Thermophilic and alkaliphilic actinobacteria are useful in bioremediation, gold nanoparticle synthesis, biofertilizers and biopesticides (**Figure 3**). In addition, they produce novel bioactive compounds and enzymes with commercial applications.

Synthesis of Gold Nanoparticles

The prokaryotes (bacteria and actinobacteria) as well as eukaryotes (algae, fungi, and yeast) have been currently being explored for the manufacturing of nanoparticles. The mechanism of gold particle synthesis involves the reduction of Au^{3+} by microbes when they are incubated with gold chloride (Beveridge and Murray, 1980). They synthesize nanoparticles either intracellularly or extracellularly. Among them, the use of prokaryotes is preferred because of their capability to tolerate high concentration of metal (Silver, 2003), leading to the production of a higher yield of nanoparticles. Moreover, the synthesis of nanoparticles by actinobacteria has an additional advantage of polydispersity property which prevents self-aggregation of nanoparticles (Ahmad et al., 2003a). The synthesis of gold nanoparticles by *Thermomonospora* sp. (Ahmad et al., 2003a) and alkalitolerant actinomycete *Rhodococcus* sp. (Ahmad et al., 2003b) was studied. The gold particles find various applications in diagnostics, therapeutic, and catalytic purposes.

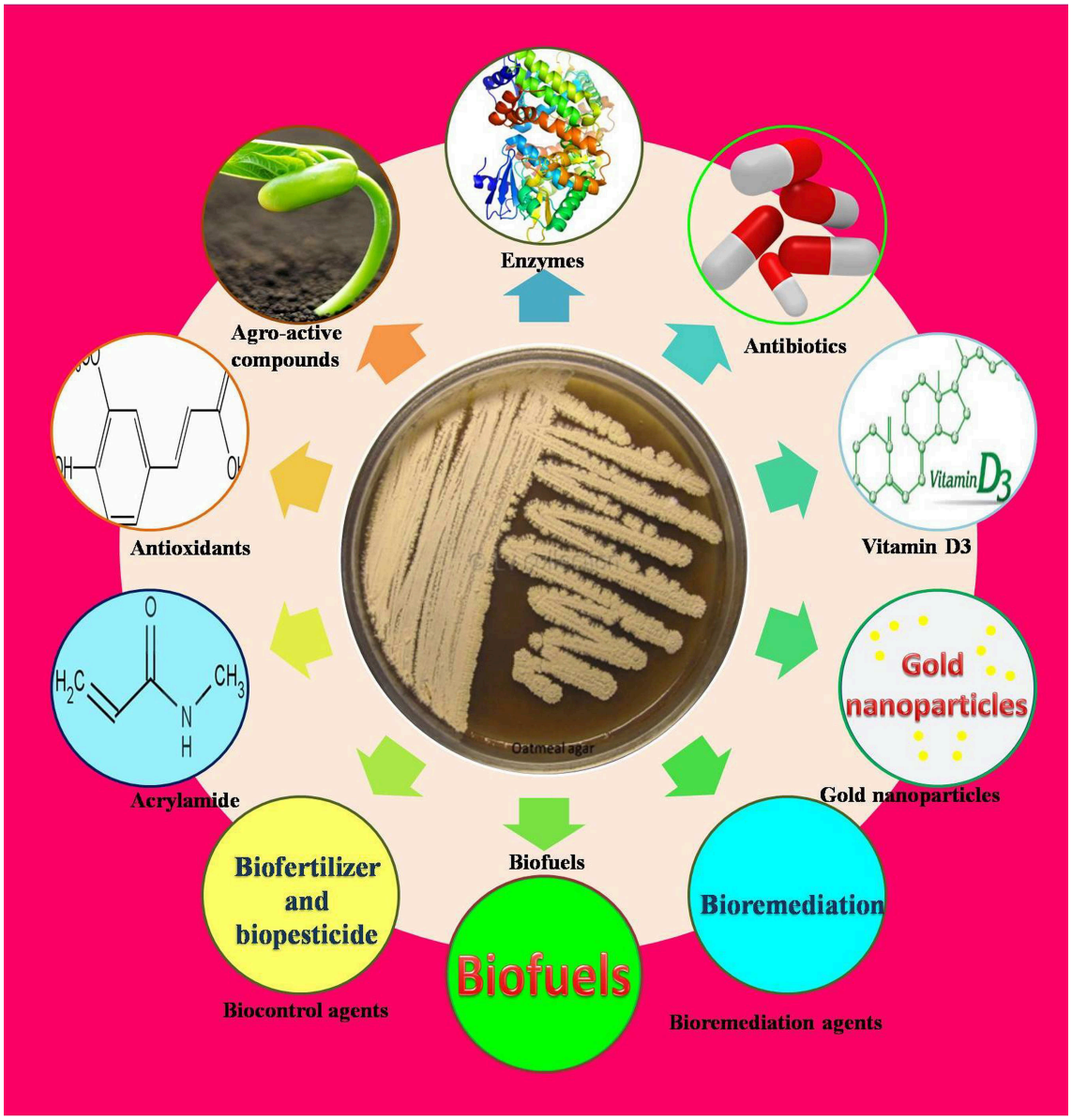

FIGURE 3 | Potential applications of Industrial thermophilic and alkaliphilic actinobacteria.

Bioremediation of Hydrocarbon Contaminated Sites

The thermophilic actinobacteria decompose a large number of biomolecules (lignin, cellulose, and hemicellulose) and recycle the nutrient back into soil which enhances the soil productivity. The process of biodegradation of interactive complex substrates necessitates actinobacteria to secrete a range of extracellular hydrolytic and oxidative enzymes. The rapid hyphal colonization and enzyme secretion enable them as being a good candidate for bioremediation process. Moreover, they are capable of metabolizing recalcitrant polymers (hydrocarbons, xenobiotic, and toxic pesticides), plastics, and rubber. Tseng et al. (2007) isolated several plastic degrading actinobacterial species belonging to the genera (*Actinomadura*, *Microbispora*, *Streptomyces,* and *Saccharomonospora*). These actinobacteria degrade various biodegradable polyesters such as poly(ethylene succinate) (PES), poly(e-caprolactone) (PCL), poly(D-3-hydroxybutyrate) (PHB), poly(tetramethylene succinate) PTMH, poly(L-lactide) (PLA), and terephthalic acid, and reduce their environmental impacts. A few other thermophilic actinobacteria are reported to act on polymer (rubber) and produce valuable chemicals such as carbonyl carbon atoms (aldehydes and ketone) and bifunctional isoprenoid species (**Table 2**). The toxic organic compounds include phenol and nitriles such as acrylonitrile and adiponitrile which are hazardous to human health. These harmful chemicals need

TABLE 2 | List of thermophilic and alkaliphilic actinobacteria degrading plastics, rubber and organic pollutants.

Actinobacteria strains	Substrate degraded	References
PLASTICS DEGRADATION		
Actinomadura miaoliensis BC 44T-5^T	PHB	Tseng et al., 2009
Actinomadura keratinilytica T16-1	PLA	Sukkhum et al., 2012
Thermobifida fusca	Terephthalic acid	Kleeberg et al., 1998
Thermobifida alba AHK119	Terephthalic acid.	Hu et al., 2010
Microbispora rosea subsp. *aerata* IFO 14046	PTMH and PCL	Jarerat and Tokiwa, 2001
Microbispora rosea subsp. *aerata* IFO 14047		
Excellospora japonica IFO 144868		
E. viridilutea JCM 339		
Streptomyces sp. strain MG	PTMH and PCL	Tokiwa and Calabia, 2004
Streptomyces thermoviolaceus subsp. *thermoviolaceus* 76T-2	PCL	Chua et al., 2013
Streptomyces bangladeshensis 77T-4	PHB	Hsu et al., 2012
Dietzia sp. Strain GS-1	Disodium terephthalate	Sugimori et al., 2000
RUBBER DEGRADATION		
Streptomyces strain La 7	Latex and natural rubber	Gallert, 2000
Actinomadura nitritigenes	Poly(cis-1,4-isoprene)	Ibrahim et al., 2006
Nocardia farcinica		
Thermomonospora curvata		
ORGANIC POLLUTANTS DEGRADATION		
Streptomyces setonii strain ATCC 39116	Phenol and benzoate	An et al., 2000
Pseudonocardia thermophila JCM3095	Acrylonitrile	Yamaki et al., 1997
Kocuria rosea HN01	DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane)	Wu et al., 2014a
Dietzia natronolimnaea JQ-AN	Aniline	Jin et al., 2012
Georgenia daeguensis	4-Chlorophenol	Woo et al., 2012
Nocardioides sp.	2,4-Dichlorophenol and 2,4,5-trichlorophenol	Maltseva and Oriel, 1997
Dietzia sp. Strain DQ12-45-1b	Petroleum hydrocarbons and crude oils	Wang et al., 2011
Dietzia cinnamea P4	Petroleum hydrocarbons	Weid et al., 2007
Dietzia sp. PD1	Azo dyes	Das et al., 2015
Dietzia sp. E1	Long chain *n*-alkane	Bihari et al., 2010
Dietzia sp. *H0B*	Prestige oil spill	Alonso-Gutierrez et al., 2011

to be degraded. Some thermophilic actinobacteria (listed in **Table 2**) are capable of metabolizing these lethal chemicals into non-toxic form by producing various enzymes such as phenol hydroxylase, polyphenol oxidase, catechol 2,3 dioxygenase, laccase, peroxidase, and nitrile converting enzymes (amidases, nitrilases, and nitrile hydratases). The pentachlorophenol is an organochlorine compound which works as a broad spectrum biocide and is used mainly in sawmills to preserve the woods. The soil and water resources of an area surrounding sawmills are contaminated with the chlorophenols causing hazardous effects on biological systems. The chlorophenols, therefore, need complete degradation. The *Saccharomonospora viridis* isolated from mushroom compost is capable of hydrolyzing this phenolic compound into non-toxic form (Webb et al., 2001).

A number of alkalitolerant and alkaliphilic actinobacteria have been reported to mineralize the hydrocarbon and other pollutants. The *Dietzia* species were found to have organic pollutant degradability and produce biosurfactants or bioemulsifiers by degrading n-alkanes (Nakano et al., 2011). The biosurfactants can be used in pharmaceuticals, detergents, textiles, and cosmetics. The species of other genera have also been reported to degrade hydrocarbons (listed in **Table 2**). A biofilm isolated from hypersaline liquids, has been shown to remove the hydrocarbon pollutants (60–70% of crude oil, pure n-hexadecane, and pure phenanthrene; Al-Mailem et al., 2015). The two alkalitolerant actinobacteria such as *Kocuria flava* and *Dietzia kunjamensis* along with other bacterial community was reported in the biofilm. A biofilm is densely packed microbial community, formed by irreversible organization, cooperation, and secretion of polymers which facilitate the adherence of microbes to the substrates and hasten the process of biodegradation of toxic compounds. The alkaliphilic and alkalitolerant actinobacteria are known to play a role in bioremediation of hydrocarbon and other organic contaminants are listed in **Table 2**.

Bioleaching

Bioleaching is a process of extracting the metals from ores. The occurrence of alkaliphiles is comparatively less than acidophiles in metal leaching sites. The two alkaliphilic actinobacteria such as

Nocardiopis sp. (Kroppenstedt, 1992) and *Nocardiopsis metallicus* strain *KBS6*^{T} (Schippres et al., 2002) have a tendency to leach metals from the alkaline slag dump, could be applied in the process of metal extraction in alkaline sites.

Bioremediation of Radionuclides Contaminated Sites

The nuclear power plants generate huge amount of radioactive wastes (radionuclides) which contaminate the land areas and water resources e.g., lakes and rivers. The radionuclides contaminated sites contain other toxic compounds as well such as heavy metals (e.g., mercury) and toxic hydrocarbons. Exposures to these lethal compounds cause cancer, birth defects, and other abnormalities. Conventionally, the chemical (solvent extraction and chemical oxidation) or physical remediation (soil washing and soil vapor extraction) methods are employed to extract these hazardous pollutants. However, these methods are quite less efficient and expensive. The microbial remediation has been found to be cost effective with high efficacy and prevents spreading of radioactive wastes over a wider area. However, the radionuclides are highly unstable and disintegrate spontaneously to emit energy in the form of harmful radiations, which act as a principle factor to limit the use of bioremediation. Since most of the microbial population is sensitive to radiations and other stresses which necessitates to search and use of radiation resistant microbes for removal or oxidation of toxic metals (Gholami et al., 2015). Some alkaliphilic (*Kocuria rosea* MG2) and alkali tolerant actinobacterial species [*Kineococcus radiotolerans* (Phillips et al., 2002), *Rubrobacter taiwanensis* (Chen et al., 2004), *Microbacterium radiodurans* (Zhang et al., 2010), and *Cellulosimicrobium cellulans* UVP1 (Gabani et al., 2012)] are resistant to lethal radiations and can sustain under harsh conditions, thus, could be potential candidates for this purpose.

Biocontrol Agent

Actinobacteria are known to improve the quality of compost and increase its nutrient content. In addition, they also reduce the odor of compost as they are able to completely digest the organic matter present in compost (Ohta and Ikeda, 1978). The thermophilic actinobacteria (*Streptomyces* sp. No. 101 and *Micromonospora* sp. No. 604) have been shown to degrade yeast debris completely and deodorize the compost (Tanaka et al., 1995). Mansour and Mohamedin (2001), reported that the *Streptomyces thermodiastaticus* produced many extracellular enzymes involved in the cell lysis of pathogenic fungi like *Candida albicans*. Some thermophilic actinobacteria are capable of suppressing plant diseases, thereby promoting good health of crop plants which leads to increase in crop yield (Iijima and Ryusuke, 1996), therefore, these thermotolerant actinobacteria could be used as alternative to commercial pesticides.

Bioactive Compounds Production

Actinobacteria are a rich source of clinically important compounds, most importantly the compounds having antitumor, antimicrobial and immunosuppressive activities (Pritchard, 2005). They are the largest antibiotic producers among all microbes, and produce approximately 55% of the total known antibiotics (Raja and Prabakarana, 2011). Among these, 75% were discovered from *Streptomyces* and remaining 25% were from non-*Streptomyces* species. The bioactive compounds discovered till date are largely of mesophilic origins. A very few natural compounds have been reported from thermophilic and alkaliphilic actinobacteria (shown in **Table 3**). Most of the antibiotics of mesophilic origin are thermolabile that is they require low temperature to sustain their effectiveness, which may be problematic for longer storage and shipping practices. Routine use of such antibiotics leads to their degradation due to repeated freezing and thawing (Eisenhart and Disso, 2012). Some antibiotics are water insoluble (Stone, 1960) and organic solvent labile, therefore, need to be dissolved in warm water to improve their solubilization; this necessitates exploring thermophilic actinobacteria that produce thermostable alternatives to currently available antibiotics.

Synthesis of Pharmaceutical Valuable Compounds

Actinobacteria synthesize a large array of secondary metabolites (antioxidant, anti-inflammatory compounds, and clinically important enzymes; shown in **Table 4**). The antioxidants produced by the thermophilic and alkaliphilic actinobacteria are melanin, ferulic acid, and canthaxanthin. These antioxidants have multiple uses in the medical field, which have been used in the treatment of cancer, heart diseases and neurodegenerative disorders such as Alzheimer and Parkinson's diseases. Ferulic acid is a component of lignin, which is linked via the ester bonds to the polysaccharides (Scalbert et al., 1985). Ferulic acid is formed upon hydrolysis of lignin by feruloyl esterase (Huang et al., 2013). Apart from functioning as antioxidants, ferulic acid can also be used as a precursor for the synthesis of vanillin (food aromatic compounds), polymers, epoxides, and aromatic compounds (alkylbenzenes, protocatechuic acid-related catechols, guaiacol, and catechol; Rosazza et al., 1995). An alkalitolerant, *Dietzia* sp. K44 produces canthaxanthin (diketocarotenoid) which has comparatively more antioxidant property than β-carotene and zeaxanthin. Canthaxanthin is naturally produced in animal and plant tissues to scavenge the free radicals (Venugopalan et al., 2013). Another important secondary metabolite, carotenoids (tetraterpenoid) is produced by *Dietzia natronolimnaea* HS-1 (Gharibzahedi et al., 2014). Carotenoids can be used as vitamin A precursor, free radicals scavenger and enhancer of the *in vitro* for the production of antibodies. *Dietzia natronolimnaea* HS-1 also produces canthaxanthin which was tested in the formulation of stable nanoemulsion (NE). The nanoemulsion system is a method to solubilize the hydrophobic antitumor compounds, which uses 2-hydroxypropyl-b-cyclodextrin (HP-β-CD) to formulate the water based drugs. The stability of NE was enhanced by mixing canthaxanthin with HP-β-CD to yield the stable inclusion complex. The stable NE has imperative therapeutical applications (Gharibzahedi et al., 2015).

Some clinically important enzymes have also been reported from thermophilic actinobacteria such as *Streptomyces* sp. (Chitte and Dey, 2002; Chitte et al., 2011) which have been shown

TABLE 3 | List of bioactive compounds produced by thermophilic and alkaliphilic actinobacteria.

Actinomyces isolates	Bioactive compounds	Activity	References
THERMOPHILIC ACTINOBACTERIA			
Excellospora viridilutea SF2315 [reclassified as *Actinomadura viridilutea* (Zhang et al., 2001)]	SF2315A and B	Antibacterial	Sasaki et al., 1988
Streptomyces thermophilus	Thermomycin	Antibacterial	Schone, 1951
Thermomonospora sp.	T-SA-125	Antibacterial	Dewendar et al., 1979
Streptomyces refuineus subsp. *thermotolerans*	Anthramycin	Antitumor Antimicrobial	Hu et al., 2007
Microbispora aerata	Diketopiperazine	Neuroprotective agents	Ivanova et al., 2013
Microbispora aerata	Microbiaeratin	Antiproliferative and cytotoxic drug	Ivanova et al., 2007
Marinactinospora thermotolerans	β-Carboline and indolactam alkaloids	Antimalarial	Huang et al., 2011
ALKALIPHILIC ACTINOBACTERIA			
Streptomyces werraensis	Erythromycin	Antibacterial	Sanghvi et al., 2014
Nocardiopsis dassonvillei WA52	WA52-A Macrolide	Antifungal	Ali et al., 2009
Streptomyces sp. No. 1543	Antimycin A	Antifungal	Sato et al., 1985
Streptomyces sp. DPTTB16	4′Phenul-1-napthyl-phenyl acetamide	Antifungal	Dhanasekaran and Panneerselvam, 2008
Streptomyces griseus Var. *autotrophicus*	Faeriefungin	Antimicrobial and insecticidal activity	Nair et al., 1989
Streptomyces strain	Pyrocoll	Antiparasitic Antitumor	Dietera et al., 2003
Nocardiopsis sp.	Griseusin D	Anticancer	Li et al., 2007a
Nocardiopsis alkaliphila YIM-80379	Nocardiopyrones A and B	Antimicrobial	Wang et al., 2013
Nocardiopsis terrae YIM 90022	Quinolone alkaloid and N-acetyl-anthranilic acid	Antimicrobial	Tian et al., 2014

to produce fibrinolytic enzymes. Fibrinolytic enzymes dissolve the blood clot (fibrin) into smaller peptides and decrease the blood viscosity, and can be used for reducing the risk of arteriosclerosis, heart attack, and stroke. Asparaginase is a well-known anticancer enzyme which inhibits the growth of uncontrolled rapidly dividing cells by hydrolyzing the amino acid asparagine which is required by the rapidly proliferating cancer cells. Hatanaka et al. (2011a) cloned and expressed the asparaginase of *Streptomyces thermoluteus* subsp. *fuscus* NBRC 14270 Another pharmaceutically valuable enzyme, X-prolyl-dipeptidyl aminopeptidase (XDAP) is known to be produced by thermophilic *Streptomyces* sp. (Hatanaka et al., 2011b), which acts on proline rich proteins and produces short peptides. These peptides act as inhibitors of dipeptidyl peptidase-4 (DPP-IV) and can regulate the blood sugar levels as DPP-IV degrades glucagon like protein-1 (GLP-1) which regulates insulin production and lowers the blood sugar level. Thus, it could be used along with GLP-1 to treat diabetes (Hatanaka et al., 2011b). Another clinically important enzyme, vitamin D3 hydroxylase converts cholecalciferol (VD_3)to its biologically active form calcitriol [$1\alpha,25(OH)_2VD_3$]. The cholecalciferol (VD_3) is an inactive form, synthesized from 7-dehydrocholesterol in the epidermal layer of skin through electrocyclic reaction on irradiance of ultraviolet. The bioconversion of VD_3 is a two step process, first it gets converted to calcidiol [$25(OH)VD_3$] by P450 in the liver, and then subsequently hydrogenated to calcitriol by P450 in the kidney. The calcitriol is a physiologically active form of vitamin D,which is involved in the regulation of calcium and phosphate concentration in the blood plasma. This calcidiol and calcitriol can be artificially synthesized from cholesterol by a multistep chemical process, but the yield is very low. There is, thus, a need of an enzyme that can catalyze the hydrogenation of VD_3 in a single step. Fujii et al. (2009) showed that *Pseudonocardia autotrophica* produces vitamin D3 hydroxylase catalyzing the conversion of VD3 into calcitriol, thus, could be used in the production of vitamin D (Fujii et al., 2009). Another important enzyme, aldose reductase catalyzes the conversion of glucose into sorbitol through polyol pathway. The high accumulation of sorbitol causes diabetes and other complications like retinopathy and neuropathy. An inhibitor YUA001 was identified from alkaliphilic *Corynebacterium* sp., that acts as a potent inhibitor of aldose reductase (Bahn et al., 1998). The two thermophilic species, *Thermomonospora alba* (Suzuki et al., 2001) and *Thermobifida alba* (Suzuki et al., 1998) produce compounds such as topostatin and isoaurostatin, respectively. These two compounds act as inhibitors of DNA topoisomerase and interfere with cellular processes like replication, transcription and translation of viruses, and therefore, could function as potential antiviral compounds.

Industrially Important Enzymes

Other than the listed uses, thermophilic and alkaliphilic actinobacteria produce a number of enzymes (amylase, proteases, lipase, cellulase, xylanase, inulinase, dextranase, and keratinase; **Table 5**) which are being produced commercially and used in industries all over the world (shown in **Figure 4**). Some important actinobacterial enzymes are briefly described below.

Amylase

A starch hydrolyzing process yields oligosacchharides and other simpler sugars (glucose, maltose, and maltotriose) which are either used in food application or syrup industry. The industrial

TABLE 4 | Pharmaceutically valuable compounds and enzymes produced by thermophilic and alkaliphilic actinobacteria.

Biological compounds	Actinobacteria isolates	Uses	References
ANTIOXIDANTS AND ANTI-INFLAMMATORY COMPOUNDS			
Melanin	*Streptomyces lusitanus* DMZ-3 *Streptomyces* sp. *Streptomyces species* D5	Cytotoxic compound Antioxidant Neurogenic disorder treatment	Madhusudhan et al., 2014 Quadri and Agsar, 2012 Diraviyam et al., 2011
Ferulic acid	*Thermobifida fusca* PU13-13	Antioxidant Anti-inflammatory	Huang et al., 2013
Canthaxanthin	*Dietzia* sp. K44	Antioxidant Feed additive Cosmetics	Venugopalan et al., 2013
Carotenoids	*Dietzia natronolimnaea* HS-1	Antioxidant Feed additive Cosmetics	Gharibzahedi et al., 2014
PHARMACEUTICALLY VALUABLE ENZYMES			
Vitamin D3 hydroxylase	*Pseudonocardia autotrophica*	Bone metabolism Immunity Cell growth regulators	Fujii et al., 2009
Fibrinolytic enzyme	*Streptomyces* sp. MCMB-379 *Streptomyces megasporus* SD5	Blood clot dissolution	Chitte et al., 2011 Chitte and Dey, 2002
Recombinant Asparaginase	*Streptomyces thermoluteus* subsp. *fuscus* NBRC 14270	Leukaemia treatment	Hatanaka et al., 2011a
L-Glutaminase	Alkaliphilic *Streptomyces* sp. SBU1	Leukaemia treatment	Krishnakumar et al., 2011
Ribonuclease	Alkaliphilic *Streptomyces* sp. M49-1	Antiviral	Demir et al., 2013
PrP^{Sc}-degrading enzyme keratinase	*Nocardiopsis* strain TOA-1	Antiprion drug	Mitsuiki et al., 2010
Recombinant X-prolyl-dipeptidyl aminopeptidases (XDAP)	*Streptomyces thermoluteus* subsp. *fuscus* NBRC 14270 *S. thermocyaneoviolaceus* NBRC 14271	Antidiabetic agents	Hatanaka et al., 2011b
ALDOSE REDUCTASE INHIBITOR			
YUA001	*Corynebacterium* sp. YUA25	Antidiabetic agents	Bahn et al., 1998
DNA POLYMERASE INHIBITORS			
Topostatin	*Thermomonospora alba* Strain No. 1520 III [reclassified as *Thermobifida alba* (Zhang et al., 1998)]	Antiviral	Suzuki et al., 1998
Isoaurostatin	*Thermomonospora alba* [reclassified as *Thermobifida alba* (Zhang et al., 1998)]	Antiviral	Suzuki et al., 2001

starch processing involves two high energy requiring steps: (1) Liquefaction (or gelatinization of starch molecules) which runs at very high temperature (105–110°C) for 5 min. (2) Saccharification (conversion of starch into simpler sugars) which requires the temperature at 55–60°C (Vieille and Zeikus, 2001). The raw starch binding thermostable amylases have become increasingly attractive to lower the process cost since they do not require gelatinized substrate for hydrolysis. The two thermophilic actinobacteria such as *Streptomyces* sp. (Kaneko et al., 2005) and *Streptomyces* sp. No. 4 (Primarini and Ohta, 2000), produce raw starch binding amylases which could be applied to reduce the energy input at industrial level making the overall process cost effective. Few other thermophilic actinobacteria are known to produce high maltotriose forming thermostable amylases which could be applicable in the food industries (listed in **Table 5**). Some alkaliphilic/alkalitolerant actinobacteria were reported to produce amylases functioning at alkaline pH, which are being used in detergent formulation to improve the detergency. At present, many modern laundries prefer amylase containing detergent for washing clothes at a

TABLE 5 | Commercially relevant enzymes produced by thermophilic and alkaliphilic actinobacteria and their potential uses.

Enzyme	Actinobacteria strains	Optimum temperature and pH	Industrial applications	References
Amylase	*Thermomonospora viridis* TF-35	60°C and 6.0	Detergent Baking Paper and pulp	Takahashi et al., 1992
	Thermomonospora curvata	65°C and 5.5–6.0	Textile industry	Glymph and Stutzenberger, 1977
Protease	*Saccharomonospora viridis SJ-21*	70°C and pH 9	Detergents Pharmaceutical	Jani et al., 2012
	Nocardiopsis prasina HA-4	55°C and pH 7–10	Leather Brewing	Ningthoujam et al., 2009
Keratinase	*Actinomadura keratinilytica* strain Cpt29	70°C and pH 10	Leather industry	Habbeche et al., 2014
	Thermomonospora curvata	65°C and pH 6	Pharamaceutical uses	Stutzenberger, 1971
Xylanase	*Thermomonospora fusca*	60°C and 7.0	Paper and pulp Baking	McCarthy et al., 1985
	Kocuria sp. RM1	30–85°C and pH 4.5–9	Animal feed	Krishna et al., 2008
	Streptomyces sp. Ab 106	60°C and pH 9.0		Techapun et al., 2002
Acetylxylan esterase	*Thermobifida fusca* NTU22	80°C and 8.0	Paper and pulp	Yang and Liu, 2008
Dextranase	*Streptomyces* sp. NK458	60°C and 9.0	Sugar mills	Purushe et al., 2012
Nitrile hydratase	*Pseudonocardia thermophila* JCM 3095	Thermostable (50–80°C)	Acrylamide production	Martinez et al., 2014
Laccase	*Thermobifida fusca* BCRC 19214	Stable at 50°C and pH 10.0	Waste treatment Textile dye treatment	Chen et al., 2013
Carbon monoxide dehydrogenase	*Streptomyces* sp. strain G26	69°C	Bioenergy generation Biofilters	Bell et al., 1988

lower temperature in order to save energy (Chakraborty et al., 2012).

Proteases

Proteases are one of the most important class of hydrolytic enzymes, which constitute >65% of the total industrial applications. A large array of actinobacterial species (including both alkalitolerant and alkaliphiles) produces alkalistable proteases and keratinase of commercial interest. The alkalistable proteases possess considerable applications in various industries such as detergent, leather, and food industries (Ellaiah et al., 2002). The alkalistable proteases are also used in the process of silver recovery from used X-ray or photographic film. The proteases of alkaliphilic actinobacteria are not only alkalistable but also thermostable (Gohel and Singh, 2012a), salt tolerant, and function actively in the presence of organic solvent (Thumar and Singh, 2009). The alkali-thermostable proteases could be a potent candidate in leather industries where the alkaline condition and high temperature are maintained during tanning process. In addition, salt and organic solvent tolerant proteases of actinobacteria find various applications in industrial processes requiring high salt concentration and solvents. The organic solvent tolerance increases the industrial value of proteases as organic solvents enhance the catalytic properties of hydrolytic enzymes (Klibanov, 2001) and preclude the occurrence of undesirable side reactions during the process.

Cellulases, Xylanase, and Acetyl Xylan Esterase

Cellulase and xylanase are the two industrially important enzymes that enable us to utilize the agricultural residues in generation of biofuel in a sustainable manner. The extreme operational conditions of industries demand highly thermostable enzymes. The two thermophilic actinobacteria, *Acidothermus cellulolyticus* (Mohagheghi et al., 1986) and *Thermobifida fusca* (Kim et al., 2005) are significantly fascinating the biofuel industry as well as several others (food, animal feed, textile, paper and pulp industry) as they are known to possess the robust enzymatic system to degrade cellulose and xylan fractions of lignocelluloic residues. The cellulases of *T. fusca* and *A. cellulyticus* have extensively been studied and are being used in bioethanol production from plant cell components. A cellulase from *T. fusca* has an additional advantage of extracting phenolics from apple peel, which can be used as antioxidants (Kim et al., 2005). This moderately thermophilic actinobacterium also secretes thermostable acetyl xylan esterase which catalyzes the removal of acetyl group from acetylxylan making easy access of xylanases to the substrate leading to its complete degradation (Yang and Liu, 2008). Thermostable and alkalistable enzymes capable of

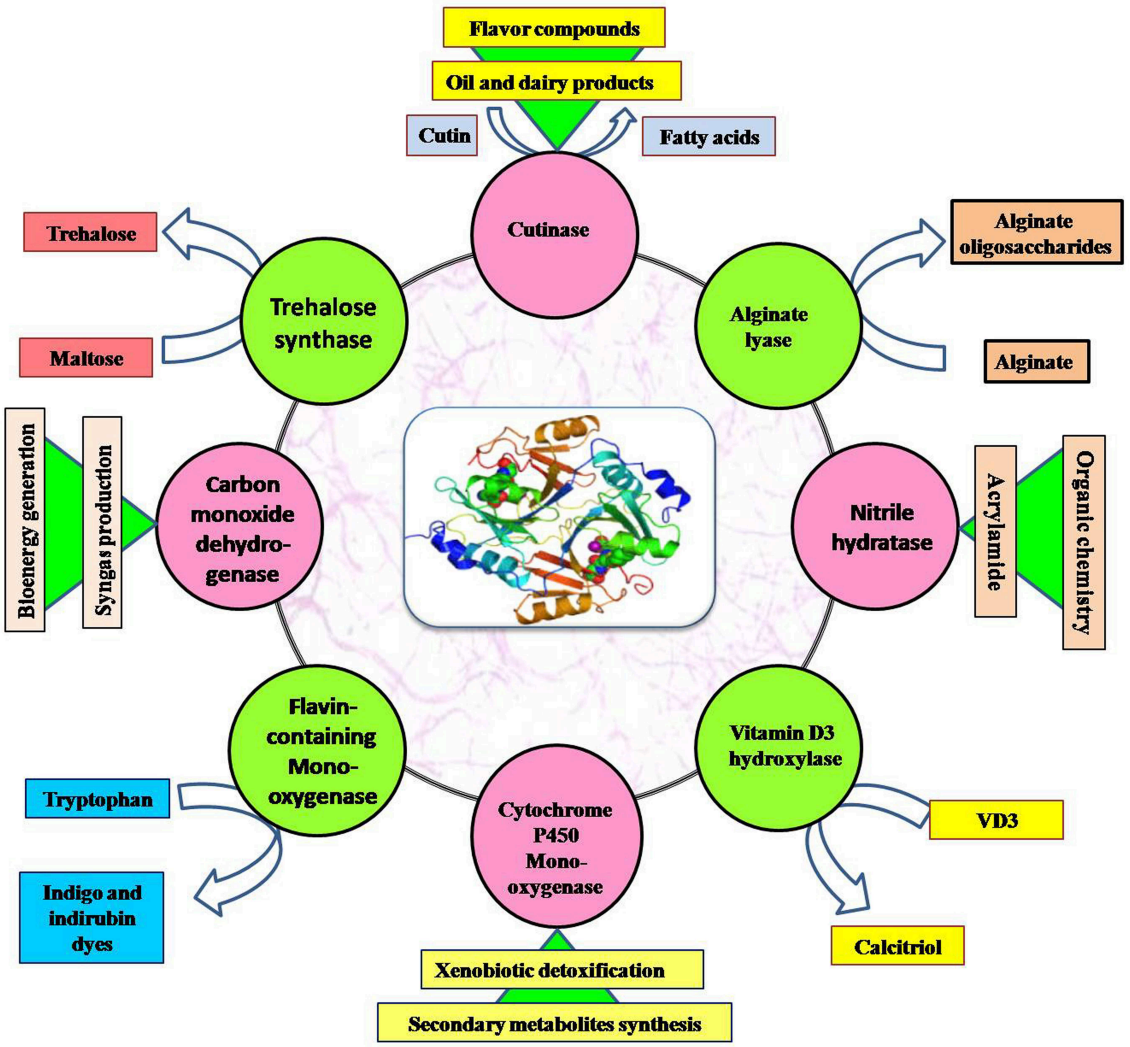

FIGURE 4 | Application of important enzymes produced by thermophilic and alkaliphilic actinobacteria.

degrading lignocelluloic substrate have also been characterized from other thermophilic and alkaliphilic actinobacteria (listed in **Table 5**).

Dextranase

The process of sugar production from sugarcane juice requires high temperature and alkaline pH. The indigenous microorganisms present in the juice may produce dextran which needs to be degraded, otherwise it blocks the filter and slows down the clarification process, thus, decreasing the yield and quality of sugar produced (Purushe et al., 2012). Since the process occurs at high temperature and alkaline pH, the addition of alkalithermostable dextranase before processing can improve the yield as well as quality of sugar produced. Therefore, dextranase produced by some thermoalkaliphilic actinobacteria such as *Streptomyces* sp. NK458 is well-suited for such application (Purushe et al., 2012).

Nitrile Hydratase

Another enzyme kown as nitrile hydratase has been reported from a large number of mesophilic and thermophilic actinobacteria, and is involved in the biotransformation of nitriles into useful compounds such as amines, amides, amidines, carboxylic acids, esters, aldehydes, and ketones (Banerjee et al., 2002). The industrial applicability of thermostable nitrile hydratases demands detailed investigation on enzymes from thermophilic actinobacteria. The thermostable nitrile hydratase from *Pseudonorcardia thermophila* has recently been immobilized in the gel matrix for acrylamide production (Martinez et al., 2014).

Laccase

Laccase catalyzes the oxidation of phenolics (2,6-dimethylphenylalanine and p-aminophenol) and produces colors, therefore, it is being used as a hair coloring agent. The

coloring occurs best at alkaline pH, as in alkaline condition, hair tends to swell up leading to easy penetration of dye molecules. Therefore, an alkalistable laccase would be the best candidate to be used for such application. Actinobacteria are known to produce thermoalkalistable laccase (e.g., *Thermobifida fusca* BCRC 19214; Chen et al., 2013). Therefore, laccase can be produced from such actinobacterial strains for hair coloring application.

Alginate Lyase

The alginate is a linear acidic polysaccharide and produced as a major component of cell wall of seaweeds. It consists of 1,4-linked α-d-mannuronate (M) and its epimer α-l-guluronate (G). These monomers polymerize in three ways: homopolymerization of G blocks [poly (G)] and homopolymerization of M blocks [poly (M)], and heteropolymerization of MG blocks [poly (MG)] (Gacesa, 1992). Alginate lyases act on these polymers to produce alginate oligosaccharides which can be used as therapeutic agents (anticoagulant, antitumor agent. and anti-inflammatory agent; Iwamoto et al., 2005). Alginate lyases are classified into two types (monofunctional and bifunctional) on the basis of their substrate specificity. Monofunctional enzymes can either act on poly(M) or poly(G) and bifunctional enzymes prefer the poly(MG) (Tondervik et al., 2010). But there are fewer reports on bifunctional and thermostable alginate lyase. An alkalitolerant actinobacterium, *Isoptericola halotolerans* CGMCC 5336 has been shown to produce moderately thermostable bifunctional alginate lyase (Dou et al., 2013).

Alditol oxidase

Oxidation of primary and secondary alcohols yields oxidative products that are used to synthesize other useful compounds. Chemical oxidation methods mediate the reaction by using heavy metals such as chromium and manganese. Interestingly, biocatalysts can also be employed to derive such oxidation reactions e.g., alcohol dehydrogenase. However, this enzyme requires $NAD(P)^+$ as cofactor for the reaction which is very costly. To overcome this demerit, the research is being focused on isolating and characterizing thermostable flavoprotein alditol oxidase (AldO) from microbial sources for industrial applications. The gene of AldO of a thermophilic actinobacterium (*Acidothermus cellulolyticus*) was identified while searching for the homologs of the well-characterized AldO of *Streptomyces coelicolor* in the genome database (Winter et al., 2012). The gene of AldO was cloned and expressed in *E. coli* and the recombinant enzyme AldO displays a high thermostability (half-life at 75°C of 112 min) and requires cheaper molecular oxygen as terminal electron acceptor. Therefore, this enzyme can be used as an alternative of chemical catalysts in industrial processes.

Carbon Monoxide Dehydrogenase

Carbon monoxide dehydrogenase is an oxidoreductase enzyme that catalyzes the interconversion between carbon monoxide and carbon dioxide. This enzyme is produced in both anaerobic and aerobic microbes during autotrophic mode of nutrition. The enzyme has a great affinity to bind CO, thereby trapping the CO from the environment, therefore, can be applied in biofilters to purify these toxic gases released by industries. *Streptomyces* sp. G26 (Bell et al., 1988) and *Streptomyces thermoautotrophicus* (Gadkari et al., 1990) have been reported to produce the thermostable carbon monoxide dehydrogenase which is well-suited for filtering the hot air released from industries. This can also be employed in the biosensor to detect and quantitate atmospheric CO concentration.

Cutinase

Cutinase is a serine esterase that acts on the ester bonds of cutin (a component of cuticle layer of plant aerial parts). *Thermobifida fusca* produces two types of cutinases which display higher thermostability than the fungal cutinases (Chen et al., 2010). The enzyme exhibits broad substrate specificity such as plant cutin and soluble/insoluble esters and hydrolyzes them into hydroxyl and hydroxy epoxy fatty acids as end products. These fatty acids can be used as substrate in the enantioselective esterification reactions or in the production of phenolic compounds as well as the oil and dairy products. The enzyme can also metabolize the synthetic polyesters and other organic pollutants (Kleeberg et al., 2005), therefore, could be used in the *in vitro* biodegradation processes.

Genome Annotation, Molecular Insights, and Genetic Manipulation of Thermophilic and Alkaliphilic Actinobacteria

The mechanisms, biosynthetic pathways and mode of action of several antibiotics of mesophilic origin have been elucidated. Classical random mutagenesis and rational genetic methods such as ribosome engineering, genome shuffling, down-regulation, and up-regulation of structural genes have been used to manipulate the genetic makeup of wild type actinobacteria strain for obtaining strains with desirable properties for e.g., enhancement in the antibiotic production titer (Olano et al., 2008). However, despite having prospective and novel characteristics, the biosynthetic pathways of bioactive compound and enzymatic system of the thermophilic and alkaliphilic actinobacteria are comparatively less explored. The inadequate information is available related to the heterologous gene expression, *in vitro* genetic engineering, structural elucidation and molecular insight on the catalysis of thermostable and alkalistable enzymes of actinobacteria. Only two thermophilic actinobacterial species, *Thermobifida fusca* and *Acidothermus cellulyticus* have been well-studied which are known to secrete a large array of highly thermostable and broad pH stable glycoside hydrolases. Their glycoside hydrolases are gaining considerable attention in the fuel biotechnology. The genes of thermo- or alkali-stable enzymes of some other thermophilic and alkaliphilic actinobacteria were cloned and expressed as well (shown in **Table 6**).

The complete genome sequence analysis reveals the presence of genes encoding industrially useful enzymes or enzymes involved in the biosynthetic pathway of novel bioactive compounds (Velásquez and van der Donk, 2011). This also

TABLE 6 | Summary of heterologous expression of proteins of thermophilic and alkaliphilic actinobacteria.

Actinobacteria	Enzymes	Expressi on Host	Optimum pH and temperature	K_m	V_{max}	References
Acidothermus cellulolyticus 11B	Thermostable endoxylanase (Xyn10A)	*E. coli* BL21	pH 6 and 90°C	0.53 mg/ml	ND	Barabote et al., 2010
Acidothermus cellulolyticus	Endoglucanase (E1)	*Pichia pastoris*	pH 5.1 and 80°C	372 ± 50 μM	0.523 ± 0.070 μM/min	Lindenmuth and McDonald, 2011
Acidothermus cellulolyticus	Glucose isomerase	*E. coli* BL21	pH 6.5 and 80°C	0.40 M	6.41	Mu et al., 2012
Acidothermus cellulolyticus	Alditol oxidase	*E. coli* BL21	pH 6–9	Varies with different substrates	Varies with different substrates	Winter et al., 2012
Saccharomonospora viridis	Xylanase (Svixyn10A)	*E. coli* BL21	8.0 and 60°C	0.68 mg/ml	217.93 U/mg	Wang et al., 2012
Nocardiopsis prasina OPC-131	Chitinase	*E. coli* BL21	pH 9.0	ND	ND	Tsujibo et al., 2003
Isoptericola jiangsuensis CLG	Chitinases *IS-chiA* and *IS-chiB*	*E. coli* BL21	pH 5 and 30°C and pH 9 and 50°C, respectively	11.66 and 17 μM, respectively	10.93 and 12.24 μmol min^{-1} mg^{-1}, respectively	Wu et al., 2011a
Brachystreptospora xinjiangensis OM-6 and *Nocardiopsis alba* OK-5	Proteases	*E. coli* BL21	pH 10	ND	ND	Gohel and Singh, 2012b
Thermobifida fusca	Cytochrome P450 monooxygenase CYP154H1	*E. coli* BL21	50°C	ND	ND	Schallmey et al., 2011
Thermobifida fusca TM51	β-D-mannosidase	*E. coli* BL21	7.17 and 53°C	180 μM	5.96 μmol min^{-1} mg^{-1},	Beki et al., 2003
Thermobifida halotolerans	Endoglucanase	*E. coli* BL21	pH 8 and 55°C	12.02 mg/ml	105.26 μM min^{-1}	Zhang et al., 2011
Thermobifida fusca	Trehalose synthase	*Pichia pastoris*	25°C and pH 6.5	ND	ND	Wei et al., 2004
Corynebacterium glutamicum	Flavin containing monooxygenase	*E. coli* BL21	pH 8 and 25°C	Varies with substrate	Varies with substrate	Ameria et al., 2015

**ND, not determined.*

provides better understanding of the genetic makeup and cellular mechanisms of an organism which enables us to engineer microbes in order to enhance their efficacy for biotechnological purposes. The genome sequence of some important thermophilic and alkaliphilic actinobacteria were annotated and analyzed which provides some valuable information related to these microbes (summarized in **Table 7**). For instance, the genome annotation of cellulolytic actinobacterium, *Thermobifida fusca* revealed the presence of additional 29 putative glycoside hydrolases (cellulose-, dextran/starch-, and xylan-degrading enzymes) than the previously characterized glycosidases (Lykidis et al., 2007). This actinobacterium has been designated as a model organism for the cellulose degradation. *Thermobifida fusca* YX has been metabolically engineered to be used in biofuel production (Deng and Fong, 2011). The gene of bifunctional butyraldehyde/alcohol dehydrogenase (adhE2) from *Clostridium acetobutylicum* ATCC 824 was introduced into the genome of *T. fusca* to enhance its efficacy for cellulose degradation. This genetically engineered strain can utilize untreated lignocellulose and convert it directly into primary alcohols (1-propanol and 1-butanol). *T. fusca* is known to produce six structurally and functionally distinct cellulases (El–E6; Irwin et al., 1993). Out of these, the three enzymes [E1 (Cel9B), E2 (Cel6A), and E5 (Cel5A)] are β-(1, 4)-endoglucanases and catalyze the conversion of insoluble cellulose into cellobiose and other simpler sugars (Hu and Wilson, 1988). The other two cellulases such as E6 (Cel48A) and E3 (Cel6B) (Zhang et al., 1995) are β-(1,4)-exoglucanases and one cellulase E4 (Cel9A) has the ability to catalyze the endo- and exo-cellulysis. These six cellulases are produced in small quantities under uninduced conditions. But

TABLE 7 | General features of thermophilic and alkaliphilic actinobacteria genome.

Actinobacteria	Genome size (Mbp)	GC content (%)	Protein-coding genes	Genes involved in secondary metabolites metabolism	Genes involved in carbohydrate transport and metabolism	Unique characteristic	References
Thermobifida fusca YX	~3.64	67.5	3117	–	–	It produces ~45 glycoside hydrolases	Lykidis et al., 2007
Acidothermus cellulolyticus 11B	~2.44	66.9	2157	–	–	It harbors a large array of industrial important enzymes	Barabote et al., 2009
Thermobispora bispora R51^T	~4.2	72.43	3596	85	221	It contains two distinct transcriptionally active 16S rRNA genes	Liolios et al., 2010
Thermomonospora curvata B9^T	~5.64	71.64	4985	181	161	It produces many bioactive compounds and thermostable enzymes	Chertkov et al., 2011
Corynebacterium efficiens	~3.15	63.4	2950	–	–	It produces industrially important amino acids	Nishio et al., 2003
Rubrobacter radiotolerans RSPS-4	~3.2 (circular genome and three plasmids)	66.91	3214	37	162	Extreme radiotolerant and moderately thermophilic actinobacterium	Egas et al., 2014
Acidimicrobium ferrooxidans ICP^T	~2.16	68.29	2038	34	87	Iron reducing acidothermotolerant	Clum et al., 2009
Saccharomonospora viridis P101^T	~4.31	67.32	3906	139	214	Causative agent of Farmer lung disease	Pati et al., 2009
Saccharopolyspora rectivirgula	~3.98	68.9	3840	–	–	Causative agent of Farmer lung disease	Pettersson et al., 2014
Arthrobacter sp. Strain IHBB 11108	3.6 (circular genome and plasmid)	58.97	3454	–	–	It produces alkalistable proteases	Kiran et al., 2015

the constitutive expression of E2 was comparatively higher than others. The cellulase E2 has been shown to play a vital role in the early growth period of *T. fusca* (Spiridonov and Wilson, 1998). A transcriptional regulator CelR (340-residue polypeptide) binds to the operator (14-base pair inverted repeat) which is present in the upstream region of genes of six cellulases and represses the transcription of the cellulase genes in *T. fusca* (Spiridonov and Wilson, 1999). The binding of CelR is controlled by the presence of cellobiose which acts as an inducer and binds with repressor protein (CelR). Binding of cellobiose brings conformational changes in CelR protein and facilitates its dissociation from operators, thereby inducing the transcription of mRNA of cellulases. The cellulase Cel9A-90 (E4) shows highest activity among other cellulases in crystalline form. It has catalytic domain (CD) of a family 9 cellulases, a cellulose binding module (CBM3c), a fibronectin III-like domain, and a family 2 CBM domain (Li et al., 2010). A active site cleft is present in the CD that consists of six glucose binding sites, numbered from −4 to +2. These residues are aligned with a flat binding surface of the CBM3c. The mutein Cel9A-51 (without CBM3c) revealed the significant role of CBM3c in processivity of the enzyme. The enzymatic activity of Cel9A was shown to be enhanced upon replacement of a conserved residue (D513) of the CBM domain (Li et al., 2007b). A mutein Cel9A-68 was constructed by deleting CBM2 domain from a Cel9A-90 gene, which showed comparatively higher cellulolytic activity (Li et al., 2010). Another mutein Cel9A-68 (T245-L251) R252K (DEL) showed slightly improved filter paper activity and increased binding affinity toward bacterial microcrystalline cellulose (Zhou et al., 2004). An enzyme E5 (Cel5A) was found to be detergent stable, which has total six cysteine residues involved in the formation of three disulfide bonds. Among them, one bond is exposed outside which gets easily reduced to free sulfahydryl group while the other two bonds are not accessible. The reduction of one accessible bond does not affect the activity of an enzyme (McGinnis and Wilson, 1993). *Thermobifida fusca* also produces other thermostable enzymes (amylase, xylanase, and mannase). Xylanase reported from *T. fusca* is thermostable. Random mutagenesis was carried out to improve catalytic efficiency (12-fold increased), substrate affinity (4.5-fold decreased) and alkalistability of this xylanase. The thermostability of the mutein, however, decreased with the improvement of other functional characteristics (Wang and Xia, 2008).

Another cellulolytic actinobacterium, *Acidothermus cellulyticus,* is reported as a potent decomposer of plant cell material. The complete genome annotation revealed that it harbors 43 genes encoding carbohydrate active enzymes. Out of 43, total 35 proteins are glycoside hydrolases and remaining eight belong to carbohydrate esterases type. The 17 plant cell wall degrading enzymes (cellulolytic and hemicellulose hydrolysis), 10 fungal cell wall degrading enzymes (chitinases, N-acetylglucosaminidase, GH16 endo-1,3-beta-glucanase and others) and 16 other proteins (glycogen and trehalose synthesizing and degrading enzymes including GH13 family α-amylase) were identified from this actinobacterium. Among 43 enzymes, only 21 are actively secreted, while others are produced intracellularly (Barabote et al., 2009). The endoglucanase

(E1 or Cel5A) of *A. cellulolyticus* is well-studied, which is ultra-thermostable, acid-stable, and displays higher substrate specificity (Tucker et al., 1989). The Cel5A belongs to glucoside hydrolases family 5 and 4/7 superfamily, and has been cloned in a number of hosts such as transgenic plants [tobacco (Dai et al., 2000), maize (Biswas et al., 2006), rice (Chou et al., 2011), and many others], and *Pichia pastoris* (Lindenmuth and McDonald, 2011). The endoglucanase producing transgenic plants ease the process of bioconversion of lignocellulosic materials into biofuels. The catalytic efficiency of Cel5A was increased by replacing of Tyr245 of WT-Cel5A with Gly (Y245G). This mutation reduces the end product inhibition and enhances the activity by 1480%. The mutein also releases 40% extra soluble sugar than wild type E1 enzyme (Baker et al., 2005). The gene of GH12 endoglucanase (not previously characterized) of *A. cellulolyticus* along with Cel5A gene were expressed into the *Zymomonas mobilis* to construct a consolidated bioprocessing (CBP) organism. Consolidated bioprocessing (CBP) is a new biotechnological approach to convert pretreated lignocellulosic materials to ethanol by using a single organism producing multiple hydrolytic enzymes (Linger et al., 2010).

Xylanase producing alkalitolerant actinobacterium, *Streptomyces viridochromogenes* strain M11 was isolated from marine sediment samples collected from the Xiaoping Island, China. This *Streptomyces* sp. produces thermostable and a broad pH stable xylanase. The xylanase production in this strain was increased (14% higher activity) by ribosome engineering. The ribosome engineering is an approach to introduce mutation in ribosome by using high concentrations of various antibiotics [10 times more concentration than minimal inhibitory concentration (MIC)]. This engineered strain produces antibiotic resistant mutants by causing mutation in the gene *rpsL* (ribosomal protein S12) and gene *rsmG* (16S rRNA methyltransferase). The K88R mutation of rpsL of this strain enhanced the xylanase production level (Liu et al., 2013). The UV mutants of *Streptomyces griseoaurantiacus* have also been shown to produce efficient cellulases (stable at high temperature and broad pH range) in relatively higher titers (Kumar, 2015). Crude oil degrading alkalitolerant actinobacterium, *Dietzia* strain DMYR9 has been isolated from oilfield and was metabolically engineered by irradiating with $^{12}C^{6+}$ heavy ions to enhance its biodegradability (Zhou et al., 2013).

Conclusions and Future Perspectives

Thermophilic and thermotolerant actinobacteria are found in 25 genera belonging to four major classes (*Actinobacteria, Acidimicrobiia, Rubrobacteria,* and *Thermoleophilia*). The taxonomic status of many thermophilic actinobacteria is ambiguous, therefore, has been revised repeatedly in the past. Bioprospecting of thermophilic actinobacteria represents an extensive pool of industrial and pharmaceutically relevant biomolecules. Their high abundance and metabolic versatility offer a new robust gateway to bioremediation of pollutants and organic residues. Although the first description on alkaliphilic actinobacteria appeared 70 years ago, the literature available on biodiversity, physiology, and ecology of alkaliphilic actinobacteria is quite inadequate. The growing industrial demand for alkalistable enzymes and biomolecules calls for further research on isolation, characterization, and bioprospecting of novel alkaliphilic actinobacteria. The use of metagenomic approaches will throw light on the novel genera of non-culturable actionobacteria and their genes in alkaline and hot environments. The availability of genome sequences of alkaliphilic and thermophilic actinobacteria is expected to encourage microbiologists and biotechnologists to go for gene mining that may lead to the discovery of novel biomolecules.

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Actinobacteria from Arid and Desert Habitats: Diversity and Biological Activity

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The lack of new antibiotics in the pharmaceutical pipeline guides more and more researchers to leave the classical isolation procedures and to look in special niches and ecosystems. Bioprospecting of extremophilic *Actinobacteria* through mining untapped strains and avoiding resiolation of known biomolecules is among the most promising strategies for this purpose. With this approach, members of acidtolerant, alkalitolerant, psychrotolerant, thermotolerant, halotolerant and xerotolerant *Actinobacteria* have been obtained from respective habitats. Among these, little survey exists on the diversity of *Actinobacteria* in arid areas, which are often adapted to relatively high temperatures, salt concentrations, and radiation. Therefore, arid and desert habitats are special ecosystems which can be recruited for the isolation of uncommon *Actinobacteria* with new metabolic capability. At the time of this writing, members of *Streptomyces*, *Micromonospora*, *Saccharothrix*, *Streptosporangium*, *Cellulomonas*, *Amycolatopsis*, *Geodermatophilus*, *Lechevalieria*, *Nocardia,* and *Actinomadura* are reported from arid habitats. However, metagenomic data present dominant members of the communities in desiccating condition of areas with limited water availability that are not yet isolated. Furthermore, significant diverse types of polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) genes are detected in xerophilic and xerotolerant *Actinobacteria* and some bioactive compounds are reported from them. Rather than pharmaceutically active metabolites, molecules with protection activity against drying such as Ectoin and Hydroxyectoin with potential application in industry and agriculture have also been identified from xerophilic *Actinobacteria*. In addition, numerous biologically active small molecules are expected to be discovered from arid adapted *Actinobacteria* in the future. In the current survey, the diversity and biotechnological potential of *Actinobacteria* obtained from arid ecosystems, along with the recent work trend on Iranian arid soils, are reported.

Keywords: *Actinobacteria*, diversity, actinomycetes, arid ecosystems, bioactive metabolites

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INTRODUCTION

The need for new bioactive structures is substantially emphasized due to the serious consequence and dynamic nature of antibiotic resistance in pathogens. Correspondingly, the need for novel bioactive compound discovery, because of their potential agricultural, pharmaceutical or industrial applications, is great (Thumar et al., 2010).

Among different resources, the privileged chemical scaffolds and metabolic potential of *Actinobacteria* have made them among the most promising bioprospecting resources (Bérdy, 2015). The rate of discovery of novel bioactive compounds has dramatically reduced in bioprospecting. As a consequence, searching for undiscovered species is imperative to address this reduction. For this purpose, either the rare genera from normal habitats or under investigated species found in unusual habitats like deserts are recommended (Harwani, 2013). Finding new actinobacterial species will presumably lead to the discovery of potentially new structural and beneficial secondary metabolites (Thumar et al., 2010). The discovery of new bioactive compounds from taxonomically unique strains of extremophilic or extremotrophic *Actinobacteria* has led to the anticipation that mining these groups could add an alternative dimension to the line of secondary metabolite resources (Thumar et al., 2010). Extremophilic and extremotolerant *Actinobacteria*, including acidtolerant and alkalitolerant, psychrotolerant and thermotolerant, halotolerant and haloalkalitolerant or xerophiles comprise the group of less investigated of this bacteria. *Actinobacteria* dwelling in deserts are capable of growing under selective conditions of pH or salinity and encompass remarkable gene clusters to produce compounds with unique antibacterial activity. However, little data is available related to the *Actinobacteria* from arid habitats, which are among the most plenteous ecosystems with regard to the occurrence of new bacterial species (Thumar et al., 2010).

By analysis of the literature data, in this review, we present the necessity of mining drought adapted *Actinobacteria,* exploring arid ecosystems for actinobacterial distribution; reporting *Actinobacteria* of arid ecosystems including studies of Iranian arid soils and bioactive metabolites of drought adapted *Actinobacteria*.

ACTINOBACTERIA AS THE OLDEST AND MOST PROMISING RESOURCE

Actinobacteria are a Gram positive group often distinguished by a high mol% G+C ratio content, filamentous or non-filamentous, among which some genera produce spores (Ludwig et al., 2012). The class *Actinobacteria* comprises 5 subclasses, 10 orders, 56 families, and 286 genera (Euzeby, 2015).

Actinobacteria are autochthonous and often among the dominant population of their ecosystems. They have a ubiquitous distribution in the biosphere, including the extremobiosphere, and are regarded as being among the predominant components of the soil microbiota (Bull, 2011). Since the discovery of Streptomycin in 1943 (Schatz et al., 1944), the greatest number of antibiotics introduced into the market, including carbapenems (Cephalosporin), macrolides (Erythromycin), ansamycins (Rifampicin), glycopeptides (Vancomycin), and Tetracyclines (Demelocyclin), have been discovered from *Actinobacteria*. The number and diversity of biosynthetic gene clusters in their genomes, attendant with respect to the fact that only a fraction of the actinobacterial bioactive chemicals have been discovered to date, justify continuing their bioprospecting as the most promising source of novel bioactive molecules discovery.

NEW SOURCE FOR EXTREMOPHILIC *ACTINOBACTERIA*

A number of environments can be considered extreme, either in terms of chemical (pH, salinity, water content) or physical parameters (temperature, pressure, radiation) (Bull, 2011). The extremophiles are evolved to thrive at or approximate to the extreme ranges of these physicochemical parameters. In contrast, a large number of microorganisms, referred to as extremotrophs, can grow but are not essentially optimized despite extreme conditions such as dilute nutrient availability that can be considered oligotroph rather than oligophile (Bull, 2011).

Members of *Actinobacteria* are recovered from a complete spectrum of extreme ecosystems. The existence of acidtolerant, alkaliphilic, psychrotolerant, thermotolerant, halotolerant, alkalitolerant, haloalkalitolerant, and xerophilous *Actinobacteria* has been reported (Lubsanova et al., 2014). Novel chemodiversity is more probable to be found in rare or recently cultivated strains. Therefore, the diversity of the extremobiosphere can resolve the challenge of rediscovery of previously known metabolites for a substantial period of time. For this reason, exploring the thriving *Actinobacteria* in extreme environments in order to obtain untapped strains is suggested. Although a few comprehensive investigations have been attempted on the bacterial diversity of arid ecosystems, the diversity of *Actinobacteria* from such environments has not been fully surveyed (Okoro et al., 2009).

ARID HABITATS AND EXISTENCE OF BIOGEOGRAPHICAL BARRIERS

Arid regions comprise the largest continental ecosystems (covering approximately 30% of all land area, of which 7% are hyper-arid) that are water-constrained. The arid areas are defined as biomes with a ratio of mean annual rainfall to mean annual evaporation of less than 0.05 and below 0.002 for extreme hyper-arid areas (Bull, 2011). The extreme desiccation condition of hyper-arid deserts is often accompanied by high temperature, nM concentrations of nutrients, low water activity, and intense radiation, while in some ecosystems, low temperature, high salinity, pH or concentrations of metals, nitrate or sulfate and inorganic oxidant anions prevail in the arid area (Bull, 2011; Koeberl et al., 2011). Among these, the availability of water and nutrients are the cardinal limiting parameters of biological activity in arid and semi-arid ecosystems (Saul-Tcherkas et al., 2013). Bacteria embedded in low water activity niches must expend rather more energy to accumulate a defined amount of water and even the most resilient bacteria usually eventuates

a state of hydrobiosis when water activity is reduced to below 0.88 aw, in which cells cease to metabolize, however, remain viable (Connon et al., 2007). Bacteria that thrive in arid habitats adjust their access to water required for their physiological requirements. Most of them are adjacent to mineral soils such as quartz, halites or gypsum; through dispersal, some water trapped in these minerals can be accessed for bacterial growth (Azua-Bustos et al., 2012).

The correlation between environmental selection or stochastic processes related to the non-random dispersal of prokaryotes indicates the existence of bacterial biogeography, however, because of the exhaustive sampling required, differentiating the endemic species is difficult. Contrary to some definite similarities, arid habitats comprise diversified local physicochemical conditions that influence community structures. As a consequence, the composition of a bacterial community is the result of local environmental selection (Ragon et al., 2012) and is therefore endemic to the arid area. However, considerable population size and cell dormancy in *Actinobacteria* may have a much more determining effect on the structure of the various microbial communities, leading to different biogeographic patterns. The phylogeny-based biogeography investigation of bacteria is scarce and their functional-trait-based evaluations are even more rarely addressed (Krause et al., 2014). In addition to strain biogeography, conserved secondary metabolome enrichment patterns that are soil type–specific are also recognized in the bacterial world (Charlop-Powers et al., 2014).

Arid regions are the interface across the often vegetated semi-arid areas and the biologically unproductive hyper-arid deserts (Neilson et al., 2012). They harbor numerous unexplored xerophilic, thermophilic, halophilic and alkaliphilic *Actinobacteria* producing new bioactive metabolites. Applications of new methods can lead to the discovery of cultivable bacteria from deserts which were supposed to be sterile (Koeberl et al., 2011). The desert habitats are among the target ecosystems for the isolation of new extremophile or extremotroph strains of *Actinobacteria* which are more likely to produce new metabolites. *Actinobacteria* have exclusive tolerance to desiccation and solute stress among bacteria and they have been isolated from diverse, hostile environments such as arid and hyper-arid deserts, which are considered analogs of potential habitats on Mars (Neilson et al., 2012; Stevenson and Hallsworth, 2014). Although high levels of germination and growth at 0.5 aw is reported for *Actinobacteria*, non-halophilic species of *Actinobacteria* are unlikely to be metabolically active below 0.80 aw, however, they may be ecologically active in water constrained soil microhabitats that contain water activity above this value (Stevenson and Hallsworth, 2014). Despite the geographical extent of arid ecosystems, little is known about the bacterial populations of these habitats and their metabolic potential (Neilson et al., 2012). In this regard, few reports are available pertaining to the isolation, screening and ecological distribution of rare *Actinobacteria* from the desert ecosystem (Harwani, 2013). Additionally, habitats other than soils are also considered as new source areas with limited water availability (Azua-Bustos et al., 2012).

XEROPHILIC STRAINS ISOLATED FROM ARID AREAS

Recovered *Actinobacteria* from extremely hot and/or acidic ecosystems or habitats with severe radiation/desiccation conditions (such as deserts and other arid regions) tend to be representative of the deepest clads of *Actinobacteria* (*Acidimicrobidae*, *Rubrobacteridae*) (Bull, 2011). The extreme desiccating condition of deserts has been the main driving force in the evolution of the DNA repair mechanisms that has generated the resistance to ionizing radiation (UV and gamma), which is a characteristic of several desert-derived *Actinobacteria* (Makarova et al., 2001). The most resistant genera of such ecosystems are strains of *Deinococcus* and *Geodermatophilus* that tolerate up to 30 Gy of irradiation. Members of these genera have not yet been recovered from non-arid soil, even using irradiation pretreatments (Bull, 2011). Xerophilic *Actinobacteria* *Geodermatophilus arenarius* and *G. siccatus* were isolated from Saharan Desert sand in Chad (Harwani, 2013; Montero-Calasanz et al., 2013). Other members of the genus *Geodermatophilus* have been isolated from Negev Desert soil and from Mojave Desert soil along the California-Nevada border, together with *Actinoplanes* and *Streptomyces* strains using selective chemoattractants (Kurapova et al., 2012). The *Geodermatophilaceae* contains only two other genera of *Blastococcus* and *Modestobacter*, which thrive in the conditions of low availability of water and nutrients. *Geodermatophilus* prefers arid soils as natural habitats and out of 15 species described in this genus, at least nine species are isolated from the desert area (Euzeby, 2015), whereas *Blastococcus* and *Modestobacter* are inhabitants of rock surfaces (Montero-Calasanz et al., 2012). An actinobacterium from a desert soil in Egypt, *Citricoccus alkalitolerans,* was recognized as alkalitolerant and that its optimum growth occurs at pH 8.0–9.0 (Li et al., 2005). Novel strains of the non-sporulating actinobacterium *Mycetocola manganoxydans* that had the ability to oxidize manganese ions were isolated from the Takalima Desert (Luo et al., 2012). Members of the *Terrabacteria* genus are also characterized by adaptations to desiccation, radiation, and high salinity (Bull, 2011). Members of the genus *Streptomyces* such as *Streptomyces deserti* from the hyper-arid Atacama Desert are also reported from arid habitats (Harwani, 2013; Santhanam et al., 2013), *Streptomyces bullii* from the hyper-arid Atacama Desert (Santhanam et al., 2013) or the moderately thermophilic xerotolerant *Streptomyces* sp. 315 from Mongolian desert soil (Kurapova et al., 2012). In addition to *Streptomyces*, strains belonging to *Micromonospora*, *Saccharothrix*, *Streptosporangium,* and *Cellulomonas* were obtained from the Qinghai-Tibet Plateau (Ding et al., 2013a), while *Micromonospora, Actinomadura,* and *Nocardiopsis* were reported from soda saline soils of the ephemeral salty lakes in Buryatiya (Lubsanova et al., 2014).

Thermotolerant and thermophilic actinomycetes were found in high abundance, exceeding that of the mesophilic forms, in Mongolian desert soils. Members of *Streptomyces*, *Micromonospora*, *Actinomadura*, and *Streptosporangium* were the most widespread thermotolerant species in desert soils (Kurapova et al., 2012). Beside *Streptomyces*, members affiliated

to the actinobacterial genera of *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Saccharopolyspora,* and *Nonomuraea* have been identified from the solar salterns of the Bay of Bengal and the Arabian Sea and inland around the Sambhar Salt Lake (Jose and Jebakumar, 2012). Interestingly, it is reported that *Actinobacteria* (20.7% of desert soil and 4.6% of agricultural soil) occur at lower concentrations in farmland compared to the surrounding desert (Ding et al., 2013b).. The genus *Rhodococcus* was among the dominant *Actinobacteria* in desert soil (Koeberl et al., 2011).

In particular, the resistance of halotolerant *Actinobacteria* (isolated from saline soils of arid territories) to alkaline conditions, high temperature and drought has experimentally been demonstrated. It was found that all the halotolerant strains (which were capable of growth at 5% NaCl), unlike unhalophilic strains, were able to grow on a medium that contained soda at pH 10, while non-halophilic strains do not possess such an ability. In this respect, a moderate thermophilic strain of *Streptomyces fumigatiscleroticus* 315 HE578745 that was isolated from the desert soil was experimentally shown to be xerotolerant (Lubsanova et al., 2014). The halotolerant alkaliphilic *Streptomyces aburaviensis* was isolated from the saline desert of Kutch in India that selectively inhibits the growth of Gram positive bacteria. It was able to grow at 15% w/v NaCl with slow growth at neutral pH, while optimum growth was in the range of 5–10% NaCl and at pH 9 (Thumar et al., 2010). Mesophilic *Actinobacteria* of the Mongolian desert soils ecosystem was represented by the genus *Streptomyces*, whereas thermotolerants were represented by the genera of *Micromonospora*, *Actinomadura,* and *Streptosporangium* (Kurapova et al., 2012).

Records of plant associated *Actinobacteria* from deserts also exist. Drought tolerant endophytic *Actinobacteria, Streptomyces coelicolor* DE07, *S. olivaceus* DE10, and *S. geysiriensis* DE27 were recovered from plants of arid and drought affected regions. These strains exhibited plant growth promotion activity and intrinsic water stress tolerance (−0.05 to −0.73 MPa) (Yandigeri et al., 2012). Some extremophilic bacteria, such as *Acidimicrobium*, *Rubellimicrobium,* and *Deinococcus-Thermus*, dramatically diminish following agricultural use. In contrary, indigenous desert bacteria can improve plant health in desert agro-ecosystems (Koeberl et al., 2011).

Actinobacteria from a low water activity area of Antarctica (similar to the situation in deserts) are also described. The bacterial diversity of Lake Hodgson, the Antarctic Peninsula, was recognized as 23% *Actinobacteria*, 21% *Proteobacteria*, 20.2% *Plantomycetes,* and 11.6% *Chlorofllexi* (Pearce et al., 2013), while from Antarctic Dry Valley soil *Cyanobacteria* (13%), *Actinobacteria* (26%), and *Acidobacteria* (16%) represented the majority of the identified resident bacteria (Smith et al., 2006). Culture-independent survey of multidomain bacterial diversity in the cold desert of the McKelvey Valley demonstrated that highly specialized communities colonize in distinct lithic niches occurring concomitantly within this ecosystem. Despite the relatively devoid soil, the greatest diversity was observed in endoliths and chasmoliths of sandstone. It indicated that the dominant communities are *Acidobacteria*, *Alphaproteobacteria,* and *Actinobacteria*. The only ubiquitous phyla in the Dry Valley zone were *Acidobacteria* and *Actinobacteria*. The overlying rock creates a favorable sub-lithic microhabitat where physical stability, desiccation buffering, water availability and irradiation protection are further provided for bactaeria (Pointing et al., 2009).

The culture independent study of *Actinobacteria* has demonstrated the dominant diversity and distribution of this phylum in arid areas. Hyper-arid soils of Yungay were shown to harbor actinobacterial OTUs (Operational Taxonomic Unit) mostly related to *Frankia* rather than the *Nitriliruptoraceae* and *Rubrobacteraceae* families that are recognized as dominant at the hyper-arid margin (Connon et al., 2007). Contrary to the fact that both regions have a sorely low level of organic substrates, higher bacterial diversity was found in the hyper-arid margin, potentially related to the mean annual rainfall and exposure to past vegetation history. Even within the hyper-arid margin, fine variations in physicochemical parameters may have a strong effect on the taxonomic diversity of actinobacterial communities (Neilson et al., 2012).

Actinobacteria comprised 94% of the 16S rRNA gene clones, represented the dominant group of high-powered soils of the Atacama Desert (Connon et al., 2007). The majority of isolates from this ecosystem belonged to the genera *Amycolatopsis*, *Lechevalieria,* and *Streptomyces* with a high incidence of non-ribosomal peptide synthase genes (Okoro et al., 2009). FISH analysis has revealed that the biomass of the metabolically active mycelial *Actinobacteria* in the prokaryotic community of Mongolian desert soils exceeded that of the unicellular *Actinobacteria* (Kurapova et al., 2012).

The overall phylum-level composition of many arid areas is shown to be dominated by *Actinobacteria.* They were shown to be the most dominant phylum (72–88%) in the case of the Atacama Desert (Crits-Christoph et al., 2013), while in other arid areas, they are among the three most abundant phyla (usually along with the Firmicutes and Proteobacteria) such as the desert soil of Aridic Calcisols in Kazakhstan (Kutovaya et al., 2015), saline–alkaline (Keshri et al., 2013), a shrub root zone of deserts (Steven et al., 2012) and high elevation desert (Lynch et al., 2014). Prevalent actinobacterial genera are not reported in almost all metagenomic studies, other than a study on the semi-arid haloalkaline ecosystem of India, in which two thirds of actinobacterial clones were recognized in the order *Rubrobacteriales* (Keshri et al., 2013).

BIOLOGICALLY ACTIVE METABOLITES REPORTED FROM XEROPHILIC *ACTINOBACTERIA*

It was hypothesized before that extremophiles can't produce secondary metabolites unless complex conditions are provided (Pettit, 2011). In contrast, now it is shown that bacteria from extreme ecosystems can produce new secondary metabolites even under regular conditions (Rateb et al., 2011). Although some antibiotic structures have been described from desert

Actinobacteria (**Table 2**), reports on the natural products of *Actinobacteria* from arid environments are rare.

Bioactive molecules of the arid inhabiting *Actinobacteria* have exhibited relatively high thermal stability, bioavailability and solubility. Two new *Streptomyces* species from Atacama Desert soils (Santhanam et al., 2011, 2013) were shown to produce new ansamycin and 22-membered macrolactones with antibacterial and antitumor activity (Rateb et al., 2011). Another *Streptomyces* strain isolated from the Chilean highland soil of the Atacama Desert produces novel aminobenzoquinones which show inhibitory activity against bacteria and dermatophytic fungi (Schulz et al., 2011).

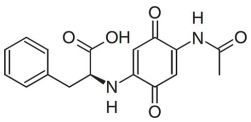
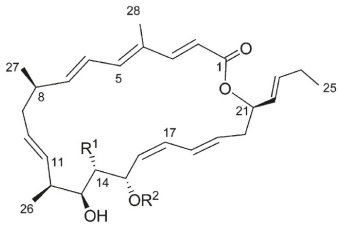
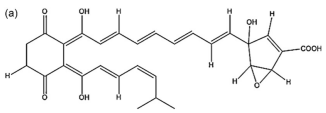
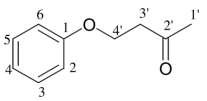
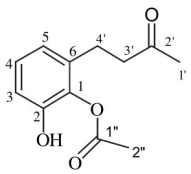
The diversity of a population comprising 52 halophilic desert actinomycetes showed the presence of strains from the *Actinopolyspora*, *Nocardiopsis*, *Saccharomonospora*, *Streptomonospora*, and *Saccharopolyspora* genera. Half of the strains were bioactive and harbored genes encoding polyketide synthetases and non-ribosomal peptide synthetases (NRPS). NRPS genes were widely distributed among these taxa, whereas PKS-I genes were detected in fewer genera (Meklat et al., 2011).

Endophytic *Actinobacteria* obtained from arid living plants belonging to the genera including *Streptomyces*, *Micromonospora*, *Nocardia*, *Nonomuraea,* and *Amycolatopsis* exhibit a high percentage of bioactivity and broad spectrum bioactivity (Huang et al., 2012). In another study, 53 *Actinobacteria* isolated from the Qinghai-Tibet Plateau were grouped into four RFLP patterns and identified as *Streptomyces*, *Micromonospora*, *Saccharothrix*, *Streptosporangium,* and *Cellulomonas*. Most of these strains had the potential to produce active compounds in addition to the detection of NRPS, PKS-I, and PKS-II genes (Ding et al., 2013a). Hence, the metagenomic analysis of the bioactive secondary metabolites (Schofield and Sherman, 2013; Wilson and Piel, 2013) can also be assessed in the future, in order to distinguish the chemical potential of drought adapted *Actinobacteria* and their conserved secondary metabolites biosynthetic pathways.

TABLE 1 | Genera of the order *Actinomycetales* containing members which are resistant to the dominant physicochemical condition in arid areas other than members of *Rubrobacteraceae* and *Acidimicrobidae*.

Suborder	Family	Genus	Physicochemical stress	References
Micrococcineae	*Intrasporangiaceae*	*Terrabacteria*	Desiccation, UV-radiation, high salinity	Battistuzzi and Hedges, 2009
	Microbacteriaceae	*Mycetocola*	Desiccation, oligotrophic	Luo et al., 2012
	Micrococcaceae	*Micrococcus*	Low temperature, UV-radiation	Miteva et al., 2009
	Microbacteriaceae	*Microbacterium*	Oligotrophic	Miteva et al., 2009
	Micrococcaceae	*Citricoccus*	Desiccation	Li et al., 2005
	Dermabacteraceae	*Brachybacterium*	Low temperature, UV-radiation	Miteva et al., 2009
Corynebacterineae	*Nocardiaceae*	*Rhodococcus*	Low temperature, high radiation, pressure	Koeberl et al., 2011
	Nocardiaceae	*Nocardia*	Low temperature, UV-radiation	Babalola et al., 2009
	Gordoniaceae	*Gordonia*	Desiccation	Brandao et al., 2001
Propionibacterineae	*Nocardioidaceae*	*Nocardioides*	Desiccation	Tuo et al., 2015
Pseudonocardineae	*Pseudonocardiaceae*	*Amycolatopsis*	Desiccation	Okoro et al., 2009
	Pseudonocardiaceae	*Lechevalieria*	Desiccation, high salinity	Okoro et al., 2010
Streptosporangineae	*Thermomonosporaceae*	*Actinomadura*	High temperature	Kurapova et al., 2012
	Streptosporangiaceae	*Streptosporangium*	High temperature	Kurapova et al., 2012
Frankineae	*Geodermatophilaceae*	*Geodermatophilus*	Desiccation, gamma-radiation, UV-radiation	Harwani, 2013
	Geodermatophilaceae	*Modestobacter*	Desiccation, low nutrient, high radiation	Chanal et al., 2006
Micromonosporineae	*Micromonosporaceae*	*Micromonospora*	High temperature	Kurapova et al., 2012
Streptomycineae	*Streptomycetaceae*	*Streptomyces*	Low/high temperature, salinity, Desiccation, pressure	Okoro et al., 2009; Santhanam et al., 2011, 2013; Kurapova et al., 2012; Harwani, 2013

TABLE 2 | Bioactive metabolites of *Actinobacteria* isolated from arid area.

Producer strain	Source of strain	Compound	Structure	Bioactivity	References
Streptomyces sp. strain DB634	Chilean highland of the Atacama Desert	Abenquines A–D		Antibacterial, antifungal and inhibition of phosphodiesterase type 4b	Schulz et al., 2011
Streptomyces sp. strain C34	Chilean hyper-arid Atacama Desert	Chaxalactins A-C		Antibacterial	Rateb et al., 2011
Saccharothrix sp. SA198	Saharan soil	Antibiotic A4		Antibacterial and antifungal	Boubetra et al., 2013
Streptomyces sp. TK-VL_333	Southwestern Algeria	4-(4-hydroxyphenoxy) butan-2-one		Antibacterial and antifungal	Kavitha et al., 2010
Streptomyces sp. TK-VL_333	Southwestern Algeria	Acetic acid-2-hydroxy-6-(3-oxo-butyl)-phenyl ester		Antibacterial and antifungal	Kavitha et al., 2010

ENZYMES REPORTED FROM XEROPHILIC *ACTINOBACTERIA*

Two thermophilic *Rhodococcus* and *Streptosporangium* were isolated from a mud volcano in India (Ilayaraja et al., 2014). According to another report, the abundance of thermotolerant *Actinobacteria* can reach the number of mesophilic ones in deserts and volcanic regions (Zenova et al., 2009) that belonged to *Thermomonospora*, *Microbispora*, *Saccharopolyspora*, *Saccharomonospora*, and *Streptomyces* (Kurapova et al., 2012). A number of hydrolytic enzymes such as amylases, xylanases and cellulase from thermotolerant *Actinobacteria* can maintain their enzymatic activity, even at high temperatures (50–65°C) (Stutzenberger, 1987). A number of *Actinobacteria* like members of *Streptomyces* have been reported that grow well at 50°C (Kim et al., 1999). Thermo stable enzymes derived from such strains can be explored for potential application in industry for enzymatic digestion purposes at higher temperatures (Ilayaraja et al., 2014). Proteolytic activity of alkaliphilic, halotolerant *Actinobacteria* is also reported. Out of 42 alkaliphilic isolates, 30 isolates were reported as halotolerant alkaliphilic *Actinobacteria* with the ability to produce extracellular protease (Ara et al., 2012).

ACTINOBACTERIA FROM ARID REGIONS OF IRAN AND THEIR POTENTIAL BIOTECHNOLOGICAL ACTIVITIES

The majority of Earth's deserts have an average annual rain (AAR) of less than 400 mm per year. In turn, "true deserts" receive less than 250 mm of AAR (Azua-Bustos et al., 2012). Iran has substantial areas of arid ecotopes, including deserts (**Figure 1**), which are presumed to harbor xerophiles including those from the phylum *Actinobacteria*. The Plateau of Iran has two plains. Dasht-e Lut (Lut Desert) and Dasht-e Kavir (Great Salt Desert) are the main deserts of this plateau. The Great Salt Desert is about 800 km long and 320 km wide (the world's 23rd largest desert) and has mosaic-like salt plates. The Lut Desert, 480 km in length and 320 km in width (the world's 25th largest desert), is a large salt desert. It is amongst the world's driest and hottest deserts (temperatures as high as 70.7°C have been recorded) and is largely considered an abiotic zone (Mildrexler et al., 2011).

These deserts are exposed to high solar radiation, including elevated UV-B. The Lut Desert is the hottest place on earth and the Great Salt Desert contains unusually high concentrations of salt deposits. It has been assumed that the Lut Desert represents the dry and high temperature limit of bacterial metabolism and

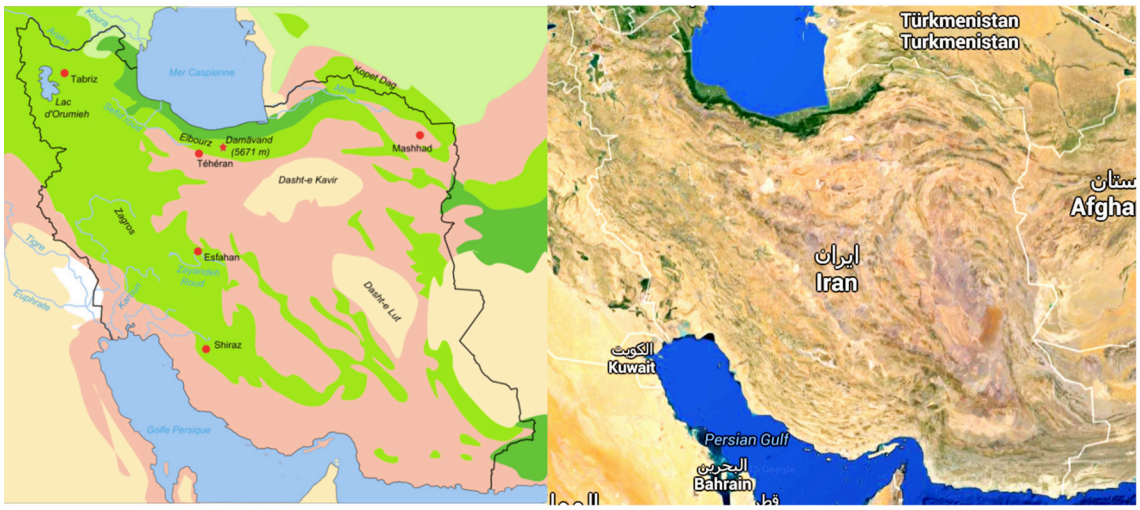


FIGURE 1 | Desert area of Iran indicated in light pink and cream in this biotope (Fabienkhan., 2006). ▮, Forests and woodlands; ▮, Forest steppe; ▮, Semi-desert; ▯, Desert lowlands; ▮, Steppe; ▯, Salted alluvial marshes.

very low or zero viable bacterial content is predicted for the Lut Desert, which should be confirmed by the inability to recover amplifiable DNA from this region in future works.

Although studies on the world's deserts are increasing, information on the diversity of *Actinobacteria* in the arid areas of Iran is scarce. Up until now, only four new species of *Actinobacteria*, which belonged to the genera *Nocardiopsis, Kribbella,* and *Promicromonospora,* have been reported from the semi-arid soil of Iran (Hamedi et al., 2011; Mohammadipanah et al., 2013, 2014). Adaptation of these strains to the extreme environmental conditions of low relative humidity, high salt concentration (including toxic ions) or high UV radiation, etc. can confer on them different metabolic potential, which may lead to the exploration of new bioactive molecules.

The diverse ecological habitats of the deserts in Iran predict diverse actinobacterial species in these ecological niches. However, the ecological habitat of Iran's deserts is underexplored and yet to be investigated for their actinobacterial diversity, as reported above. Only a few actinobacterial members have been introduced from the arid areas of Iran and their secondary metabolite potential is still under investigation. Seven new species of halophilic and alkaliphilic *Actinobacteria* are described and a number of them are in the pipeline of polyphasic identification at University of Tehran. Nevertheless, their comprehensive exploitation and utilization is underinvestigated.

Application of drought adapted *Actinobacteria* in the discovery of unique bioactive compounds, enzymes, or environmental protection and sustainable agricultural application is recommended. For instance, production of the metabolite from the radiation resistant strains, halotolerant microorganisms and enzymes from thermotolerant and alkaliphilc *Actinobacteria* of these ecosystems are encouraged. Further focus on indigenous *Actinobacteria* from the deserts of Iran would increase our knowledge of their occurrence, distribution, ecology, taxonomy and biotechnological potential.

DISCUSSION

Diverse chemical structure, wide taxonomical spectrum, and environmental dispersal have kept *Actinobacteria* among the most reliable sources for new antibiotic discovery. Drought, extreme temperature, salinity and alkalinity and oligotrophy led to the isolation of halophilic, alkaliphilic, thermophilic and radiation resistant *Actinobacteria* (Pan et al., 2010). Designing competent culture conditions for extreme environments is an approach to exploit more biodiversity from such habitats. Additionally, their extensive stress tolerance makes them more amenable to biotechnological applications (Ding et al., 2013a).

Actinobacteria from Salar and extreme hyper-arid soils have been isolated using the application of pretreatment or selective media and members of at least 12 genera have been reported. A remarkable proportion of these isolates belonged to rare genera and represented new species. Members of the *Streptomyces* genus are reported as being remarkably abundant in Atacama Desert habitats and a distinguished clade with a widespread range of antibacterials and differing modes of action has been isolated from this desert. These *Streptomyces* strains are in fact Salar adapted ecovars (Bull and Asenjo, 2013). By application of a confined type of isolation media, strains of genera, including *Nocardia*, *Microlunatus*, *Prauserella,* and *Streptomyces* were recovered, and around 50% of them produced carotenoids with antibacterial activity, even against Gram negative bacteria (Namitha and Neqi, 2010). Aminobenzoquinones (rare combinations of benzoquinones and a range of amino acids) are reported from *Streptomyces* strains isolated from the Salar de Tara. Despite the poor antibacterial and antifungal activities of abenquines, inhibitory activity against type 4 phosphodiesterase (PDE4b) was revealed for them, suggesting that they can be further assessed for their anti-inflammatory activities (Schulz et al., 2011; Bull and Asenjo, 2013). The bacterial communities of another high altitude Salar,

the Salar de Huasco (3800 m) were reported to be prevailed by members of *Alphaproteobacteria*, specifically, the *Roseobacter* clade. Radiation protection, sulfur cycling or regulation of the community structure by quorum sensing and the production of bioactive compounds are among the ecological functions of these bacteria in such ecosystems (Bull and Asenjo, 2013).

It is postulated that extremotolerants may have larger genetic and metabolic plasticity. Drought and radiation are life-controlling determinants, while habitat availability, temperature, pH and toxicants (high localized concentrations of elements such as arsenic) are among other principal determinants. Avoidance strategies to desiccation and intense radiation are evolved by bacteria, such as growth niche (hypo- and endo-lithic), extracellular polymer synthesis and pigmentation that protect the cell during epilithic colonization. Melanins are produced by many *Actinobacteria* thriving in extreme hyper-arid ecosystems. The dominant abundance of bacteria in a hyper-saline habitat was detected at a depth of about 2 m where water films had been formed by the aid of halite, nitrate and perchlorate salts. These suggest enough evidence to show that microorganisms in desert environments can be metabolically functional and not necessarily dormant or non-functional cells (Bull and Asenjo, 2013).

In desert habitats, the availability of water and organic substrates are among the main parameters limiting the ability of bacteria to maintain their metabolic functions (Saul-Tcherkas et al., 2013). Organic substrates can originate from the chemical profile of the plant root exudates, which induces variability in the associated bacterial composition of the arid soil (Saul-Tcherkas et al., 2013). These ecophysiological conformities such as excretion of chemicals, support an allelopathic habitat by altering the levels of organic matter and soil moisture. The significant differences in plant ecophysiological allelopathic adaptation reflect a strong influence on the soil bacterial community composition.

FUTURE PERSPECTIVE

The current focus of the natural product discovery is mainly on marine ecosystems (Bull and Asenjo, 2013), and arid habitats are underinvestigated habitats for this purpose. Microorganisms thriving in deserts are evolved to be less dependent on water. Other than the metabolic potential for pharmaceutical, environmental or agricultural purposes, diversity assessment of the desert ecosystems can advance our knowledge on actinobacterial ecology under extreme stress (Pointing et al., 2009).

There is a need for the development of new approaches and conditions to recover the actinobacterial strains from arid areas, nevertheless, in some cases *Actinobacteria* are the only bacteria that can be isolated (Okoro et al., 2009). The results obtained using metagenomic approaches to *Actinobacteria* in extreme environments has not yet been adequate to clearly indicate the dominant taxa in these habitats. Consequently, this level of data is not extensive enough to lead us toward their functional ecology in order to deduce their metabolic state of being metabolically active or dormant (Bull, 2011).

The ability of actinobacterial spores to germinate in very low available water environments (−96.4 MPa, 0.50 aw) enables their adaptation to drought conditions. Investigation of the desert soils demonstrates a high abundance of mycelial *Actinobacteria*, with actinobacterial isolates often adapted to high temperature, high salt concentration, and radiation (Kurapova et al., 2012). A broader spectrum of selective techniques used for the isolation of *Actinobacteria* from desert soils and of specific primers for molecular biological investigation will improve our knowledge of the diversity of *Actinobacteria* from the above mentioned ecosystems.

Desert habitats are especially rich in *Actinobacteria*, not necessarily extensive in taxonomic diversity (**Table 1**), and also in the genetic diversity of their biosynthetic pathways for synthesizing novel new secondary metabolites. Mining the natural habitats of the arid areas in Iran and designing improved procedures for selective isolation of key taxa is encouraged, as the inhabitants of the extreme areas are likely to produce new chemical entities.

Advanced or more targeted investigations are required to more fully explore and exploit the abundance, diversity, or even the plasticity and function of actinobacterial members in desert habitats.

AUTHOR CONTRIBUTIONS

FM wrote the manuscript and JW revised for its integrity and accuracy. FM and JW approved the final version of this manuscript and take responsibility for its contents.

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Endophytic Actinobacteria and the Interaction of *Micromonospora* and Nitrogen Fixing Plants

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For a long time, it was believed that a healthy plant did not harbor any microorganisms within its tissues, as these were often considered detrimental for the plant. In the last three decades, the numbers of studies on plant microbe-interactions has led to a change in our view and we now know that many of these invisible partners are essential for the overall welfare of the plant. The application of Next Generation Sequencing techniques is a powerful tool that has permitted the detection and identification of microbial communities in healthy plants. Among the new plant microbe interactions recently reported several actinobacteria such as *Micromonospora* are included. *Micromonospora* is a Gram-positive bacterium with a wide geographical distribution; it can be found in the soil, mangrove sediments, and freshwater and marine ecosistems. In the last years our group has focused on the isolation of *Micromonospora* strains from nitrogen fixing nodules of both leguminous and actinorhizal plants and reported for the first time its wide distribution in nitrogen fixing nodules of both types of plants. These studies have shown how this microoganism had been largely overlooked in this niche due to its slow growth. Surprisingly, the genetic diversity of *Micromonospora* strains isolated from nodules is very high and several new species have been described. The current data indicate that *Micromonospora saelicesensis* is the most frequently isolated species from the nodular tissues of both leguminous and actinorhizal plants. Further studies have also been carried out to confirm the presence of *Micromonospora* inside the nodule tissues, mainly by specific *in situ* hybridization. The information derived from the genome of the model strain, *Micromonospora lupini*, Lupac 08, has provided useful information as to how this bacterium may relate with its host plant. Several strategies potentially necessary for *Micromonospora* to thrive in the soil, a highly competitive, and rough environment, and as an endophytic bacterium with the capacity to colonize the internal plant tissues which are protected from the invasion of other soil microbes were identified. The genome data also revealed the potential of *M. lupini* Lupac 08 as a plant growth promoting bacterium. Several loci involved in plant growth promotion features such as the production of siderophores, phytohormones, and the degradation of chitin (biocontrol) were also located on the genome and the functionality of these genes was confirmed in the laboratory. In addition, when several host plants species were inoculated with *Micromonospora* strains, the plant growth enhancing effect was evident under greenhouse conditions. Unexpectedly, a high number of plant-cell wall degrading enzymes were also detected, a trait usually found only in pathogenic bacteria.

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Thus, *Micromonospora* can be added to the list of new plant-microbe interactions. The current data indicate that this microorganism may have an important application in agriculture and other biotechnological processes. The available information is promising but limited, much research is still needed to determine which is the ecological function of *Micromonospora* in interaction with nitrogen fixing plants.

Keywords: *Micromonospora*, legumes, PGPB, actinorhizal, endophytic, nodule

INTRODUCTION

Bacteria, archaea, and viruses are present in every niche present in our planet and have a great impact on other forms of life. Since the appearance of plants on Earth, their capacity to adapt to different ecosystems and their evolutionary process have inherently been associated to microorganisms (Reid and Greene, 2012).

Microbial communities present in soil account for the richest reservoir of biological diversity in our planet (Berendsen et al., 2012). Microorganisms that live in the rhizosphere, the soil region influenced by plant roots, are of great importance as this is where most plant-microbe interactions occur (Schenk et al., 2012) and this complex plant-associated microbial community is for the most part beneficial to the plant (Berendsen et al., 2012). Despite the importance of microorganisms for plants, these extremely complex microbial communities have remained largely uncharacterized mainly due to our lack of culturing most microorganisms under laboratory conditions (Schenk et al., 2012). Fortunately, our awareness of mutually beneficial relationships and their potential application in biotechnological processes is expanding, in part due to the new sequencing technologies and information derived from their use.

Microbes that interact with plants are termed rhizospheric or endophytic depending on their localization outside or inside the plant, respectively, and many endophytes originate from the rhizosphere or phyllosphere (Dudeja et al., 2012). These organisms can accelerate seed germination, promote plant establishment under adverse conditions, enhance plant growth or prevent pathogen infections (Hurek et al., 2002; Ryan et al., 2008). Thus, a complex and invisible ecosystem sustains plant growth and health (Reid and Greene, 2012). The potential application of beneficial microbes in different fields (e.g., agriculture, biotechnology, medicine, etc.) is immense provided progress is made in understanding these complex plant-microbe interactions in a global context.

Hitherto, plant associated Gram-negative bacteria are the best studied given their relative facility to be recovered from internal plant tissues and also because mutants can be easily generated for interaction studies (Francis et al., 2010). However, many Gram-positive bacteria included in the phyla *Firmicutes* and *Actinobacteria* (e.g., *Bacillus*, *Micromonospora*, *Streptomyces*, etc.) have excellent biocontrol, plant growth-promoting and bioremediation activities. In addition, several characteristics observed including pigment and spore production, biosynthesis of secondary metabolites and unique lifestyles present in these microorganisms can be advantageous for different biotechnological applications, including agriculture.

In this review, the diversity and interaction between actinobacteria and plants will be discussed, focusing on their ecological aspects and potential applications in agriculture. The second part of this revision will focus on the specific interaction of the genus *Micromonospora* with nitrogen fixing plants.

PLANT-ASSOCIATED ACTINOBACTERIA

Actinobacteria represent approximately 20–30% of the rhizospheric microbial community (Bouizgarne and Ben Aouamar, 2014). They are Gram-positive and show a wide morphological spectrum ranging from unicellular organisms to branching filaments that form a mycelium. A unique feature is their high guanine plus cytosine content (>50%) in their genome. These microorganisms are for the most part saprophytic, soil-dwelling organisms with an important role in the turnover of organic matter. In addition, many species are sporulated and spend the majority of their life cycles as semidormant spores (Coombs and Franco, 2003a).

Several taxa are well-known to interact with plants and these include examples of both endophytic and plant-pathogenic species. The first actinobacterial endophyte isolated, *Frankia* (Callaham et al., 1978), is a nitrogen-fixing microorganism that induces nodulation on several angiosperm plant families and has received a lot of attention due to its role in the nitrogen economy of its hosts (Verma et al., 2009). Several plant-pathogenic taxa include *Streptomyces acidiscabies*, *Streptomyces europaeiscabiei*, *Streptomyces scabies*, and *Streptomyces turgidiscabies* which cause potato scab (Loria et al., 2006; Bignell et al., 2010); *Clavibacter michiganensis* with several subspecies and pathogen for alfalfa (*C. michiganensis* subsp. *insidiosus*), maize (*C. michiganensis* subsp. *nebraskrensis*), potato (*C. michiganensis* subsp. *michiganensis*) and wheat (*C. michiganensis* subsp. *tessellarius*); *Leifsonia xyli* subsp. *xyli* which causes ratoon stunting disease of sugarcane (Young et al., 2006); *Curtobacterium flaccumfaciens* which affects several *Phaseolus* and *Vigna* species, *Beta vulgaris* species (red and sugar beet), *Ilex opaca* (American holly), *Tulipa* species (tulips), and *Euphorbia pulcherrima* (poinsettia) (Saddler and Messenber-Guimaraes, 2012); *Rathayibacter iranicus* and *Rathayibacter tritici* which cause gumming in several grasses and wheat (Evtushenko and Dorofeeva, 2012).

In the last decade, many reports on the isolation and diversity of plant-associated and endophytic actinobacteria from wild plants and crops have been published. In many of these studies, a neutral or a plant growth promotion effect was observed. The isolation and identification of actinobacteria in healthy internal root tissues of wheat was reported by Coombs and Franco

(2003a); these authors further demonstrated the colonization of germinating wheat by one of the isolated strains, *Streptomyces* sp. EN27 (Coombs and Franco, 2003b). A *Streptomyces* strain, WYEC108, isolated from linseed rhizosphere soil in Great Britain (Crawford et al., 1993) was able to colonize the roots of *Pisum sativum,* increased the number and size of root nodules, and enhanced the assimilation of iron and other nutrients by the plant (Tokala et al., 2002). Several actinobacterial strains recovered from wild plants adapted to poor soil and severe climate conditions of the Algerian Sahara desert were reported by Goudjal et al. (2013). Some of these strains produced the auxin indol acetic acid (IAA), which promoted seed germination and root elongation when tomato seeds were treated with bacterial supernatants.

The search of endophytic actinobacteria as biological control agents of plant disease is also of interest given their ability to colonize healthy plant tissues and produce antibiotics *in situ* (Kunoh, 2002; Cao et al., 2004). Maize (*Zea mays*), an important crop cultivated in many countries, especially in tropical areas, was also screened for the presence of bioactive actinobacteria (de Araújo et al., 2000). Endophytic streptomycetes isolated from healthy banana plants (*Musa* sp.), were studied for the ability to produce antifungal molecules that inhibited the growth of *Fusarium oxysporum*, which causes fusarium wilt (Cao et al., 2005). Similarly, *Streptomyces* strains were isolated from tomato and native plants of the Algerian Sahara and screened for biocontrol activity against *Rhizotocnia solani* (Goudjal et al., 2014).

Several studies have focused on the diversity and distribution of actinobacterial communities in plants, these works have provided information about the most common taxa found, e.g., the genus *Streptomyces,* but have also discovered new plant-actinobacteria associations as those represented by the interaction *Micromonospora*-nitrogen fixing plants.

Members of the genera *Microbispora, Micromonospora*, *Nocardia*, *Streptosporangium*, and *Streptoverticillium* were recovered from the surface of sterilized roots of different plant species in Italy (Sardi et al., 1992) and of maize in Brazil (de Araújo et al., 2000). Interestingly, the genus *Microbispora* was the most abundant genus recovered in maize (44%), followed by *Streptomyces* and *Streptosporangium*. A diverse collection of 11 native Korean plants were screened for the presence of endophytic actinobacteria. *Streptomyces* was the most common taxon accounting for almost 50% of the strains isolated and followed by the genera *Microbacterium, Microbispora*, *Micrococcus*, *Micromonospora*, *Rhodococcus*, and *Streptacidiphilus*. Single isolates representing the genera *Arthrobacter*, *Dietzia*, *Herbiconiux*, *Kitasatospora*, *Mycobacterium*, *Nocardia*, *Rathayibacter*, and *Tsukamurella* were also recovered (Kim et al., 2012).

Kaewkla and Franco (2013) demonstrated the high diversity of actinobacterial strains distributed in native Australian plants using highly designed isolation protocols which included low concentration isolation media, plating larger quantities of plant sample and long incubation times (up to 16 weeks). These authors reported the isolation of >500 actinobacterial strains that were identified in 16 different genera. Again, the genus *Streptomyces* accounted for >60% of the isolates.

Although the percentage of plant species sampled at present is very low, medicinal plants have received special attention given their importance as potential reservoirs of actinobacterial communities that produce compounds with biotechnological application. Qin et al. (2009, 2012) conducted a thorough study screening medicinal plants growing in the tropical rain forests in Xishuangbanna, China. These authors focused on the isolation of non-streptomycetes and found that the genus *Pseudonocardia* was the predominant taxon, followed by *Nocardiopsis*, *Micromonospora*, and *Streptosporangium* while almost 25% of the strains could not be identified at the genus level. An in depth analysis of the plant *Maytenus austroyunnanensis* applying culture- dependent and independent methods revealed an immense diversity reporting genera such as *Actinostreptospora*, *Amnibacterium*, *Catenuloplanes*, *Quadrisphaera*, and *Pseudokineococcus* which were previously unknown to reside inside plant tissues (Qin et al., 2012).

A list of endophytic and plant-associated actinobacteria recovered from different plant species and their potential application in agriculture is presented in **Table 1**.

In recent years, metagenomic analyses have been used to determine the bacterial communities of several agriculturally important crops. These studies have shown that actinobacteria are present in many of these plant microbiomes. Okubo et al. (2014) demonstrated that while the shoots of two field-grown rice cultivars collected in Nipponbare and Kasalath were dominated by *Alphaproteobacteria* (approximately 52%), the actinobacterial populations made up to 15% of the bacterial community structure. The characterization of the natural microbiome of *Vitis vinifera* leaves in Portugal reported a high diversity of proteobacteria, firmicutes, and actinobacteria, where the latter group accounted for approximately 19% of the microbial community composition and members of the families *Corynebacteriaceae*, *Microbacteriaceae*, and *Kineosporiaceae* were identified (Pinto et al., 2014).

A recent study to determine the bacterial communities of *Olea europaea* L. cultivars collected from different regions in the Mediterranean basin also confirmed the presence of actinobacterial populations on the olive leaf endosphere. An interesting conclusion of this work was that soil, climate conditions, and geographical distances had little effect on the endophytic microbial community composition (Müller et al., 2015). In another study, the root microbiota of *Lactuca sativa* cultivars and its wild ancestor *Lactuca serriola* were analyzed, the lettuce microbiota was dominated by *Proteobacteria* and *Bacteriodetes*, but *Chloroflexi* and *Actinobacteria* were also abundant (Cardinale et al., 2015). The composition of the actinobacterial population included members of the families *Micromonosporaceae* and *Nocardioaceae* but also the genera *Actinoplanes, Aeromicrobium*, *Arthrobacter*, *Demequina*, and *Streptomyces*. Interestingly, the domesticated cultivar (*L. sativa*) was richer in species diversity than its wild counterpart *L. serriola*. Unfortunately for most of the above studies, the function of these microorganisms on their host plants is unknown. In the case of lettuce, which is one of the raw foods widely consumed,

TABLE 1 | Endophytic and plant-associated actinobacteria reported in the literature.

Genus	Host plant	Isolation source	References	Potential use
*Frankia**	*Comptonia*	Root nodule	Callaham et al., 1978	Nitrogen fixation
Actinosynnema	*Grass blade*	–	Hasegawa et al., 1978	Not determined
Streptomyces	*Allium porrum, Amaryllis belladona, Betula pendula, Brassica oleracea, Calluna vulgaris, Chelidonium majusCichonum intybus, Euphorbia* sp., *Fragaria vesca, Lactuca scariola, Quercus* sp., *Rubus idaeus*	Roots	Sardi et al., 1992	Not determined
Streptomyces	*Linum usitatissimum*	Rhizosphere soil	Crawford et al., 1993	Growth promotion
Microbispora, Streptomyces, Streptosporangium	*Zea mays*	Roots	de Araújo et al., 2000	Biocontrol
Microbispora, Micromonospora, Nocardioides, Streptomyces	*Triticum aestivum*	Roots and leaves	Coombs and Franco, 2003a	Biocontrol agent
Streptomyces	*Licopersicon esculentum*	Roots	Cao et al., 2004	Biocontrol
Streptomyces, Streptoverticillium, Streptosporangium	*Musa* sp.	Roots	Cao et al., 2005	Biocontrol of *Fusarium oxysporum*
Agromyces, Microbacterium	*Retama taetam, Ononis natrix, Argyrolobium uniflorum, Astragalus armatus*	Root nodules	Zakhia et al., 2006	Not determined
Actinoplanes, Micromonospora, Streptomyces	*Cucumis sativus*	Roots	El-Tarabily et al., 2009	Biocontrol; growth promotion
Microbispora, Nocardia Sacchromonospora, Streptomyces, Streptosporangium, Streptoverticillium	*Azadirachta indica*	Leaves, stems, roots	Verma et al., 2009, 2011	Siderophore production; biocontrol
Pseudonocardia, Nocardiopsis, Micromonospora, Streptosporangium	*Phyllanthus urinaria, Kadsura heteroclita, Maesa indica, Rauvolfia verticillata, Paris yunnanensis, Maytenus austroyunnanensis, Gloriosa superba, Scoparia dulcis, Tadehagi triquetrum, Goniothalamus* sp., *Cephalotaxus* sp., *and Azadirachta* sp.	Leaves, stems, roots	Qin et al., 2009	Secondary metabolites
Arthrobacter, *Dietzia Herbiconiux, Intrasporangium, Kitasatospora, Microbacterium, Microbispora, Micrococcus Micromonospora Mycobacterium, Nocardia Rathayibacter, Rhodococcus, Streptacidiphilus, Streptomyces, Tsukamurella*	*Artemisia princeps, Capsella bursa-pastoris, Chelidonium majus, Conyza canadensis, Erigeron annuus, Iris rossii, Lamium purpureum, Physostegia virginiana, Rudbeckia bicolor, Setaria viridis, Viola mandshurica*	Roots	Kim et al., 2012	Growth promotion, biocontrol
Actinomadura, Amycolatopsis, Cellulosimicrobium, Gordonia, Glycomyces, Janibacter, Jiangella, Microbacterium, Micromonospora, Mycobacterium, Nocardia, Nocardiopsis, Nonomuraea, Plantactinospora, Polymorphospora, Promicromonospora, Pseudonocardia, Streptosporangium, Streptomyces, Saccharopolyspora, Tsukamurella	*Maytenus austroyunnanensis*	Root, stem, leaves	Qin et al., 2012	Not determined
Actinomadura, Actinomycetospora, Actinopolymorpha, Amycolatopsis, Gordonia, Kribbella, Micromonospora, Nocardia, Nocardioides, Nocardiopsis, Nonomuraea, Polymorphospora, Promicromonospora, Pseudonocardia, Streptomyces, Williamsia	*Callitris preissii, Eucalyptus camaldulensis, Eucalyptus microcarpa,Pittosporum phylliraeoides*	Leaves, stems, roots	Kaewkla and Franco, 2013	Not determined

(Continued)

TABLE 1 | Continued

Genus	Host plant	Isolation source	References	Potential use
Actinomadura, Kibdelosporangium, Kitasatospora, Micromonospora, Microtetraspora, Nocardia, Nocardioides, Nocardiopsis, Promicromonospora, Pseudonocardia, Saccharopolyspora, Streptoalloteichus, Streptomyces	*Achillea fragrantissima*, *Artemisia judaica*, *Centaurea scoparia*, *Chiliadenus montanus*, *Echinops spinosus*, *Iphiona mucronata*, *Pulicaria crispa*, *Scariola orientalis*, *Seriphidium herba-album*, *Tanacetum sinaicum*	Not specified	El-Shatoury et al., 2013	Growth promotion
Streptomyces	*Cleome arabica, Solanum nigrum, Astragallus armatus, Aristida pungens, Panicum turgidum*	Roots	Goudjal et al., 2013, 2014	Biocontrol, IAA production, growth promotion
Amycolatopsis, Isoptericola, Micromonospora, Microbispora, Nocardia, Nonomuraea, Promicromonospora, Pseudonocardia, Streptomyces	*Acacia auriculiformis, Bauhinia purpurea, Canavalia gladiate, Cassia fistula, Clitoria ternatea, Erythrina variegata, Leucaena leucocephala, Mimosa pudica, Peltophorum pterocarpum, Pithecellobium dulce, Poinciana pulcherrima, Pterocarpus macrocarpus, Samanea saman*, *Sesbania grandiflora*, *Tamarindus indica*	Roots, rhizosphere	Mingma et al., 2014	Biocontrol
Microbacterium	*Trichilia elegans*	Leaves	Rhoden et al., 2015	Not determined

The data presented is based on the references provided in column 4.
**Frankia is known to induce root nodules on a diverse group of angiosperm plants termed actinorhizals.*

it has been suggested that bacteria present in the plant's root such as *Streptomyces*, may serve as biological control agents by producing antibiotics to eliminate potential human pathogens (e.g., enterobacteria) (Cardinale et al., 2015).

Several soil microbiomes related to *Andropogon gerardii, Schizachyrium scoparium, Lespedeza capitata*, and *Lupinus perennis* grown in communities which varied in plant richness (1–16 species) were determined (Bakker et al., 2014). In this study the antagonistic activity and community structure of *Streptomyces* populations was assessed in relation to the species plant richness. The authors reported that the diversity and richness of bacterial and *Streptomyces* communities displayed different relationships with biotic and abiotic soil characteristics, therefore influencing bacterial communities.

The roots, leaves, and stems are the main plant tissues that have been screened for the presence of bacteria, however, nitrogen fixing nodules produced by legumes and actinorhizal plants are also an important reservoir of microorganisms. Nodules are rich in nutrients and therefore can also be colonized by bacteria unrelated to rhizobial or *Frankia* symbiotic nitrogen fixation.

Actinobacterial strains identified in the genera *Agromyces*, *Curtobacterium*, *Microbacterium*, *Micromonospora*, and *Streptomyces* have been reported from nodule tissues (Sturz et al., 1997; Trujillo et al., 2006, 2007, 2010; Zakhia et al., 2006; Muresu et al., 2008; Stajkoviæ et al., 2009; Deng et al., 2011; Hoque et al., 2011; Li et al., 2011; Carro et al., 2012a). Of these, the genera *Microbacterium* and *Micromonospora* were the most frequently isolated. Host plants inoculated with some of these strains showed better growth and development in comparison with non-inoculated controls suggesting a beneficial effect (Trujillo et al., 2010, 2014b; Deng et al., 2011; Martínez-Hidalgo et al., 2014). However, our knowledge about these new plant-microbe interactions is still very poor given the limited data currently available.

In light of their ecological importance, *Frankia* as a provider of nitrogen to actinorhizal plants, and *Streptomyces* as a plant pathogen for important crops such as potato, these bacteria have been under research for many decades, but this is not the case for most of other reported plant-actinobacteria interactions. However, in the last 10 years the interaction *Micromonospora*-nitrogen fixing plants is gaining attention due its potential application in downstream biotechnological applications, especially in the area of agriculture. In the following sections we will provide a general overview on the past and present status of *Micromonospora* and its close interaction with legumes and actinorhizal plants.

MICROMONOSPORA AND NITROGEN FIXING NODULES: A UNIVERSAL PLANT-MICROBE INTERACTION?

The actinobacterium *Micromonospora* was first described in 1923 (Ørskov, 1923). The first strains originated from soil and Jensen (1932) pointed out the importance of this microorganism in this niche. This bacterium belongs to the family *Micromonosporaceae* and includes aerobic, filamentous, spore-producing and mesophilic microorganisms. *Micromonospora* colonies are usually pigmented and range in color from orange, red, or brown. In many old cultures a brown-black, or black mucous mass of spores is observed. The formation of single spores is the main morphological characteristic of the genus *Micromonospora*; however, spores are also produced in dense clusters on the

surface or completely embedded in the substrate mycelium (**Figure 1**) (Genilloud, 2012; Trujillo et al., 2014a).

The presence of *Micromonospora* has been reported from many geographical sites worldwide and although soil is the most frequent source of isolation, marine, aquatic sediments and mangrove environments are also inhabited by this microorganism (Maldonado et al., 2009; Genilloud, 2012; Trujillo et al., 2014a). In recent years *Micromonosporae* have been reported as major components of nitrogen fixing root nodules of both leguminous and actinorhizal plants (Valdés et al., 2005; Trujillo et al., 2006, 2007, 2010; Garcia et al., 2010; Carro et al., 2012a, 2013a). Isolation of *Micromonospora* strains from internal nodular tissues has been reported from the legumes *Arachis hypogaea, Cicer arietinum, Glycine max, Lens culinaris, Lupinus angustifolius, Lupinus gredensis, Medicago sativa, Melilotus* sp., *Mucuna* sp., *Ononis* sp., *Ornithopus* sp., *Phaseolus* sp., *Trifolium* sp., and *Vicia* sp. The isolation of *Micromonospora* strains usually requires selective isolation procedures to favor its slow growth, however, in all the above examples, the same isolation protocol as that used for the isolation of rhizobia was applied (Cerda, 2008; Rodríguez, 2008; Carro, 2009; Alonso de la Vega, 2010; Trujillo et al., 2010).

Actinorhizal plants that have been sampled to date in Mexico, Spain, Canada, and France include the species *Alnus viridis*, *Casuarina equisetifolia*, *Coriaria myrtifolia*, *Elaeagnus x ebbingei*, *Hippophae rhamnoides*, *Myrica gale*, and *Morella pensylvanica* (Valdés et al., 2005; Trujillo et al., 2006; Carro et al., 2013a). Except for the study of Valdés et al. (2005), the isolation of *Micromonospora* from actinorhizal nodules also followed the same isolation protocols as that of legumes, using yeast-mannitol agar as isolation medium (Vincent, 1970). Currently our group maintains a collection of ∼2000 isolates recovered from diverse legume and actinorhizal plants species collected in Spain, France, Germany, Ecuador, Nicaragua, and Australia but our hypothesis is that *Micromonospora* is also present in those plant species which have not been sampled to date. In the case of legumes, the above examples indicate how *Micromonospora* had been largely overlooked in this niche due to its slow growth as compared to rhizobial strains which can be readily recovered from isolation plates after 3–5 days while *Micromonospora* strains usually appear after 7–10 days on the same plates. While the work carried by Carro et al. (2013a) strongly suggests that this microorganism is also a normal occupant of actinorhizal nodules. Thus, the systematic recovery of *Micromonospora* populations strongly suggests that this bacterium closely interacts with the host plant and nitrogen-fixing bacteria occupying the same niche.

The biogeographical and species distribution of *Micromonosporae* isolated from nitrogen fixing nodules of legumes and actinorhizal plants sampled hitherto is presented in **Table 2**.

DISTRIBUTION, LOCALIZATION AND GENETIC DIVERSITY OF *MICROMONOSPORA* IN NITROGEN FIXING NODULES

The distribution of *Micromonospora* strains in the nitrogen fixing nodules sampled so far indicate that its distribution is not homogeneous and it varies from nodule to nodule and plant to plant (Trujillo et al., 2010; Carro et al., 2012a).

The distribution pattern of *Micromonospora* in *Lupinus* spp. is highly variable with no isolates for some nodules to as many as approximately 30 (Alonso de la Vega, 2010; Trujillo et al., 2010). Variation is also reported from plant to plant and from different nodules of the same plant (Trujillo et al., 2010). A comparison of the species *Lupinus angustifolius* and *Lupinus gredensis* collected in the same geographical area in Spain, indicated that 67 and 60% of the plant samples screened (17 in total) contained the target microorganism, respectively. Out of the 45 nodules chosen for isolation, 95 *Micromonospora* strains were recovered, 74 from *L. angustifolius* and 21 from *L. gredensis*. Interestingly, 48% of the nodules did not appear to contain any *Micromonospora* strains (Alonso de la Vega, 2010).

In terms of the bacterial species distribution, *Micromonospora saelicesensis* and *Micromonospora lupini* were the most abundant, nevertheless the diversity determined on the basis of 16S rRNA gene sequencing was very high (Alonso de la Vega, 2010; Trujillo et al., 2010). These authors also screened lupine plants at different growth stages which corresponded to young, maximum growth, and flowering plants. In this case, the number of bacteria increased in parallel to the plant growth and decreased as the plants became old.

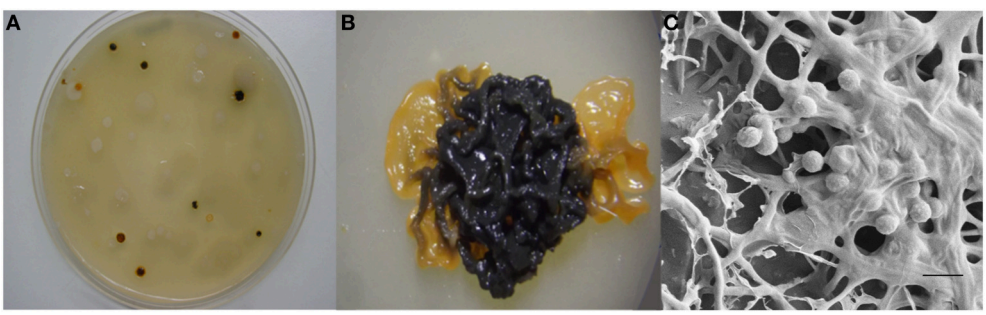

FIGURE 1 | Morphological features of *Micromonospora*. (A) *Micromonosporae* isolates recovered from a nitrogen fixing nodule. **(B)** 14 day old colony producing brown-black spores. **(C)** Scanning electron micrograph of a mucous mass of spores. Bar, 1 μm (Carro, 2009; Alonso de la Vega, 2010).

TABLE 2 | Biogeographical and species distribution of *Micromonosporae* in nitrogen fixing nodules of legumes and actinorhizal plants sampled.

Host plant (Legumes)	Common name	Geographical origin	Closest species identification (16S rRNA gene)	References
Arachys sp.	Peanut	Nicaragua	*M. chaiyapumensis, M. endolithica*	Cerda, 2008
Cicer arietinum	Chickpea	Spain	ND	Trujillo et al., 2010
Glycine max	Soy	Nicaragua	ND	Trujillo et al., 2010
Lens culinarium	Lentil	Spain	ND	Trujillo et al., 2010
Lupinus angustifolius	Blue lupine	Spain	*M. aurantiaca, M. auratinigra, M. chaiyapumensis, M. coriariae, M. coxensis, M. echinospora, M. fulviviridis, M. lupini, M. matsumotoense, M. narathiwatensis, M. olivasterospora, M. sagamiensis, M. saelicesensis*	Trujillo et al., 2007; Rodríguez, 2008; Alonso de la Vega, 2010
Lupinus gredensis	Lupine	Spain	*M. chaiyapumensis, M. chersina, M. coxensis, M. echinofusca, M. echinospora, M. lupini, M. olivasterospora, M. saelicesensis, M. viridifaciens*	Alonso de la Vega, 2010
Lupinus sp.	Lupine	Germany	*M. saelicesensis*	Trujillo et al., 2010
Medicago sp.	Alfalfa	Australia, Spain	*M. aurantiaca, M. chokoriensis, M. lupini, M. saelicesensis, M. schwarzwaldensis,M. tulbaghiae, M. viridifaciens*	Martínez-Hidalgo et al., 2014
Mucuna sp.	Mucuna	Ecuador	ND	Trujillo et al., 2010
Ononis sp.	–	Spain	ND	Trujillo et al., 2010
Ornithopus sp.	–	Spain	ND	Trujillo et al., 2010
Phaseolus vulgaris	Bean	Nicaragua	*M. chaiyapumensis, M. chersina, M. endolithica*	Cerda, 2008
Pisum sativum	Sweet pea	Spain	*M. aurantica, M. auratinigra, M. chaiyapumensis, M. chersina, M. coerulea, M. coriariae, M. coxensis, M. fulviviridis, M. lupini, M. matsumotoense, M. pattaloongensis, M. saelicesensis, M. sagamiensis,, M. siamensis*	Carro, 2009; Carro et al., 2012a
Trifolium sp.	Clover	Spain	ND	Trujillo et al., 2010
Vicia sp.	Vetch	Spain	ND	Trujillo et al., 2010
HOST PLANT (ACTINORHIZALS)				
Alnus glutinosa	Alder	France	*M. cremea, M. coxensis, M. lupini, M. matsumotoense*, *M. olivasterospora, M. saelicesensis, M. siamensis*	Carro et al., 2013a
Alnus viridis	Alder	France	*M. chokoriensis, M. coriariae, M. lupini, M. matsumotoense, M. pisi, M. rifamycinica, M. saelicesensis*	Carro et al., 2013a
Casuarina equisetifolia	Coast sheoak	Mexico	*M. aurantiaca*	Valdés et al., 2005
Coriaria myrtifolia	Redoul	Spain, France	*M. coriarie, M. saelicesensis, M. peucetia*	Trujillo et al., 2006; Carro et al., 2013a
Elaeagnus x ebbingei	–	France	*M. aurantiaca, M. auratinigra, M. chaiyaphumensis, M. coriariae, M. coerulea, M. cremea, M. coxensis, M. equina, M. lupini, M. matsumotoense, M. mirobrigensis, M. peucetia, M. saelicesensis, M. siamensis*	Carro et al., 2013a
Hippophae rhamnoides	Sandthorne	France	*M. chaiyapumensis, M. chersina, M. coxensis, M. equina, M. lupini, M narathiwatensis, M. saelicesensis, M. siamensis, M. viridifaciens*	Carro et al., 2013a
Morella pensylvanica	–	France	*M. coriariae, M. cremea, M. olivasteraspora, M. peucetia, M. saelicesensis*	Carro et al., 2013a
Myrica gale		Canada	*M. lupini*, *M. tulbaghiae*	Carro et al., 2013a

As for the legume *Pisum sativum*, a similar pattern of distribution was observed. However, for this plant, at least one *Micromonospora* strain was recovered from every nodule sampled (Carro et al., 2012a). It is also important to note that while lupine plants were collected in the field, all *Pisum sativum* samples originated from cultivation fields where chemical fertilizers are applied periodically (Carro et al., 2012a).

In a recent study, Carro et al. (2013a) screened several actinorhizal plants and recorded the number of *Micromonospora* strains and species found. *Micromonospora* strains were recovered from all plants sampled, and, as in the case of legumes, the number of isolates also varied significantly. High numbers of *Micromonospora* strains were isolated from *Alnus*, *Elaeagnus*, and *Hippophae* nodules, while the number of isolates was much lower in *Myrica*, *Morella*, and *Coriaria* nodules. Similarly to legumes, most isolates were related to *M. saelicesensis* and *M. lupini* but *M. coriariae* was also isolated in high numbers. The latter species was first reported from *Coriaria myrtifolia* nodules (Trujillo et al., 2006).

The first *Micromonospora* strains isolated from nitrogen fixing nodules were considered contaminants because it was assumed that the spores produced by this microorganism were

soil contaminants that had resisted the sterilization protocols. However, the absence of other fast-growing sporulating microorganisms, e.g., fungi or *Streptomyces* strongly indicated that the strains had originated from the internal plant tissues (Trujillo et al., 2010). Applying fluorescent *in situ* hybridization (FISH) and transmission electronic microscopy (TEM), *Micromonospora lupini* Lupac 08 was localized inside the nodular tissues of lupin suggesting a close interaction between the host plant and the bacterium (Rodríguez, 2008; Trujillo et al., 2010). Further experiments using a *Micromonospora* strain tagged with green fluorescent protein to trace the microorganism *in planta* are in the process of completion.

The degree of genetic variation of *Micromonospora* strains recovered from the nitrogen-fixing nodules of various plants was analyzed using several molecular typing techniques (e.g., BOX–PCR, ARDRA, RFLP, RAPDS) (Cerda, 2008; Carro, 2009; Alonso de la Vega, 2010; Trujillo et al., 2010; Carro et al., 2012a; Martínez-Hidalgo et al., 2014). Highly diverse genetic fingerprint profiles were found among the isolates studied, indicating that they were not clones; the diversity found was unexpectedly high considering that in some cases, the strains analyzed were isolated from the same nodule (Alonso de la Vega, 2010). Subsequently, taxonomic studies carried for some of these isolates confirmed that many of these bacterial strains represented new species and include *Micromonospora coriariae* (Trujillo et al., 2006); *Micromonospora lupini* and *Micromonospora saelicesensis* (Trujillo et al., 2007); *Micromonospora pisi* (Garcia et al., 2010); *Micromonospora cremea, Micromonospora zamorensis*, and *Micromonospora halotolerans* (**Figure 2**). The latter three strains were isolated from the rhizospheric soil of the sampled plants (Carro et al., 2012b, 2013b).

The species *M. saelicesensis* is the most frequently isolated from the nodule tissues in both legume and actinorhizal plants, followed by the species *M. lupini* (Cerda, 2008; Carro, 2009; Alonso de la Vega, 2010; Trujillo et al., 2010; Carro et al., 2012a). Furthermore, the number of new species found in this niche also appears to be very high as commented above. To expand the taxonomic studies of the genus *Micromonospora*, Carro et al. (2012a) carried out a multilocus sequence analysis study based on five loci and over 90 *Micromonospora* isolates recovered from the rhizosphere and plant tissues (nodules) of *P. sativum*. These studies were complemented with DNA-DNA hybridization analyses to confirm the high diversity at the species level (Carro et al., 2012a) and revealed that many of the new isolates represent new species (Carro et al., 2012b, 2013b).

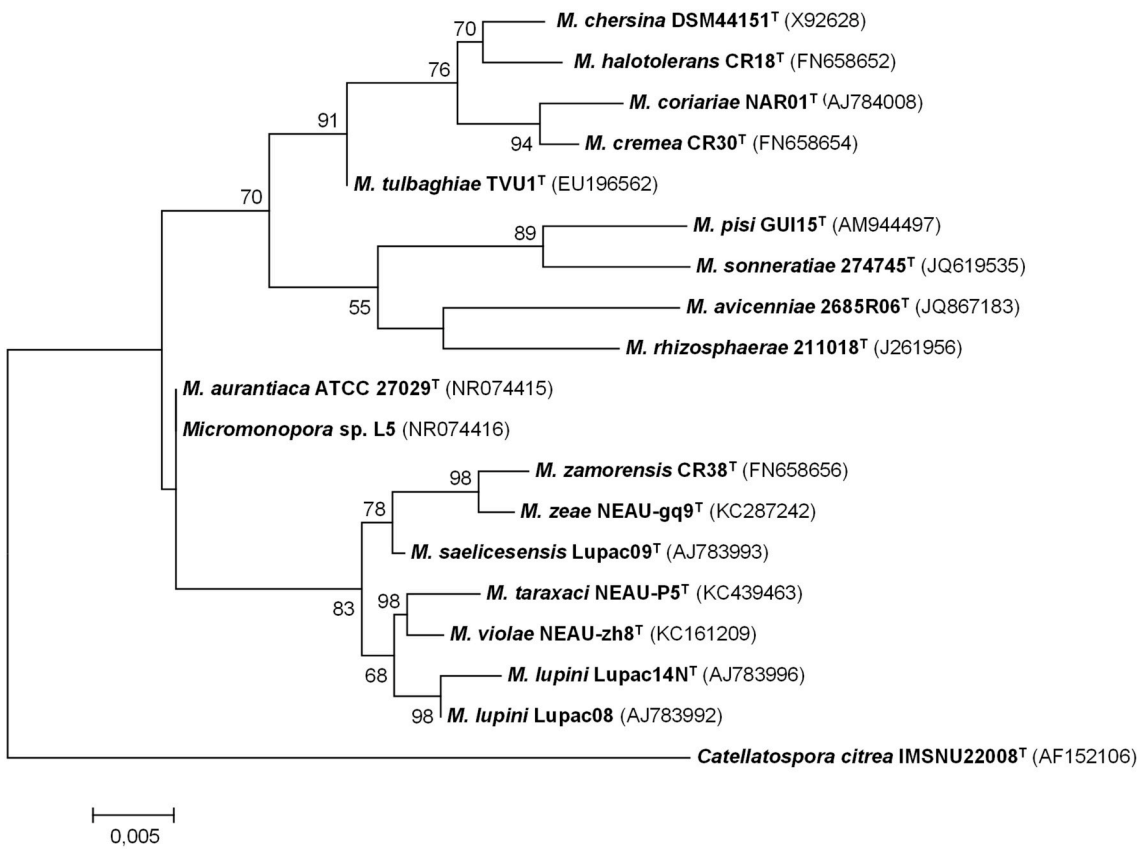

FIGURE 2 | Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of *Micromonospora* species isolated from plant material and rhizospheric soil. There were 1408 nucleotides in the final dataset. Analyses were carried in MEGA 6 software. Bar indicates 0.005 substitutions per nucleotide position (Based on references provided in **Table 2**).

GENOME FEATURES OF *MICROMONOSPORA* ISOLATED FROM NODULES

Very few *Micromonospora* strains have been sequenced. At present, only five *Micromonospora* genomes are available in the public databases: *Micromonospora* sp. strain L5 and *M. lupini* Lupac 08 and isolated from nodules of *Casuarina equisetifolia* and *Lupinus angustifolius*, respectively (Alonso-Vega et al., 2012; Hirsch et al., 2013). The remaining are the soil isolates *Micromonospora aurantiaca* ATCC 27029^T (Hirsch et al., 2013), *Micromonospora* sp. ATCC 39149 (Accession No. GCF_000158815.1) and *Micromonospora carbonacea* JXNU-1 (Jiang et al., 2015). Several genomic characteristics of the strains are presented in **Table 3**. Actinobacterial genomes are usually larger than those of most other bacteria, e.g., proteobacteria and *Micromonospora* is no exception, the currently available genomes range from 6.9 to 7.3 Mb and share a similar GC content (72–74%).

The genome sequence of strain Lupac 08 was determined to identify genomic traits potentially involved in this plant-microbe interaction (Alonso-Vega et al., 2012; Trujillo et al., 2014b). The annotated genome disclosed various traits potentially involved in the capacity of this bacterium to alternate a lifestyle as a saprophyte in the soil and as an endophyte inside the root nodules (Trujillo et al., 2014b). The genome of strain Lupac 08 has a circular chromosome of 7.3 Mb with a GC content of 71.9% and lacking plasmids. A total of 10 rRNA genes were identified, specifically 3 5S rRNA, 4 16S rRNA, and 3 23S rRNA genes. In addition 77 tRNA genes were predicted (Alonso-Vega et al., 2012). Approximately, 62% (4338 CDSs) of the genes were assigned a biological function while 38% were annotated hypothetical open reading frames with unknown biological activities (Alonso-Vega et al., 2012). The genome of *Micromonospora* sp. L5 is smaller, 6.9 Mb, a GC content of 72.9% and 6332 open reading frames (Hirsch et al., 2013). This strain is highly related to *M. aurantiaca* ATCC 27029^T and average nucleotide identity values (ANI) of their genomes strongly suggest that *Micromonospora* sp. L5 belongs to this species. The number of tRNAs identified in *Micromonospora* sp. L5 is 52 (Hirsch et al., 2013) which is much lower when compared to the 77 tRNAs identified in *M. lupini* 08. Indeed, the latter strain has one of the largest numbers of tRNAs reported for actinobacteria sequenced to date. The number of rRNA and tRNA genes in a genome appear to be correlated and is an indication of positive selection related to the time of response of a bacterium to adapt to its environment (Dethlefsen and Schmidt, 2007; Yano et al., 2013).

The core genome of the strains *M. lupini* Lupac 08, *M. aurantiaca* ATCC 27029^T and *Micromonospora* sp. L5 was determined and the results indicated that the strains shared a common gene pool of only approximately 32% suggesting a high degree of genomic diversity (Trujillo et al., 2014b). As expected, the strains *M. aurantiaca* and *Micromonospora* L5 with 85% genome similarity confirm their close relationship. *M. lupini* on the other hand appears to be very different, with 66.6% of its genome being strain specific. As more *Micromonospora* genomes are sequenced the core genome should be better defined.

A number of genomic traits that probably participate in the plant/soil life style of endophytic *Micromonospora* include transport and secretion systems. Several genes coding for transport and secretion systems which may be involved in plant colonization were also identified. The number of transporters is slightly higher in *M. lupini* Lupac 08 than in *Micromonospora* L5, and included ATP dependent (mainly of the ABC family type), ion channels, PTS (phosphotransferase) and secondary transporters (Trujillo et al., 2014b).

MICROMONOSPORA LUPINI LUPAC 08: A FRIENDLY BACTERIUM HIGHLY EQUIPPED WITH PLANT CELL WALL DEGRADING ENZYMES

Micromonosporae are well-known for their capacity to produce high numbers of cellulases, these enzymes very likely contribute to the turn-over of decayed material in different habitats (de Menezes et al., 2008, 2012). However, the presence of high numbers of these molecules and other plant-cell wall degrading enzymes in beneficial endophytic bacteria is usually very low (Krause et al., 2007; Mastronunzio et al., 2008; Taghavi et al., 2010; Pujic et al., 2012).

The genome of strain Lupac 08 contains a high number of genes encoding enzymes potentially involved in plant cell wall degradation. Approximately 10% of the genome codes for carbohydrate metabolism, and almost 200 out of the 685

TABLE 3 | Genomic features of sequenced *Micromonospora* strains available in the databases.

Feature	*M. lupini* Lupac 08	*M. aurantiaca* ATCC 27029^T	*Micromonospora* sp. L5	*Micromonospora* sp. ATCC 39149	*Micromonospora carbonacea* JXNU-1
Size (Mb)	7.3	7	6.9	6.8	7.6
GC%	72	73	73	72	74
rRNA Operon	10	9	9	6	7
tRNA	77	52	53	51	50
CDS number	7054	6676	6617	5633	6247
Genes in COGs (%)	70.20%	68.30%	69%	nd	nd

nd, not determined.

genes have a putative hydrolytic function. Hydrolytic activities for cellulose, pectin, starch, and xylan, were confirmed in the laboratory and indicate that this strain could degrade plant cell wall components in a way similar to that of phytopathogen bacteria (Trujillo et al., 2014b). Plant-polymer degrading enzymes are thought to be involved in internal plant colonization (Compant et al., 2005). Plant pathogenic fungi and bacteria usually enter plant tissues by degrading plant cell wall components using several hydrolases which include cellulases and endoglucanases. On the other hand, genome data show that non-pathogenic (endophytic or symbiotic) microorganisms contain a low set of plant-polymer degrading enzymes (Krause et al., 2007; Mastronunzio et al., 2008; Taghavi et al., 2010). In the case of *M lupini*, the genome of this microorganism revealed a high number of hydrolytic enzymes (e.g., cellulases, xylanases, endoglucanases) with the potential to degrade plant tissues (**Figure 3**). However, green-house experiments show that when host plants are inoculated with strain Lupac 08 no damage is produced. On the contrary, *M. lupini* stimulates nodulation and plant growth (Cerda, 2008; Trujillo et al., 2014b). Therefore, if the plant does appear to be negatively affected by these enzymes, what is their potential function when the bacterium interacts with its host plant? Our group is currently working on this subject, some of the loci, especially those related to cellulose metabolism may participate in other processes such as cellulose biosynthesis (Robledo et al., 2008, 2012; Mba Medie et al., 2012). Several genes coding for plant cell-wall degrading enzymes were also located in the genome of *Micromonospora* sp. L5 (Hirsch et al., 2013). Similarly to strain Lupac 08, target substrates include cellulose, hemicellulose, pectin, starch, and xylan, however, the number of loci involved in carbohydrate transport and metabolism are slightly lower in strain L5 (8.9%), as compared to strain Lupac 08 (9.7%) (Trujillo et al., 2014b).

Bacterial endophytic colonization is still a poorly understood process, in part because it is very complex. For microorganisms that colonize the roots, plant exudates appear to play a crucial role (Badri et al., 2009). Molecules present in root exudates may serve as carbon sources for microorganisms and therefore, these are attracted to the plant roots (Shidore et al., 2012). Thus, plant exudates may act as signals that influence the ability of a bacterium to colonize the root or survive in the rhizosphere. These signals may induce the alteration of specific gene expression patterns in the bacterium, which in turn may influence its interaction with the plant (Morrissey et al., 2004; Mark et al., 2005; Shidore et al., 2012). While it is considered that plant exudates affect the behavior of rhizospheric microorganisms, our knowledge as to how these molecules influence bacterial gene expression is still very limited (Mark et al., 2005). Furthermore, it is not known how these altered bacterial genes affect the plant-microbe interaction process and only a few studies are available (Morrissey et al., 2004; Mark et al., 2005; Shidore et al., 2012).

In the case of the *Micromonospora*-plant interaction, it could be that the plant's root exudates might be involved in the repression of hydrolytic enzyme genes (e.g., cellulases, xylanases, etc.) from the bacterium which, if expressed during its interaction with the plant would be detrimental upon infection. The effect on *Azoarcus* sp. gene expression upon exposure to plant root exudates was recently reported (Shidore et al., 2012). This study concluded that the genes expressed by *Azoarcus* strain

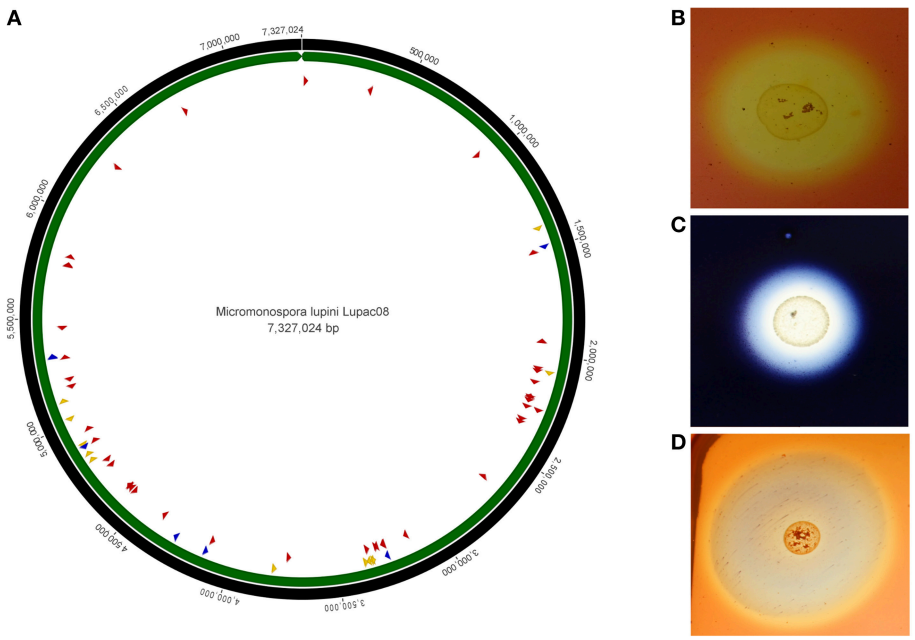

FIGURE 3 | Circular genome representation of *Micromonospora lupini*, Lupac 08. (A) Distribution of various plant-cell wall hydrolytic enzyme loci. Red, cellulases, and cellulose-binding sites; blue, pectinases; yellow, xylanases. **(B)** *In vitro* cellulase degradation. **(C)** *In vitro* starch degradation. **(D)** *In vitro* xylanase degradation (Based on Trujillo et al., 2014b).

BH72 upon exposure to the plant's root exudates influenced the colonization of the roots (Shidore et al., 2012). In this sense, the genome of *M. lupini* contains many regulatory genes located near plant cell wall degrading loci suggesting that these genes are under strong regulation, which in turn, may be directly related to the surrounding environment, soil, or plant tissues (Trujillo et al., 2014b).

MICROMONOSPORA, A PLANT GROWTH PROMOTER WITH WIDE APPLICATION IN AGRICULTURE

Plant growth promoting bacteria (PGPB) are defined as soil bacteria that facilitate plant growth and are often found in association with plant roots, leaves, flowers, or within plant tissues. Many of these bacteria are found in the plant rhizoplane and rhizosphere but other are endophytic and able to colonize the internal plant tissues (Glick, 2015). Plant growth promoting bacteria have been reported to positively affect plants in a number of ways, directly by facilitating resource acquisition (e.g., nitrogen fixation, phosphorous, iron) or controlling plant hormone levels, or indirectly by lowering the inhibitory effects of plant pathogen microorganisms (e.g., biocontrol agents).

The current data about the interaction of *Micromonospora* with legume and actinorhizal plants is limited, and therefore the bacterium's ecological role inside the roots nodules and its interaction with the nitrogen fixing bacteria (rhizobia/*Frankia*) is unknown. Plant co-inoculation studies indicate that *Micromonospora* acts as a plant growth promoting bacterium with a positive effect on the plant (Martínez-Hidalgo et al., 2014; Trujillo et al., 2014b). Nodulation and nitrogen tests were carried out on *Lupinus* and *Phaseolus*, these studies showed that *Micromonospora* is not able to induce nodules or fix nitrogen but a positive effect on the growth of the plant was observed by an increase in the number of nodules and the height of the plants which had been inoculated with both microorganisms when compared to the plants treated with only one of the two strains (Cerda, 2008). Furthermore, when *Micromonospora* and the nitrogen-fixing bacterium (*Bradyrhizobium* or *Rhizobium*, respectively) were grown together, they were compatible and did not inhibit the growth of each other. Interestingly, *Micromonospora* did inhibit the growth of several *Frankia* strains; furthermore the latter strains came from different plant species (Carro et al., 2013a). On the other hand no inhibition was observed between *Micromonospora* and *Frankia* when the strains originated from the same plant (Carro et al., 2013a).

Studies carried out with *Trifolium* plants yielded similar results. *Micromonospora lupini* Lupac 08 stimulated plant growth when it was co-inoculated with *Rhizobium* sp. on clover plantlets and these were grown in a greenhouse (Trujillo et al., 2014b). In general, the number of nitrogen-fixing nodules increased in plants treated with both bacteria as compared to the plants inoculated only with the *Rhizobium* strain. Overall, the plants inoculated with both bacteria exhibited better growth and increased shoot length compared to single-strain treatments (Trujillo et al., 2014b).

Solans (2007) studied the plant promotion effect of three actinobacterial strains isolated from the plant species *Discaria trinervis* which included a *Micromonospora* strain. The inoculation experiments of *D. trinervis* grown in glass tubes with vermiculite-sand was done using pure mycelia suspensions and/or supernatants obtained from the actinobacterial cultures grown for 8 days. Plants inoculated with mycelium plus supernatant from *Micromonospora* strain BCRU-MM18 had a higher shoot length than the control plants and it was proposed that this effect was probably due to the presence of several plant hormones such as zeatin, IAA, and gibberellic acid. Further studies confirmed that strain BCRU-MM18 produced significant amounts of IAA (9.03 ng/ml), giberellic acid (9.03 ng/ml), and zeatin (270 μg/ml); in all cases these amounts were higher than those produced by the nitrogen fixer *Frankia* sp. BCU110501 (Solans et al., 2011). The same *Micromonospora* strain (BCRU-MM18) was co-inoculated in *Medicago sativa* which had also been inoculated with the nitrogen fixer *Sinorhizobium meliloti* in the presence of high nitrogen content. Unexpectedly, a promotion of nodulation was observed despite the high amounts of nitrogen present (7 mM) which usually inhibit nodulation (Solans et al., 2009). The above studies showed the positive effect that *Micromonospora* had on the symbiosis of both leguminous and actinorhizal plants, especially in increasing nodulation rates.

Recently, *Micromonospora* strains isolated from wild alfalfa plants collected in several sites in Spain were studied for their plant growth and nutrient content effect on this legume. Selected strains significantly increased the nodulation of *Medicago* sp. inoculated with *Ensifer meliloti* and also the plant's efficiency for nitrogen uptake. Furthermore, aerial growth, shoot-to-root ratio and increase in levels of key nutrients was also reported (Martínez-Hidalgo et al., 2014). These authors also discussed the importance of choosing the most effective strains.

The wide distribution of *Micromonospora* among nitrogen fixing plants (both legumes and actinorhizals) differs from that of rhizobia or *Frankia* which are limited to a narrow host range of legumes and angiosperms, respectively. The capacity of infection by *Micromonospora* with a positive effect for its host plant may be regarded as an advantage for downstream biotechnological applications and the potential to use this bacterium as a plant growth promoter in combination with rhizobia or *Frankia*.

THE *MICROMONOSPORA* METABOLOME AND ITS POTENTIAL ROLE IN PLANT-MICROBE COMMUNICATION SIGNALS

Microbial secondary metabolites have been the subject of many research projects, mainly with the aim to discover new compounds with biotechnological application (Miao and Davies, 2010; Genilloud, 2014). However, our knowledge about the ecological role of these compounds is very limited. It is proposed, that in the environment, these natural products serve as allelochemicals and signaling molecules to communicate with organisms, in this case, with the plant (Badri et al., 2009). Udwary et al. (2011) recently reported the identification of several

biosynthetic gene clusters coding for secondary metabolites in the genome of *Frankia*. In this work, it was proposed that some of these compounds could function as communication molecules to establish the symbiotic interaction between *Frankia* and the host plant (Udwary et al., 2011). The potential role of lectins produced by *Frankia alni* ACN14a to permit binding of the bacterial cells to the roots of the host plant was suggested by Pujic et al. (2012). In another study, a hybrid (PKS)/NRPS protein produced by *Trichoderma virens* was proposed to induce the defense mechanisms of maize (Mukherjee et al., 2012).

Moreover, Conn et al. (2008) demonstrated that culture filtrates obtained from *Micromonospora* sp. strain EN43 isolated from healthy wheat tissues were able to induce several plant defense systems in *Arabidopsis thaliana*. When the bacterium was grown in a minimal medium, the culture filtrate applied to the plant induced the systemic acquired system pathway; however, when grown in a complex medium, the jasmonic acid/ethylene pathway was activated (Conn et al., 2008). Based on these results, the authors suggested that different metabolites were produced under the two conditions tested and that these compounds were responsible for the activation of the different defense mechanisms in the plant (Conn et al., 2008). In addition, it was also proposed that a physical contact of the bacterium and the plant may be required for the defense mechanisms to be activated. Overall, the above examples show the potential ecological role of secondary metabolites in plant-microbe interactions.

The information derived from sequenced actinobacterial genomes have revealed that these microorganisms have the biosynthetic potential to make far more natural products than was realized before genome sequences were available (Genilloud, 2014). Only a small fraction of endophytic bacteria have been characterized and they remain as an untapped resource of novel bioactive small molecules (Qin et al., 2011; Brader et al., 2014). As mentioned above, some of these metabolites are speculated to affect the physiological conditions of host plants including growth and disease resistance (Conn et al., 2008; Udwary et al., 2011). *Micromonosporae* strains are also a good source for obtaining natural products (Weinstein et al., 1963; Thawai et al., 2004; Antal et al., 2005; Anzai et al., 2010; Kyeremeth et al., 2014). In this sense, the model strain *Micromonospora lupini* Lupac 08 is no exception and a family of new anthraquinone molecules with antitumoral activity were isolated and identified (Igarashi et al., 2007, 2011). Moreover, 15 clusters involved in the biosynthesis of secondary metabolites were identified in the genome of *M. lupini* Lupac 08. These included siderophores, terpenes, butyrolactones, polyketides (PKS), non-ribosomal peptides (NRPS), chalcone synthases and bacteriocins. Approximately 7.4% of the genome was related to genes coding for secondary metabolites.

The production of siderophores by endophytic bacteria is suggested to promote plant growth by sequestering iron from the environment and providing the nutrient to the plant. Alternatively, plant growth promoting bacteria can protect plants by binding the available iron surrounding the roots and limiting access to the nutrient by phytopathogen microorganisms (Glick, 2015). Recently it was shown that a siderophore-producing endophytic streptomyces strain significantly increased root and shoot biomass as compared to a siderophore deficient mutant strain (Rungin et al., 2012). Furthermore, Misk and Franco (2011) reported the capacity of several endophytic siderophore producing *Streptomyces* strains to suppress root rot in chickpea produced by *Phytophtora*. In this case, the streptomycete strains were isolated from several legumes. Several gene loci related with the synthesis of siderophores were identified in the genome of *M. lupini* Lupac 08 and the strain was shown to produce these molecules in the laboratory (Trujillo et al., 2014b). Siderophores produced by *Micromonospora* may also contribute to the increased root and shoot biomass observed when host plants are inoculated with this bacterium (Martínez-Hidalgo et al., 2014; Trujillo et al., 2014b).

The characterization and identification of secondary metabolites produced by *Micromonospora* strains isolated from nitrogen fixing plants is at present reduced to three anthraquinones, lupinacidins A, B, and C (Igarashi et al., 2007, 2011). However, the genome of strain Lupac 08 revealed that other metabolites are potentially produced (e.g., terpenes, butyrolactones, polyketides, non-ribosomal peptides etc.). These compounds may act as communication molecules between the microorganism and the plant to allow bacterial colonization (Udwary et al., 2011). Alternatively, as suggested by other studies these metabolites may provide protection against pathogens, either by producing specific control agents or by activating plant defense systems (Conn et al., 2008). Furthermore, some metabolites may be necessary for nutrient uptake (Barry and Challis, 2009; Rungin et al., 2012) All these areas remain to be studied in the interaction *Micromonospora*-nitrogen fixing plants.

CONCLUDING REMARKS

Our knowledge of the interaction between *Micromonospora* with legumes and actinorhizal plants is in its infancy and a lot more work is required to fully understand this ecological process. Apart from the studies presented above, there is no other information regarding the molecular interaction between *Micromonospora* and its host plants and how it interacts with other bacteria present in the nitrogen fixing nodules. The current data is promising as it strongly suggests that *Micromonospora* provides a benefit to the plant. The genome of strain Lupac 08 revealed many features that make this microorganism an excellent candidate as a plant-growth promoter which could be applied to a large number of agriculturally important crops.

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Actinobacterial diversity in limestone deposit sites in Hundung, Manipur (India) and their antimicrobial activities

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Studies on actinobacterial diversity in limestone habitats are scarce. This paper reports profiling of actinobacteria isolated from Hundung limestone samples in Manipur, India using ARDRA as the molecular tool for preliminary classification. A total of 137 actinobacteria were clustered into 31 phylotypic groups based on the ARDRA pattern generated and representative of each group was subjected to 16S rRNA gene sequencing. Generic diversity of the limestone isolates consisted of *Streptomyces* (15 phylotypic groups), *Micromonospora* (4), *Amycolatopsis* (3), *Arthrobacter* (3), *Kitasatospora* (2), *Janibacter* (1), *Nocardia* (1), *Pseudonocardia* (1) and *Rhodococcus* (1). Considering the antimicrobial potential of these actinobacteria, 19 showed antimicrobial activities against at least one of the bacterial and candidal test pathogens, while 45 exhibit biocontrol activities against at least one of the rice fungal pathogens. Out of the 137 actinobacterial isolates, 118 were found to have at least one of the three biosynthetic gene clusters (PKS-I, PKS-II, NRPS). The results indicate that 86% of the strains isolated from Hundung limestone deposit sites possessed biosynthetic gene clusters of which 40% exhibited antimicrobial activities. It can, therefore, be concluded that limestone habitat is a promising source for search of novel secondary metabolites.

Keywords: actinobacterial diversity, limestone habitat, Hundung, antibacterial, biocontrol, biosynthetic genes, *Streptomyces*

Introduction

Actinobacteria are major producers of secondary metabolites such as antimicrobial compounds, anticancer molecules and immunosuppressant agents (Takahashi and Omura, 2003). Since the beginning of antibiotic revolution, actinobacteria especially the genus *Streptomyces* have played major roles as antibiotic producers (Bérdy, 2005). However, the discovery of new antibiotics has not been in pace with the increase in demand for new antibiotics. The exhaustion of the usual

Abbreviations: ARDRA, Amplified Ribosomal DNA Restriction Analysis; DGGE, Denaturing Gradient Gel Electrophoresis; GM1, Gauze Medium No. 1; NRPS, Non-Ribosomal Peptide Synthetase; PKS, Polyketide Synthase; RFLP, Restriction Fragment Length Polymorphism; SCNA, Starch Casein Nitrate Agar.

terrestrial sources and the rise of resistant pathogens dictate the search for new antibiotics. To meet urgent clinical needs, screening for secondary metabolites from actinobacteria residing in unexplored habitats is warranted to, possibly, generate novel compounds.

Limestone habitats have high deposition of $CaCO_3$ salts and may be considered a special habitat. Limited studies have been done for systematically exploring such habitats for novel actinobacterial strains (Kim et al., 1998; Groth et al., 1999a, 2001; Jurado et al., 2009; Nakaew et al., 2009; Niyomvong et al., 2012). Some reports are available on actinobacterial diversity in hypogean environments but the studies were focused on biodeterioration and conservation of paleolithic cave art. Actinobacteria implicated in deterioration of art work are considered serious risk factors if environmental changes promote their massive proliferation (Groth et al., 1999b; Portillo et al., 2009). To date, four new genera *Beutenbergia, Fodinibacter, Hoyosella,* and *Knoellia* have been reported from limestone habitats and related limestone ecosystems such as cave biofilms (Groth et al., 1999b, 2002; Jurado et al., 2009; Wang et al., 2009).

Manipur has a huge reserve of good quality limestone suitable for use in the manufacture of cement. The major limestone reserves have been located by Geological Survey of India near Ukhrul district, Manipur. Other limestone deposit sites include areas in Hundung, Phungyar, Meihring, Mova, Khonggoi, Lambui, and Paoyi. This paper reports the actinobacterial diversity profiling of the Hundung limestone deposit sites using ARDRA as the molecular tool for preliminary classification. ARDRA has been originally designed to decrease selection of duplicate clones in molecular analysis. It has been less frequently used in the study of bacterial diversity profiling unlike techniques such as DGGE. The paper also incorporates the results of antimicrobial screening of the Hundung actinobacterial strains.

Materials and Methods

Sampling

Samples for the isolation of actinobacteria were collected from limestone deposit sites, Manipur, India (25.05°N, 94.33°E). The samples included limestones from the quarry site and rice field soil from the adjoining areas. These samples were aseptically packed in polyethylene bags and taken to the laboratory at the earliest possible time. Samples were then kept refrigerated till processing for isolation.

Isolation of Actinobacteria

Two synthetic media, Gauze's Medium No. 1 (GM1, pH 5.3) (Atlas, 1997) and Starch Casein Nitrate Agar (SCNA, pH 8.5) (Kûster and Williams, 1964), were used for the isolation of actinobacteria. Isolation was done using the procedure as described earlier (Nimaichand et al., 2012). The strains were preserved as lyophilized cultures and as glycerol suspension (20% w/v) at −80°C.

Amplified Ribosomal DNA Restriction Analysis (ARDRA; Heyndrickx et al., 1996)

Genomic DNA extraction and amplification of the 16S rRNA gene was done as described by Li et al. (2007). The amplified products were checked and purified by HiPurA™ 96 PCR product purification kit (HiMedia, India). Restriction digestion of the amplified 16S rRNA gene product was done using the enzymes *Hha*I and *Hin*fI (New England Biolabs, UK). The reaction mixture containing 10 μl amplified 16S rRNA gene product, 2 μl NEB buffer 4 (10X), 1 μl restriction enzyme (10 U/μl) and 7 μl deionized water was incubated at 37°C for 2 h and inactivated by heating at 70°C for 10 min. To 20 μl of the restriction digest, 4 μl loading dye (6X) (Promega) was added. Each sample was loaded in a well in agarose gel (3%, w/v) and the gel was run at 100 V for 90 min. In another well, 1 μl DNA ladder (100 bp) (Promega) was loaded to estimate the size of the restriction fragment. The gel was visualized in a gel documentation system (BIORAD Gel Doc EZ Imager). Bands between 100 and 1000 bp were used as reference points and banding patterns were analyzed by scoring the prominent bands. ARDRA band profiles for all the strains were scored with the help of GelBuddy software (Zerr and Henikoff, 2005) for the presence or absence of restriction fragments. A dendrogram was generated using the software package NTSYSpc version 2.02. The phylogenetic relationship was determined according to the method of unweighted pair group method with arithmetic mean (UPGMA; Sneath and Sokal, 1973). Based on the similarity indices (70% and above) in the dendrogram, all the strains were clustered into different phylotypic groups.

Sequencing of 16S rRNA Genes

Sequencing of a randomly-selected representative strain for each phylotypic group was done. The partial 16S rRNA gene sequence of the strain was identified using the EzTaxon-e server database (Kim et al., 2012).The phylogenetic tree of these strains based on neighbor-joining method (Saitou and Nei, 1987) along with related type species were constructed using the software package MEGA version 5.2 (Tamura et al., 2011). Distances were calculated according to Kimura's two-parameter model (Kimura, 1983). To determine the support of each clade, bootstrap analysis was performed with 1000 resamplings (Felsenstein, 1985).

Nucleotide Accession Numbers

The partial 16S rRNA gene sequences were deposited in GenBank with the following accession numbers: KP883248-KP883278.

Antimicrobial Screening

The indicator pathogens used for antimicrobial screening were: *Bacillus subtilis* MTCC 121, *Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* DN1, *Candida albicans* MTCC 227, *Candida vaginitis* CV, *Curvularia oryzae* MTCC 2605, *Fusarium oxysporum* MTCC 287, *Helminthosporum oryzae* MTCC 3717, *Pyricularia oryzae* MTCC 1477, *Rhizoctonia oryzae-sativae* MTCC 2162 and *Rhizoctonia solani* MTCC 4633. All the test pathogens were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India except for DN1 (lab collection) and CV (clinical isolate gifted from the Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad).

Antimicrobial assays against the bacterial and candidal strains were performed by agar well diffusion method (Hugo and Russell, 1983). Antifungal bioassay was done by dual culture technique

(Khamna et al., 2009). The mycelial growth inhibition was calculated using the formula:

$$\text{Percentage growth inhibition} = [(C - T)/C] \times 100\%$$

where, C = Radial growth of the test pathogen in the control plate, and T = Radial growth of the test pathogen in the test plate.

Screening for Biosynthetic Genes

Three sets of degenerate primers were used for amplification of PKS-I, PKS-II and NRPS specific domains (Metsä-Ketelä et al., 1999; González et al., 2005). The primers used are listed in **Table 1**. PCR amplifications were performed in eppendorf mastercycler in a final volume of 25 μl containing 5 μl reaction buffer (with Mg^{2+}) (10x) (Bioline, USA), 0.5 μl of each primer (100 μM) (IDT, USA), 2.0 μl of dNTPs mixture (2.5 mM) (Bioline, USA), 0.15 μl of *Taq* DNA polymerase (2.5 U/μl) (Bioline, USA), 2.5 μl DMSO (HiMedia, India), 11.85 μl deionized water and 2.5 μl of extracted DNA. Amplification was done using the following protocol: one denaturation step of 94°C for 5 min; 30 amplification cycles of 94°C for 1 min, 57°C (for K1F-M6R and A3F-A7R) or 58°C (for KSαF-KSαR) for 1 min, and 72°C for 2 min; and a final extension at 72°C for 5 min. Amplification products were analyzed in agarose gel (1%) using DNA ladder (100 bp) (Promega) as reference.

Results

Description of Sampling Sites

For the actinobacterial isolation, six Hundung samples were collected and used. The sample collection sites included: an abandoned cement factory site (Sample 1), quarry sites (Sample 2–5) and a rice field adjoining the quarry site (Sample 6). The limestones in Hundung, with color ranging from light gray to brown, are of good quality grade which are suitable for production of cement (Bhatt and Bhargava, 2005). The estimated reserve of this Hundung limestone is about 1.88 million tons (Sadangi, 2008; Lisam, 2011). The general characteristics of the samples used for isolation are highlighted in **Table 2**.

Actinobacterial Isolation

Among the isolates obtained, 137 morphologically distinct putative actinobacterial strains were selected for further studies. These included 51 strains from Sample 1, 48 from Sample 2, 10 from Sample 3, 17 from Sample 4, 2 from Sample 5 and 9 from Sample 6. The coding scheme for the actinobacteria from the Hundung samples is shown in **Table 2**.

Diversity Analysis of Hundung Actinobacteria

Upon analysis of the ARDRA-based dendrogram (Supplementary Figure S1), the isolates were classified into 31 phylotypic groups (see Supplementary Table S1 for classification pattern of the Hundung actinobacteria based on ARDRA-dendrogram). The 16S rRNA gene sequence profile for these 31 phylotypic groups is given in **Table 3**. Fifteen of these phylotypes belong to the genus *Streptomyces*. In addition, four phylotypes belong to the genus *Micromonospora*, three each to *Arthrobacter* and *Amycolatopsis* and two to *Kitasatospora*. Remaining phylotypes comprise of the genera *Janibacter, Rhodococcus, Nocardia,* and *Pseudonocardia*. Among the different sites, sample 2 gave the highest diversity (16 phylotypes), followed closely by sample 1 (15 phylotypic groups). Sample 1 yielded the genera *Streptomyces*, *Janibacter*, *Arthrobacter*, *Amycolatopsis,* and *Micromonospora* while sample 2 generated *Streptomyces*, *Rhodococcus*, *Amycolatopsis*, *Micromonospora*, *Arthrobacter*, *Nocardia,* and *Pseudonocardia*. Sample 3 which contained 5 phylogenetic groups yielded the genera *Streptomyces* and *Micromonospora*. Four genera viz., *Streptomyces*, *Janibacter*, *Amycolatopsis,* and *Kitasatospora* were present in sample 4 while sample 6 contained 3 genera: *Streptomyces*, *Amycolatopsis,* and *Arthrobacter*. Sample 5 yielded *Streptomyces* strains only though this may not reflect the true actinobacterial diversity in this sample, as we have selected only 2 strains from the isolates obtained from this sample. Nonetheless, overall analysis of the Hundung sites (1–6) indicated *Streptomyces* to be the dominant genus in these habitats. **Figures 1**, **2** depict the dendrograms based on the 16S rRNA gene sequences of the *Streptomyces* and rare actinobacterial strains obtained from Hundung limestone habitats.

Antimicrobial Activities

Antibacterial and Anticandidal Activities

Antibacterial and anticandidal activity was assessed against a set of indicator organisms. The antibacterial and anticandidal profiles of the Hundung actinobacteria are shown in **Table 4**. Of 137 actinobacterial isolates, 19 exhibited antimicrobial activities against at least one of the test pathogens. In case of *Bacillus subtilis*, 18 strains showed inhibition, of which 5 (MBRL 5, MBRL 10, MBRL 201, MBRL 204, MBRL 251) showed inhibition zones above 17 mm diameter. Against *Escherichia coli*, 5 strains

TABLE 1 | PCR primers for screening the biosynthetic genes.

Primer name	Sequence (5′–3′)	Target gene	Length of target gene fragment (bp)	References
K1F M6R	TSA AGT CSA ACA TCG GBC A CGC AGG TTS CSG TAC CAG TA	PKS-I	1200–1400	González et al., 2005
KSαF KSαR	TSG CST GCT TGG AYG CSA TC TGG AAN CCG CCG AAB CCG CT	PKS-II	600	Metsä-Ketelä et al., 1999
A3F A7R	GCS TAC SYS ATS TAC ACS TCS GG SAS GTC VCC SGT SCG GTA S	NRPS	700–800	González et al., 2005

TABLE 2 | Profile of the Hundung limestone samples and coding scheme for the actinobacterial strains.

Sample	Sampling sites	pH of the sample	Isolation medium	No. of strains	Isolate code
1	Cement factory location	9.26	GM1	29	MBRL 1–MBRL 29
			SCNA	22	MBRL 200–MBRL 221
2	Quarry site 1	8.70	GM1	33	MBRL 30–MBRL 61
			SCNA	15	MBRL 222–MBRL 237
3	Quarry site 2	7.45	GM1	–	–
			SCNA	10	MBRL 238–MBRL 247
4	Quarry site 3	6.50	GM1	14	MBRL 62–MBRL 75
			SCNA	3	MBRL 248–MBRL 250
5	Quarry site 4	7.91	GM1	–	–
			SCNA	2	MBRL 251–MBRL 252
6	Soil sample from the adjoining rice field	4.89	GM1	6	MBRL 76–MBRL 81
			SCNA	3	MBRL 253–MBRL 255
		Total number of strains		137	

TABLE 3 | Sequence analysis profile of representative strain of each phylotypic group.

Phylotypic group	Strain	Accession number	Closest homolog	Pairwise similarity (%)
I	MBRL 216	KP883268	*Streptomyces badius* NRRL B-2567^T	100.00
II	MBRL 221	KP883270	*Janibacter limosus* DSM 11140^T	99.52
III	MBRL 6	KP883248	*Streptomyces phaeofaciens* NBRC 13372^T	99.20
IV	MBRL 77	KP883262	*Streptomyces rubiginosohelvolus* NBRC 12912^T	100.00
V	MBRL 207	KP883264	*Streptomyces roseolus* NBRC 12816^T	99.74
VI	MBRL 241	KP883276	*Streptomyces drozdowiczii* NBRC 101007^T	100.00
VII	MBRL 26	KP883252	*Streptomyces omiyaensis* NBRC 13449^T	99.30
VIII	MBRL 243	KP883278	*Streptomyces roseofulvus* NBRC 13194^T	100.00
IX	MBRL 213	KP883266	*Arthrobacter nitroguajacolicus* G2-1^T	100.00
X	MBRL 219	KP883269	*Arthrobacter subterraneus* CH7^T	98.96
XI	MBRL 46	KP883255	*Rhodococcus canchipurensis* MBRL 353^T	98.45
XII	MBRL 57	KP883256	*Amycolatopsis lurida* DSM 43134^T	97.46
XIII	MBRL 222	KP883271	*Streptomyces scabiei* ATCC 49173^T	100.00
XIV	MBRL 76	KP883261	*Amycolatopsis thailandensis* CMU-PLA07^T	98.95
XV	MBRL 32	KP883253	*Micromonospora kangleipakensis* MBRL 34^T	98.42
XVI	MBRL 64	KP883259	*Kitasatospora phosalacinea* JCM 3340^T	99.35
XVII	MBRL 70	KP883260	*Kitasatospora cheerisanensis* KCTC 2395^T	99.40
XVIII	MBRL 210	KP883265	*Micromonospora coxensis* 2-30-b/28^T	99.27
XIX	MBRL 240	KP883275	*Micromonospora schwarzwaldensis* HKI0641^T	99.48
XX	MBRL 8	KP883249	*Streptomyces violacerectus* NBRC 13102^T	100.00
XXI	MBRL 18	KP883251	*Streptomyces exfoliatus* NBRC 13191^T	100.00
XXII	MBRL 14	KP883250	*Streptomyces fragilis* NRRL 2424^T	99.44
XXIII	MBRL 63	KP883258	*Amycolatopsis keratiniphila* subsp. *keratiniphila* DSM 44409^T	99.92
XXIV	MBRL 34	KP883254	*Micromonospora coerulea* DSM 43143^T	98.52
XXV	MBRL 242	KP883277	*Streptomyces griseorubiginosus* NBRC 13047^T	100.00
XXVI	MBRL 226	KP883272	*Streptomyces shaanxiensis* CCNWHQ 0031^T	99.52
XXVII	MBRL 215	KP883267	*Streptomyces rubiginosohelvolus* NBRC 12912^T	100.00
XXVIII	MBRL 79	KP883263	*Arthrobacter defluvii* 4C1-a^T	100.00
XXIX	MBRL 230	KP883273	*Nocardia asteroides* NBRC 15531^T	98.64
XXX	MBRL 235	KP883274	*Pseudonocardia carboxydivorans* Y8^T	100.00
XXXI	MBRL 59	KP883257	*Streptomyces olivaceoviridis* NBRC 13066^T	99.49

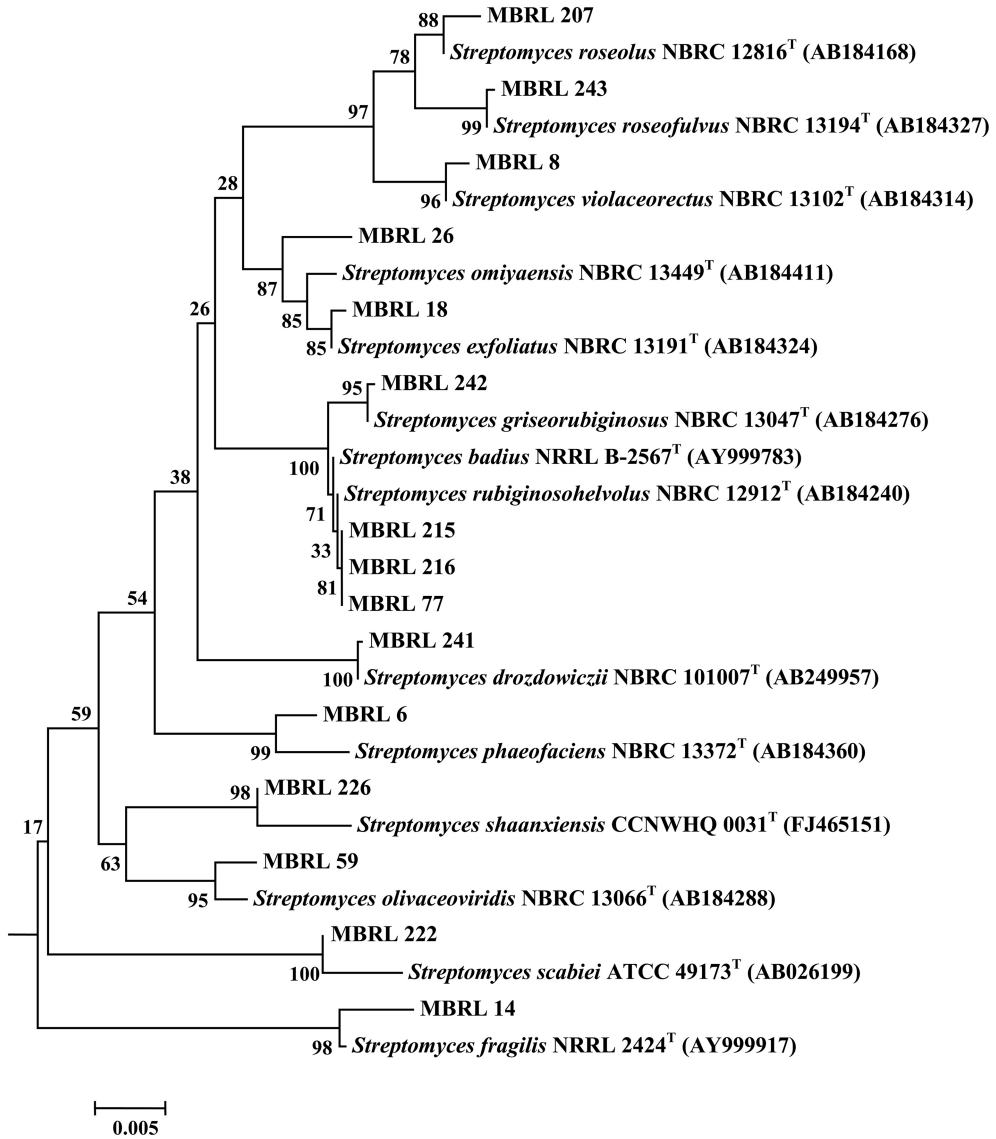

FIGURE 1 | Dendrogram of the representative *Streptomyces* strains based on the 16S rRNA gene sequences. Numbers at nodes are levels of bootstrap support (%) for branch points (1000 resamplings). Bar, 0.002 substitutions per nucleotide position.

exhibited antagonistic activities of which 2 (MBRL 5, MBRL 10) showed inhibition zone sizes above 17 mm diameter. No strain had activity against *Pseudomonas aeruginosa*. Against *Candida albicans*, 5 strains showed inhibitory activity and 4 against *Candida vaginitis* (See Supplementary Table S2 for complete antibacterial and anticandidal profile).

Biocontrol Activities

Several actinobacterial strains exhibited biocontrol potential against rice fungal pathogens. Forty five actinobacterial strains from Hundung limestone habitat showed biocontrol activities against at least one of the rice fungal pathogens. Frequencies of biocontrol activities against the indicator fungal pathogens were as follows: *Helminthosporum oryzae* MTCC 3717 (22.6%), *Rhizoctonia solani* MTCC 4633 (18.2%), *Rhizoctonia oryzae-sativae* MTCC 2162 (16.8%), *Pyricularia oryzae* MTCC 1477 (14.6%), *Curvularia oryzae* MTCC 2605 (10.9%) and *Fusarium oxysporum* MTCC 287 (9.5%) respectively. **Table 4** summarizes the biocontrol profile of the Hundung actinobacteria (See Supplementary Table S3 for complete biocontrol activity profile).

Screening for Biosynthetic Genes

It is well known that many bioactive metabolites in actinobacteria are produced by PKS and NRPS gene clusters. Screening for genes associated with secondary metabolism is helpful in evaluating the biosynthetic potential of actinobacteria. Of 137 Hundung actinobacterial strains, 118 possessed at least one of the three biosynthetic gene clusters. A total of 43 strains had a single type of biosynthetic gene cluster (PKS-I, 5 strains; PKS-II, 27; NRPS,

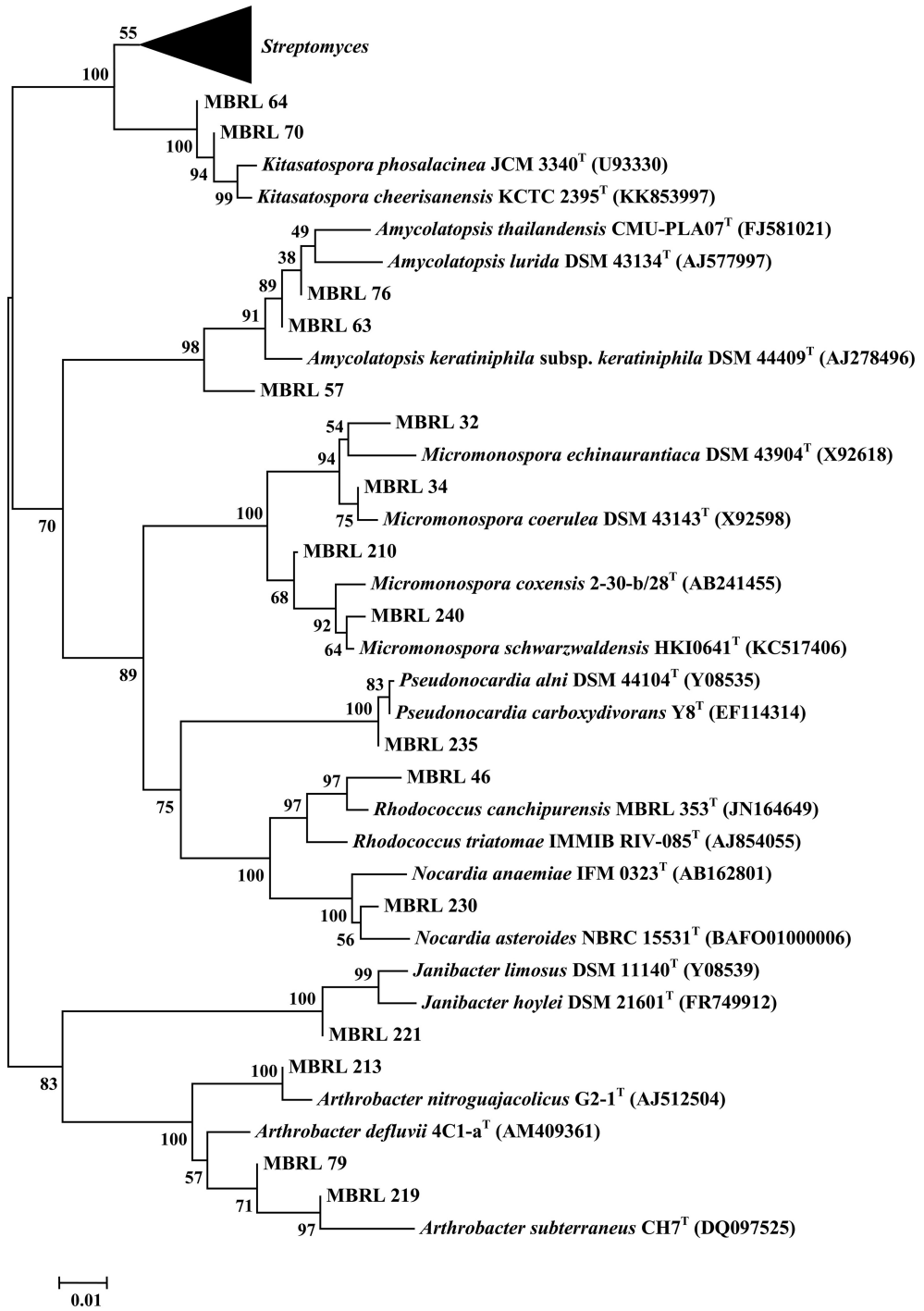

FIGURE 2 | Dendrogram of the representative rare actinobacterial strains based on the 16S rRNA gene sequences. Numbers at nodes are levels of bootstrap support (%) for branch points (1000 resamplings). Bar, 0.01 substitutions per nucleotide position.

11). The remaining 75 strains had two or more of the biosynthetic gene clusters: 9 strains possessed both PKS-I and PKS-II; 8 had both PKS-I and NRPS; 36 had both PKS-II and NRPS while 22 strains had all the three biosynthetic gene clusters. **Table 4** shows the amplication profile for biosynthetic genes in the Hundung actinobacteria (see Supplementary Table S4 for complete PCR profile of biosynthetic genes).

Discussion

Diversity profiling focused on actinobacteria in limestone habitats started when Kim et al. (1998) reported the diversity of actinobacteria antagonistic to phytopathogenic fungi in caves of Korea. They reported the presence of *Streptomyces, Micromonospora*, Nocardioform actinobacteria, *Actinomyces*,

TABLE 4 | Antimicrobial, biocontrol and biosynthetic genes profile of the Hundung actinobacteria.

Phylotypic group	Total number of isolates	Genus classified	Test pathogens											Biosynthetic gene		
			MTCC 121	MTCC 739	DN1	MTCC 227	CV	MTCC 2605	MTCC 287	MTCC 3717	MTCC 1477	MTCC 2162	MTCC 4633	PKS-I	PKS-II	NRPS
I	29	*Streptomyces*	4	4	0	4	3	5	7	12	8	10	10	4	24	23
II	2	*Janibacter*	1	0	0	0	0	1	0	1	1	1	1	0	1	1
III	27	*Streptomyces*	4	0	0	1	1	3	2	4	5	5	6	14	24	19
IV	1	*Streptomyces*	1	0	0	0	0	0	0	1	1	0	0	0	1	1
V	2	*Streptomyces*	0	0	0	0	0	0	0	0	0	0	0	2	2	2
VI	4	*Streptomyces*	0	0	0	0	0	0	0	1	0	0	0	1	3	2
VII	1	*Streptomyces*	0	0	0	0	0	0	0	1	0	0	0	1	1	1
VIII	1	*Streptomyces*	0	0	0	0	0	0	0	0	0	0	0	1	2	0
IX	1	*Arthrobacter*	0	0	0	0	0	0	0	0	0	0	0	1	1	1
X	1	*Arthrobacter*	0	0	0	0	0	0	0	0	0	0	0	0	0	0
XI	1	*Rhdococcus*	0	0	0	0	0	1	0	0	0	0	0	0	0	0
XII	17	*Amycolatopsis*	6	0	0	0	0	2	2	4	3	5	5	9	9	16
XIII	1	*Streptomyces*	0	0	0	0	0	0	0	0	0	0	0	0	0	1
XIV	1	*Amycolatopsis*	0	0	0	0	0	0	0	0	0	0	0	0	0	1
XV	2	*Micromonospora*	0	0	0	0	0	0	0	0	0	0	0	0	1	0
XVI	1	*Kitasatospora*	0	0	0	0	0	0	0	0	0	0	0	0	1	0
XVII	2	*Kitasatospora*	1	0	0	0	0	0	0	0	0	0	0	0	2	2
XVIII	1	*Micromonospora*	0	0	0	0	0	0	0	0	0	0	0	0	0	0
XIX	1	*Micromonospora*	0	0	0	0	0	0	0	0	0	0	0	2	0	0
XX	1	*Streptomyces*	0	0	0	0	0	1	1	1	0	0	0	1	0	0
XXI	2	*Streptomyces*	0	0	0	0	0	1	0	1	0	0	1	1	1	1
XXII	7	*Streptomyces*	0	0	0	0	0	0	0	2	0	1	1	3	4	1
XXIII	1	*Amycolatopsis*	0	0	0	0	0	1	0	1	1	1	1	1	1	1
XXIV	1	*Micromonospora*	0	0	0	0	0	0	0	0	0	0	0	0	1	0
XXV	18	*Streptomyces*	1	1	0	0	0	0	1	2	0	0	0	3	11	3
XXVI	1	*Streptomyces*	0	0	0	0	0	0	0	0	0	0	0	0	1	0
XXVII	2	*Streptomyces*	0	0	0	0	0	0	0	0	0	0	0	1	1	1
XXVIII	2	*Arthrobacter*	0	0	0	0	0	0	0	0	0	0	0	0	3	1
XXIX	1	*Nocardia*	0	0	0	0	0	0	0	0	0	0	0	0	0	0
XXX	2	*Pseudonocardia*	0	0	0	0	0	0	0	0	1	0	0	0	4	0
XXXI	3	*Streptomyces*	0	0	0	0	0	0	0	0	0	0	0	2	2	2

MTCC 121, Bacillus subtilis; MTCC 739, Escherichia coli; DN1, Pseudomonas aeruginosa; MTCC 227, Candida albicans; CV, Candida vaginitis; MTCC 2605, Curvularia oryzae; MTCC 287, Fusarium oxysporum; MTCC 3717, Helminthosporum oryzae; MTCC 1477, Pyricularia oryzae; MTCC 2162, Rhizoctonia oryzae-sativae; MTCC 4633, Rhizoctonia solani.

Dactylosporangium, *Saccharomonospora,* and *Streptosporangium* in these habitats. Groth et al. (1999a) studied the actinobacterial diversity in Karstic caves (Altamira and Tito Bustillo) located in northern Spain and reported members of the genera *Streptomyces*, *Nocardia*, *Rhodococcus*, *Nocardioides*, *Amycolatopsis*, *Saccharothrix*, *Brevibacterium*, *Microbacterium,* and coccocid actinobacteria of the family *Micrococcaceae*. Groth et al. (2002) reported the isolation of a new genus *Knoellia* from limestone caves. To the repertoire of the actinobacterial diversity in caves, Nakaew et al. (2009) added the genera *Nonomuraea*, *Actinocorallia*, *Catellatospora*, *Microbispora,* and *Sprillospora.* Jurado et al. (2009) reported the new genus *Hoyosella* from cave biofilms in Spain. Niyomvong et al. (2012) found the presence of the genera *Streptomyces*, *Actinomadura*, *Actinoplanes*, *Gordonia*, *Microbispora*, *Micromonospora*, *Nocardia*, *Nonomuraea,* and *Saccharopolyspora* in the tropical limestone caves of Khao No-Khao Kaeo karst in Thailand.

Considering the rich diversity of actinobacteria in limestone habitats, the present study on actinobacterial diversity of limestone deposit sites in Hundung, Manipur, India, has special significance. As per our findings, the genus *Streptomyces* is predominantly present in these limestone habitats. This is also indicated by the presence of phylotypic group III (represented by the genus *Streptomyces*) in all the six samples used for actinobacterial isolation. Apart from *Streptomyces*, we also observed the presence of rare actinobacteria *Micromonospora*, *Arthrobacter*, *Amycolatopsis*, *Kitasatospora*, *Janibacter*, *Rhodococcus*, *Nocardia,* and *Pseudonocardia*. This work forms the first report of the isolation of *Janibacter* and *Kitasatospora* from limestone and related habitats.

ARDRA is preferable to other molecular genome typing methods for preliminary phylogenetic grouping as it is faster and more cost effective than the other approaches. Moreover, as ARDRA is based on the presence of restriction sites within the ribosomal DNA, duplicate strains will most likely have the same restriction pattern. The use of ARDRA in this study, therefore, helped reduce the number of duplicate strains among the isolates from the community, indicating the true diversity of the community even when the sample size is small.

In the course of a screening program for novel antibiotics from strains obtained from Grotta dei Cervi, a cave in Italy, Herold et al. (2004) identified a bioactive complex, Cervimycins A–D, from a strain of *Streptomyces tendae.* Cervimycins are potent antibiotics against multidrug resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE) strains (Herold et al., 2005). Quadri and Agsar (2012) have investigated antimicrobial activities of actinobacteria of limestone quarries located at Deccan traps, India. Of 63 actinobacteria from this habitat, six strains (belonging to the genera *Streptomyces*, *Micromonospora*, *Nonomuraea*, *Kribbella*, *Lechevalieria,* and *Saccharothrix*) showed potent antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi,* and *Candida albicans*. Carlsohn (2011) found novel strains of *Amycolatopsis saalfeldensis*, *Kribbella aluminosa,* and *Streptomyces* strains from a mine in Germany and they were strongly inhibitory to *Stapthylococcus aureus*, *Mycobacterium smegmatis,* and *Candida albicans*, and moderately antagonistic to *Escherichia coli*. Rule and Cheeptham (2013) reported some *Streptomyces* strains from a volcanic cave in Canada (Cheeptham et al., 2013) as antagonistic to *Micrococcus luteus*, MRSA, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans.*

In the present study, 5 Hundung actinobacteria were found to be potent antimicrobial strains. Of these, 2 *Streptomyces* species MBRL 201 and MBRL 251 showed strong antimicrobial activity against *Bacillus subtilis*, but less bioactivity against *Escherichia coli, Candida albicans,* and *Candida vaginitis*. Besides these two, two other Hundung *Streptomyces* species (MBRL 5 and MBRL 10) also exhibited promising antimicrobial activities. MBRL 204, another Hundung *Streptomyces* strain, exhibited relatively lesser antimicrobial activity compared to the other 4 isolates (MBRL 5, MBRL 10, MBRL 201 and MBRL 251).

Soil actinobacteria have been proposed as promising biocontrol agents (Goodfellow and Williams, 1983; Chater, 1993). However, actinobacteria from limestone habitats have not been investigated for their biocontrol potential. Quadri and Agsar (2012) have reported that only 9.5% of the strains isolated from limestone habitats have antifungal activities against *Aspergillus fumigates*, *Aspergillus niger,* and *Fusarium solani*. In the current investigation, many strains belonging to the genera *Streptomyces* and *Amycolatopsis* (e.g., Phylotypic group I, III and XII) were found to have biocontrol activities against selected rice fungal pathogens *Curvularia oryzae*, *Fusarium oxysporum*, *Helminsthosporum oryzae*, *Pyricularia oryzae*, *Rhizoctonia oryzae-sativae,* and *Rhizoctonia solani.* Rare actinobacteria belonging to genera *Janibacter* and *Pseudonocardia* obtained from Hundung limestone habitats also exhibited significant biocontrol potential against some fungal pathogens.

The biosynthetic gene clusters play a crucial role in microbial natural product biosynthesis. The biosynthesis of cervimycin complex (metabolites reported from limestone related habitats) involved the type II PKS system. Other antibacterial metabolites such as Ravidomycins from *Streptomyces rabidus* are biosynthesized by type II PKS system (Kharel et al., 2010). Hence, it is imperative to screen for the presence of these biosynthetic gene clusters in the actinobacterial isolates. In our studies, 118 of the 137 actinobacterial isolates were found to have at least one of the three biosynthetic gene clusters. Of these 118 actinobacteria possessed the biosynthetic gene clusters, 47 exhibited antimicrobial and/or biocontrol activities indicating that less than 50% of the strains possessing biosynthetic gene clusters were bioactive under the screening condition. The findings of the various experiments indicate that 86% of the strains isolated from Hundung limestone rocks possessed biosynthetic gene clusters of which 40% exhibited antimicrobial activities. It can, therefore, be concluded that limestone habitats is a promising source for search of novel secondary metabolites.

Author Contributions

SN planned, conducted the experiments, analyzed the data, and prepared the manuscript, AD performed, analyzed and interpreted the ARDRA data, KT performed the biocontrol assay, DN and WL supervised the experiments.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00413/abstract

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Ubiquity, diversity and physiological characteristics of *Geodermatophilaceae* in Shapotou National Desert Ecological Reserve

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The goal of this study was to gain insight into the diversity of culturable actinobacteria in desert soil crusts and to determine the physiological characteristics of the predominant actinobacterial group in these crusts. Culture-dependent method was employed to obtain actinobacterial strains from desert soil samples collected from Shapotou National Desert Ecological Reserve (NDER) located in Tengger Desert, China. A total of 376 actinobacterial strains were isolated and 16S rRNA gene sequences analysis indicated that these isolates belonged to 29 genera within 18 families, among which the members of the family *Geodermatophilaceae* were predominant. The combination of 16S rRNA gene information and the phenotypic data allowed these newly-isolated *Geodermatophilaceae* members to be classified into 33 "species clusters," 11 of which represented hitherto unrecognized species. Fermentation broths from 19.7% of the isolated strains showed activity in at least one of the six screens for antibiotic activity. These isolates exhibited bio-diversity in enzymatic characteristics and carbon utilization profiles. The physiological characteristics of the isolates from different types of crusts or bare sand samples were specific to their respective micro-ecological environments. Our study revealed that members of the family *Geodermatophilaceae* were ubiquitous, abundant, and diverse in Shapotou NDER, and these strains may represent a new major group of potential functional actinobacteria in desert soil.

Keywords: ***Geodermatophilaceae*, 16S rRNA, diversity, physiological characteristics, desert**

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Introduction

It has become increasingly clear that the overuse of antibiotics and the subsequent rise in antibiotic-resistant pathogens will force us to search for new antibiotics to meet urgent clinical needs (Talbot et al., 2006). Previous studies have indicated that environments considered to be extreme habitats are rich sources of novel actinobacteria (Subramani and Aalbersberg, 2013). It has been hypothesized that unusual climate conditions and ecological factors may endow the organisms in such habitats with the unique capacity to produce novel bioactive compounds (Bull et al., 2005; Okoro et al., 2008).

The Shapotou desert region (latitude 36°39′-37°41′N, elevation 104°25′-105°40′E) is recognized as the first "National Desert Ecological Reserve" (NDER) in China. This NDER is renowned worldwide as a teaching and scientific research base for studying controlled desertification. It is

located on the southeast edge of the Tengger Desert, south of the Yellow River, in the northwest part of China. This region is at an altitude of 1300–1700 m, has an annual average precipitation of 186.2 mm, an annual mean temperature of 9.7°C, and an annual average wind speed of 2.8 m/s with a typical temperate desert climate.

In desert regions, microbiotic crusts play a significant role in controlling desertification by providing surface stability. Microbiotic crusts are important in stabilization of the sandy surface, soil formation, and in carbon and nitrogen assimilation (Evans and Johansen, 1999). Microbiotic crusts in Shapotou NDER are generally categorized into the following three typical types: Cyanobacteria-dominated crusts (CC), Moss-dominated crusts (MC), and Lichen-dominated crusts (LC). Samples were therefore, collected from these three types of crusts and bare sands. Culture-dependent method was employed to evaluate the diversity of culturable actinobacteria in Shapotou NDER, and to explore the potential functional actinobacterial resources from this extreme environment.

Actinobacterial strains were discovered and identified from the three types of soil crusts and bare sands samples from the Shapotou NDER. We found that the members of the family *Geodermatophilaceae* were ubiquitous in the different types of crusts, as well as the bare sands samples. Based on the physiological characteristics of these diverse *Geodermatophilaceae* members, we characterized the influence of micro-ecological niche environments on the phenotypic characteristics of these isolates.

Materials and Methods

Sample Collection

A total of 50 samples for isolation of actinobacteria were collected from four different micro-ecological environments in Shapotou NDER (latitude 36°39′-37°41′N, elevation 104°25′-105°40′E). The detailed information regarding the sample number, type of sample, and specific collection location of the 50 samples is displayed in **Table 1**. All the samples were placed in sterilized envelopes following collection and taken to the laboratory within 1 week of collection. All samples were immediately processed for isolation after arriving at the laboratory.

Actinobacteria Isolation and Maintenance

The following four types of media were prepared to isolate the actionbacterial strains. The main components of the media were as follows: M1 (g l^{-1}): glucose 10, yeast extract 1, beef extract 1, casein (enzymatic hydrolysate) 2, agar 15; M2 (g l^{-1}): 1/5 strength R2A (Difco); M3 (g l^{-1}): cellobiose 2, yeast extract 5, $CaCO_3$2, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.5, agar 15; M4 (g l^{-1}): sodium propionate 2, NH_4NO_3 0.1, KCl 0.1, $MgSO_4 \cdot 7H_2O$ 0.05, agar 15. The isolation media were adjusted to pH 7.2–7.5 using 1 M NaOH and/or 1 M HCl. In addition, betaine (0.125% w/v), sodium pyruvate (0.125% w/v), compound trace salts solution (0.1% v/v), and compound vitamins (0.1% w/v) were added to the media to facilitate the isolation of strains that are difficult to culture (Yue et al., 2006). Aztreonam (25 mg l^{-1}) and potassium dichromate (50 mg l^{-1}) were also added to the media to prevent or stymie the growth of Gram-stain negative bacteria and fungi that may be present.

The procedure for actinobacteria isolation was carried out as described in Zhang et al. (2010). Briefly, 0.3 ml of 10^{-3} soil suspension was spread on each isolation plate and the plates were incubated at 28°C for 3 weeks. Single colonies were transferred to freshly prepared PYG plates [(g l^{-1}) (peptone 3, yeast extract 5, glycerol 10, glycine betaine 1.25, sodium pyruvate 1.25, agar 15, pH 7.5), supplemented with compound trace salts solution ($FeSO_4 \cdot 7\ H_2O$ 0.2 g, $MnCl \cdot 2\ H_2O$ 0.1 g, $ZnSO_4 \cdot 7\ H_2O$ 0.1 g, 0.1% v/v) and compound vitamins (vitamin B1 1 mg, vitamin B2 1 mg, vitamin B3 1 mg, vitamin B6 1 mg, phenylalanine 1 mg, biotin 1 mg, alanine 0.3 mg, 0.1% w/v)] and subsequently purified. The pure cultures were maintained on PYG slants at 4°C and also as glycerol suspensions (20%, v/v) at −80°C.

Identification of *Geodermatophilaceae*

Purified isolates were transferred to PYG medium and International Streptomyces Project (ISP) medium 2 for observation of the morphological characteristics. Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were performed as described in the methods section of Xu et al. (2003). The purified PCR products were sequenced with an ABI PRISM automatic sequencer. The sequences obtained were compared with available 16S rRNA gene sequences from GenBank using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim et al., 2012). The server was used to determine an approximate phylogenetic affiliation of each strain. Multiple alignments with sequences of the related strains and calculations of levels of sequence similarities were carried out using MEGA version 5 (Tamura et al., 2011). A phylogenetic tree was constructed using the neighbor-joining method described in Saitou and Nei (1987). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

Bioactivity Screening

Antimicrobial activities of the isolated strains were investigated by using media containing *Enterococcus faecalis* HH22, *Klebsiella pneumonia* ATCC 700603, *Mycobacterium smegmatis* CPCC240556, *Sporobolomyces salmonicolor* SS04, and *Xanthomonas campestris* pv. *oryzae* PXO99A, respectively, all at a concentration of 10^8 colony forming units (CFU) per ml. The anti-viral activity against the human immunodeficiency virus (HIV) was investigated using the procedure described in Yang et al. (2013). Results were considered positive if the HIV inhibition ratio was above 90% and at least 80% of the cells survived. This assay was performed under conditions where the sample concentration was 1% (v/v).

Physiological Characteristics Determination

From the 70 newly-isolated *Geodermatophilaceae* members, the physiological characteristics were determined for 34 representative strains. Carbohydrate utilization tests were carried out using API 50 CH test kits (bioMérieux) and Biolog GEN III MicroPlates (Biolog Inc.) according to the manufacturer's instructions. Enzymatic activities were determined using API

TABLE 1 | Samples collected in the Shapotou region.

Sample number	Sample type	Site information	Sample number	Sample type	Site information	Sample number	Sample type	Site information
BCL12001	BS	37°25′38.72″N 104°35′8.13″E 1701 mH	MSY12018	MS	37°27′38.50″N 104°59′58.79″E 1329 mH	BSY12035	BS	37°27′38.66″N 104°59′58.52″E 1329 mH
MCL12002	MS	37°25′37.85″N 104°35′8.26″E 1701 mH	MSY12019	MS	37°27′38.36″N 104°59′59.10″E 1329 mH	CSY12036	CC	37°27′38.12″N 104°59′58.79″E 1329 mH
BCL12003	BS	37°25′38.89″N 104°35′9.01″E 1701 mH	MSY12020	MS	37°27′38.18″N 104°59′59.54″E 1329 mH	CSY12037	CC	37°27′37.81″N 104°59′59.47″E 1329 mH
CCL12004	CC	37°25′38.65″N 104°35′7.67″E 1701 mH	MSY12021	MS	37°27′38.04″N 104°59′59.84″E 1329 mH	CSY12038	CC	37°27′37.76″N 104°59′59.72″E 1329 mH
MCL12005	MS	37°25′39.09″N 104°35′7.97″E 1701 mH	MSY12022	MS	37°27′37.86″N 105°00′0.17″E 1329 mH	CSY12039	CC	37°27′37.60″N 104°59′59.72″E 1329 mH
CYW12006	CC	37°25′30.86″N 104°43′52.00″E 1698 mH	MSY12023	MS	37°27′37.89″N 105°00′0.51″E 1329 mH	CSY12040	CC	37°27′37.40″N 104°59′59.86″E 1329 mH
LYW12007	LC	37°25′30.76″N 104°43′53.52″E 1698 mH	MSY12024	MS	37°27′38.09″N 105°00′0.28″E 1329 mH	CSY12041	CC	37°27′37.45″N 105°00′0.13″E 1329 mH
LYW12008	LC	37°25′29.83″N 104°43′53.65″E 1698 mH	MSY12025	MS	37°27′38.29″N 105°00′0.04″E 1329 mH	BSY12042	BS	37°27′37.29″N 104°59′59.86″E 1329 mH
BYW12009	BS	37°25′30.14″N 104°43′51.19″E 1698 mH	BSY12026	BS	37°27′38.63″N 104°59′59.68″E 1329 mH	BSY12043	BS	37°27′37.32″N 104°59′59.59″E 1329 mH
MYW12010	MS	37°25′31.17″N 104°43′51.13″E 1698 mH	CSY12027	CC	37°27′38.87″N 104°59′59.44″E 1329 mH	CSY12044	CC	37°27′37.34″N 104°59′59.18″E 1329 mH
BYW12011	BS	37°25′31.35″N 104°43′52.09″E 1698 mH	MSY12028	MS	37°27′39.09″N 104°59′59.24″E 1329 mH	BSY12045	BS	37°27′37.36″N 104°59′58.79″E 1329 mH
BHW12012	BS	37°27′3.06″N 104°47′41.19″E 1619 mH	MSY12029	MS	37°27′39.16″N 104°59′59.63″E 1329 mH	CSY12046	CC	37°27′37.55″N 104°59′58.97″E 1329 mH
BHW12013	BS	37°27′3.45″N 104°47′42.21″E 1619 mH	CSY12030	CC	37°27′39.03″N 104°59′59.89″E 1329 mH	CSY12047	CC	37°27′37.76″N 104°59′58.65″E 1329 mH
MHW12014	MS	37°27′3.67″N 104°47′40.92″E 1619 mH	MSY12031	MS	37°27′38.83″N 105°00′0.15″E 1329 mH	BSY12048	BS	37°27′37.96″N 104°59′58.32″E 1329 mH
LHW12015	LC	37°27′4.05″N 104°47′41.63″E 1619 mH	BSY12032	BS	37°27′38.54″N 105°00′0.61″E 1329 mH	BSY12049	BS	37°27′37.90″N 104°59′58.03″E 1329 mH
LHW12016	LC	37°27′3.24″N 104°47′41.36″E 1619 mH	MSY12033	MS	37°27′38.38″N 105°00′0.81″E 1329 mH	CSY12050	CC	37°27′37.99″N 104°59′59.11″E 1329 mH
MSY12017	MS	37°27′38.52″N 104°59′59.89″E 1329 mH	BSY12034	BS	37°27′38.17″N 105°00′0.06″E 1329 mH			

CC, Cyanobacteria-dominated soil crusts; MC, Moss-dominated soil crusts; LC, Lichen-dominated soil crusts; BS, Bare sand.

ZYM test kits (bioMérieux) according to the manufacturer's instructions. Bacterial growth was tested at 4, 10, 20, 28, 30, 32, 37, 40, and 45°C on PYG agar medium incubated for 15–30 days. The ability of the strains to grow at different concentrations of NaCl was tested at the following concentrations: 0, 1, 3, and 5–20%, w/v, with 5–20% being tested at intervals of 1.0%. Growth ability in this experiment was determined according to the protocol described by Wang et al. (2001). The pH tolerance was assayed in PYG medium at pH values from 5.0 to 11.0 at intervals of 0.5 pH units. Other physiological and biochemical tests were performed according to the methods established by Williams et al. (1983) and Kämpfer et al. (1991).

The sensitivity of the bacteria to 33 different antibiotics was tested on PYG agar using the following concentrations: amikacin (1500 μg/ml), ampicillin (510 μg/ml), aztreonam (1500 μg/ml), cephalothin (1500 μg/ml), cefazolin (1500 μg/ml), cefepime (1500 μg/ml), cefoperazone (3700 μg/ml), cefotaxime (1500 μg/ml), ceftazidime (1500 μg/ml), ceftriaxone (1500 μg/ml), cefuroxime (1500 μg/ml), chloromycetin (1500 μg/ml), ciprofloxacin (250 μg/ml), clarithromycin (750 μg/ml), clindamycin (100 μg/ml), erythromycin (765 μg/ml), gentamycin (515 μg/ml), gentamycin (6000 μg/ml), levofloxacin (250 μg/ml), macrodantin (15,000 μg/ml), minocycline (1500 μg/ml), norfloxacin (500 μg/ml), ofloxacin (250 μg/ml), oxacillin (50 μg/ml), penicillin G (500 μg/ml), piperacillin (5000 μg/ml), streptomycin (540 μg/ml), streptomycin (15,000 μg/ml), sulfamethoxazole/trimethoprim (1187.5 μg/ml and 62.5 μg/ml), sulfanilamide (15,000 μg/ml), tetracycline (1500 μg/ml), tobramycin (500 μg/ml), and vancomycin (1500 μg/ml).

Numerical comparative analysis of the physiological and biochemical characteristics tested was performed using the NTSYSpc package (version 2.2 for Windows; Exeter Software) (Rohlf, 2000). A binary 0/1 matrix was created based on the positive or negative respective values of 173 physiological characteristics, some of which are described above.

Results

Isolation of Actinobacteria

A total of 470 purified isolates were obtained in the present study. The 16S rRNA gene sequences revealed that 376 actinobacterial strains were isolated from the 50 samples. These isolates belonged to 18 families and 29 genera, among which the members of *Geodermatophilaceae* were predominant, including 70 strains of three genera. (**Supplementary Figure S1**). Among the four types of isolation media, M2 resulted in the most successful isolation of actinobacterial strains. Specifically, 35% of the actinobacterial strains were obtained from M2. While 29, 26, and 10% of the actinobacterial isolates were purified from M1, M4, and M3, respectively (**Supplementary Figure S2**).

The actinobacterial strains, measured in number of isolates per sample, accounted for 35, 30, 24, and 11%, from cyanobacteria-dominated soil crusts, lichen-dominated soil crusts, moss-dominated soil crusts, and bare sands respectively. At the genus level, the diversity of the isolates from the lichen-dominated soil crusts was higher (33%) than cyanobacteria-dominated soil crusts (30.8%) moss-dominated soil crusts (23.6%), and bare sands (12.6%).

Diversity of *Geodermatophilaceae*

In total, 70 *Geodermatophilaceae* strains, including 34 *Blastococcus* spp., 11 *Geodermatophilus* spp., and 25 *Modestobacter* spp. were collected from the 50 samples (**Table 2**). In the phylogenetic dendrogram based on 16S rRNA gene sequences analysis of the isolates and the type stains of 25 validly described species in the family *Geodermatophilaceae*, these 70 newly-isolated members of the family *Geodermatophilaceae* fell into 23 "species clusters," with the 16S rRNA gene sequence similarity below 98.65% to the closest homolog as the threshold for differentiating two species (Kim et al., 2014) (**Figure 1**). As indicated in the phylogenetic dendrogram, six *Modestobacter* "species clusters," two *Blastococcus* "species clusters" and three *Geodermatophilus* "species clusters" may represent hitherto unrecognized species.

Bioactivities of Newly-isolated Strains

Among the 70 *Geodermatophilaceae* strains, 3 exhibited activity against Enterococcus faecalis (4.3%), 3 against Klebsiella pneumonia (4.3%), 4 against Mycobacterium smegmatis (5.7%), 6 against Sporobolomyces salmonicolor (8.6%), 2 against Xanthomonas campestris pv. oryzae PXO99A (2.9%), and 6 against HIV (8.6%), respectively. Additionally, 9 of the isolates exhibited activities in more than one of these screening models. In total, 19.7% of the newly-isolated *Geodermatophilaceae* strains showed activity in at least one of the six antibiotic screens.

Physiological Characteristics of Newly-isolated Strains

The strains assayed for physiological characteristics were similar in their physiological characteristic profiles in the following capacity: more than 60% of the strains tested could utilize dextrin, D-fructose, D-fructose-6-PO_4, D-galactose, α-D-glucose, glucuronamide, α-keto-glutaric acid, D-malic acid, D-maltose, D-mannose, D-trehalose, D-turanose and sucrose as their sole carbon source, and 91% of the strains tested assimilated esculin ferric citrate and potassium 5-ketogluconate and produced acid. In the API ZYM assay, none of the strains tested was positive for β-fucosidase, N-acetyl-β-glucosaminidase, or α-mannosidase. Twenty-nine strains showed the enzymatic activities of acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, lipase (C14), and valine arylamidase. Most of the tested strains were resistant to aztreonam (1500 μg/ml), sulfanilamide (15,000 μg/ml), and sulfamethoxazole/trimethoprim (1187.5 μg/ml and 62.5 μg/ml). The phylogenetic dendrogram based on 173 physiological characteristics of the tested strains showed that the micro-ecological environment from which the strains were isolated was an important factor correlating with the physiological characteristic profiles of the isolates. The strains exhibited characteristics specific to the micro-ecological environment where they were found (**Figure 2**).

TABLE 2 | Newly-isolated *Geodermatophilaceae* members.

Strain number	Accession number	The closest homolog	16S rRNA gene similarity with the closest homolog (%)	Sample number	Sample type	Geographical location	Isolationmedium
I12A-02628	KR184357	*Blastococcus aggregatus* ATCC 25902(T)	98.1	MSY12029	MC	G4	M1
I12A-02647	KR184375	*Blastococcus aggregatus* ATCC 25902(T)	98.3	MYW12010	MC	G2	M1
I12A-02683	KR184408	*Blastococcus aggregatus* ATCC 25902(T)	98.4	CSY12047	CC	G4	M3
I12A-02696	KR184418	*Blastococcus aggregatus* ATCC 25902(T)	98.5	CSY12044	CC	G4	M2
I12A-02698	KR184420	*Blastococcus aggregatus* ATCC 25902(T)	99.4	CSY12040	CC	G4	M3
I12A-02663	KR184391	*Blastococcus aggregatus* ATCC 25902(T)	99.4	MSY12019	MC	G4	M3
I12A-02636	KR184365	*Blastococcus aggregatus* ATCC 25902(T)	99.4	MSY12028	MC	G4	M1
I12A-02691	KR184415	*Blastococcus aggregatus* ATCC 25902(T)	99.5	CSY12030	CC	G4	M3
I12A-02992	KR184448	*Blastococcus aggregatus* ATCC 25902(T)	99.5	CSY12040	CC	G4	M1
I12A-02653	KR184381	*Blastococcus aggregatus* ATCC 25902(T)	99.7	BYW12011	BS	G2	M2
I12A-02672	KR184399	*Blastococcus aggregatus* ATCC 25902(T)	99.7	CSY12038	CC	G4	M3
I12A-02689	KR184413	*Blastococcus aggregatus* ATCC 25902(T)	99.7	CSY12040	CC	G4	M3
I12A-02692	KR184416	*Blastococcus aggregatus* ATCC 25902(T)	99.7	CCL12004	CC	G1	M2
I12A-02936	KR184433	*Blastococcus aggregatus* ATCC 25902(T)	99.7	CSY12040	CC	G4	M2
I12A-02941	KR184436	*Blastococcus aggregatus* ATCC 25902(T)	99.7	CSY12040	CC	G4	M1
I12A-02999	KR184469	*Blastococcus aggregatus* ATCC 25902(T)	99.7	CSY12040	CC	G4	M2
I12A-02654	KR184382	*Blastococcus aggregatus* ATCC 25902(T)	99.7	LHW12015	LC	G3	M2
I12A-02660	KR184388	*Blastococcus aggregatus* ATCC 25902(T)	99.7	MHW12014	MC	G3	M2
I12A-02639	KR184368	*Blastococcus aggregatus* ATCC 25902(T)	99.7	MSY12025	MC	G4	M3
I12A-02626	KR184355	*Blastococcus aggregatus* ATCC 25902(T)	99.7	MSY12029	MC	G4	M3
I12A-02666	KR184394	*Blastococcus endophyticus* YIM 68236(T)	98.9	BHW12013	BS	G3	M1
I12A-02971	KR184446	*Blastococcus endophyticus* YIM 68236(T)	98.9	MCL12005	MC	G1	M3
I12A-02953	KR184441	*Blastococcus endophyticus* YIM 68236(T)	99	CSY12027	CC	G4	M2
I12A-02649	KR184377	*Blastococcus endophyticus* YIM 68236(T)	99.1	BHW12012	BS	G3	M2
I12A-02599	KR184501	*Blastococcus endophyticus* YIM 68236(T)	99.1	MSY12019	MC	G4	M1
I11A-00338	KR184318	*Blastococcus endophyticus* YIM 68236(T)	99.1	MSY12025	MC	G4	M2
I12A-02986	KR184467	*Blastococcus endophyticus* YIM 68236(T)	99.2	BSY12034	BS	G4	M1
I12A-02609	KR184338	*Blastococcus endophyticus* YIM 68236(T)	99.6	LHW12015	LC	G3	M2
I12A-02939	KR184434	*Blastococcus jejuensis* KST3-10(T)	98.6	CSY12040	CC	G4	M2
I12A-02700	KR184422	*Blastococcus jejuensis* KST3-10(T)	98.8	CSY12040	CC	G4	M1
I12A-02972	KR184463	*Blastococcus jejuensis* KST3-10(T)	98.8	CSY12040	CC	G4	M2
I12A-02929	KR184429	*Blastococcus jejuensis* KST3-10(T)	98.8	MSY12029	MC	G4	M2
I12A-02646	KR184374	*Blastococcus jejuensis* KST3-10(T)	98.8	MYW12010	MC	G2	M1
I12A-02985	KR259823	*Blastococcus saxobsidens* BC448(T)	99.7	CSY12040	CC	G4	M3
I12A-02622	KR184351	*Geodermatophilus amargosae* G12(T)	99.8	CSY12050	CC	G4	M1
I12A-02606	KR184335	*Geodermatophilus normandii* CF5/3(T)	99.8	MYW12010	MC	G2	M3
I12A-02614	KR184343	*Geodermatophilus nigrescens* YIM 75980(T)	99.5	CSY12030	CC	G4	M4
I12A-02620	KR184349	*Geodermatophilus nigrescens* YIM 75980(T)	100	CSY12044	CC	G4	M2
I12A-02675	KR184402	*Geodermatophilus obscurus* DSM 43160(T)	97.8	CSY12039	CC	G4	M2
I12A-02940	KR184435	*Geodermatophilus obscurus* DSM 43160(T)	97.8	CSY12040	CC	G4	M1
I12A-02924	KR184427	*Geodermatophilus obscurus* DSM 43160(T)	98	CCL12004	CC	G1	M2
I12A-02624	KR184353	*Geodermatophilus obscurus* DSM 43160(T)	99.1	MSY12029	MC	G4	M3
I12A-02694	KR184417	*Geodermatophilus ruber* CPCC 201356(T)	97.8	CSY12030	CC	G4	M3
I12A-02611	KR184340	*Geodermatophilus ruber* CPCC 201356(T)	97.8	LHW12016	LC	G3	M3
I12A-02630	KR184359	*Geodermatophilus ruber* CPCC 201356(T)	98.4	CSY12050	CC	G4	M1
I12A-02982	KR184447	*Modestobacter marinus* 42H12-1(T)	97.8	CSY12040	CC	G4	M1
I11A-00468	KR184323	*Modestobacter marinus* 42H12-1(T)	97.8	CSY12040	CC	G4	M1
I12A-02690	KR184414	*Modestobacter marinus* 42H12-1(T)	98.1	CSY12030	CC	G4	M1
I12A-02938	KR184455	*Modestobacter marinus* 42H12-1(T)	98.1	CSY12040	CC	G4	M1

(Continued)

TABLE 2 | Continued

Strain number	Accession number	The closest homolog	16S rRNA gene similarity with the closest homolog (%)	Sample number	Sample type	Geographical location	Isolationmedium
I12A-02915	KR184423	*Modestobacter marinus* 42H12-1(T)	98.1	MSY12017	MC	G4	M2
I12A-02627	KR184356	*Modestobacter marinus* 42H12-1(T)	98.2	CSY12027	CC	G4	M1
I12A-02662	KR184390	*Modestobacter marinus* 42H12-1(T)	98.2	MCL12002	MC	G1	M3
I12A-02951	KR184459	*Modestobacter marinus* 42H12-1(T)	98.3	CSY12040	CC	G4	M1
I11A-00199	KR184503	*Modestobacter marinus* 42H12-1(T)	98.3	CSY12050	CC	G4	M2
I12A-02657	KR184385	*Modestobacter marinus* 42H12-1(T)	98.6	MHW12014	MC	G3	M1
I12A-02575	KR184483	*Modestobacter marinus* 42H12-1(T)	98.6	MSY12029	MC	G4	M4
I12A-02993	KR184449	*Modestobacter marinus* 42H12-1(T)	99.4	LYW12008	LC	G2	M2
I12A-02588	KR184494	*Modestobacter marinus* 42H12-1(T)	99.5	BSY12032	BS	G4	M4
I12A-02613	KR184342	*Modestobacter multiseptatus* AA-826(T)	96.1	CSY12040	CC	G4	M2
I12A-02616	KR184345	*Modestobacter multiseptatus* AA-826(T)	96.3	CSY12040	CC	G4	M2
I12A-02615	KR184344	*Modestobacter multiseptatus* AA-826(T)	97	CSY12040	CC	G4	M1
I12A-02617	KR184346	*Modestobacter multiseptatus* AA-826(T)	97.1	CSY12040	CC	G4	M2
I12A-02988	KR184468	*Modestobacter multiseptatus* AA-826(T)	97.2	CCL12004	CC	G1	M2
I12A-02577	KR184485	*Modestobacter multiseptatus* AA-826(T)	97.5	CSY12027	CC	G4	M1
I12A-02573	KR184481	*Modestobacter multiseptatus* AA-826(T)	97.8	BSY12026	BS	G4	M2
I12A-02618	KR184347	*Modestobacter multiseptatus* AA-826(T)	97.8	MSY12029	MC	G4	M2
I11A-00478	KR184324	*Modestobacter multiseptatus* AA-826(T)	98.8	MSY12029	MC	G4	M1
I12A-02991	KR259822	*Modestobacter roseus* KLBMP 1279(T)	100	MSY12025	MC	G4	M4
I12A-02955	KR184442	*Modestobacter versicolor* CP153-2(T)	98.6	MHW12014	MC	G3	M1
I12A-02641	KR184370	*Modestobacter versicolor* CP153-2(T)	98.8	BYW12009	BS	G2	M4

CC, Cyanobacteria-dominated soil crusts; MC, Moss-dominated soil crusts; LC, Lichen-dominated soil crusts; BS, Bare sand. G1, 37°25′37″–37°25′40″N, 104°35′7″–104°35′10″ E, ∼1700 mH; G2, 37°25′29″–37°25′32″N, 104°43′51″–104°43′54″ E, ∼1700 mH; G3, 37°27′3″–37°27′5″N, 104°47′40″–104°47′43″ E, ∼1620 mH; G4, 37°27′37″–37°27′40″N, 104°59′58″–105°0′1″ E, ∼1330 mH.

Discussion

The family *Geodermatophilaceae* is a newly-established actinobacterial taxon. Normand et al. (1996) proposed the family *Geodermatophilaceae* in 1996, which was regarded as an invalid taxon at that time. In 2006, based on the common characteristics of the genera *Geodermatophilus*, *Blastococcus,* and *Modestobacter*, Normand (2006) summarized the typical characteristics of *Geodermatophilaceae*. Subsequently, the family *Geodermatophilaceae* was finally accommodated as a validly described taxon in the phylum *Actinobacteria*. To date, the family *Geodermatophilaceae* consists of three genera: *Geodermatophilus*, *Blastococcus,* and *Modestobacter*, that includes 25 validly described species.

The members of *Geodermatophilaceae* were found from various environments, including soil samples (Zhang et al., 2011; Jin et al., 2013), soil crusts (Reddy et al., 2007), deep subseafloor sediment (Ahrens and Moll, 1970), even stone habitats (Salazar et al., 2006; Chouaia et al., 2012; Gtari et al., 2012; Normand et al., 2012), dry-hot valley (Nie et al., 2012), and arid sand from desert (Montero-Calasanz et al., 2012, 2013a,b,c). In this study, we found *Geodermatophilaceae* members ubiquitously in desert soil samples, and we obtained *Geodermatophilaceae* cultures from three different types of desert soil crusts, as well as from the bare sands. These four environments represent typical micro-ecological environments in the Shapotou region. As we have observed, most *Geodermatophilaceae* members could form tiny motile spores or dormant spores, allowing them to spread around and survive long periods of desiccation. Moreover, most of the *Geodermatophilaceae* members we tested formed pink to black colonies on different types of agar plates. The pigmentation, cell wall composition and a high G+C content may increase protection of these strains from UV damage in the desert environments, where the UV transparency is often high.

The abundance and ubiquitous distribution of the *Geodermatophilaceae* in desert environments exhibited in relation to their resident microbiota, and in turn, the micro-ecological environments endowed the microorganisms with some specific metabolic characteristics. We found that the abundance and diversity of the *Geodermatophilaceae* in lichen- and cyanobacteria-dominated soil crusts were much higher than those of the bacteria found in moss-dominated soil crusts or bare sands. In the desert, the moisture, organic, and nitrogen content of the soil were the vital factors in determining physiological characteristics of the organisms. The lichen- and cyanobacteria-dominated soil crusts may contain a much higher proportion of clay and humic colloidal material, which can markedly affect the physiological activities of the strains from different micro-ecological environments.

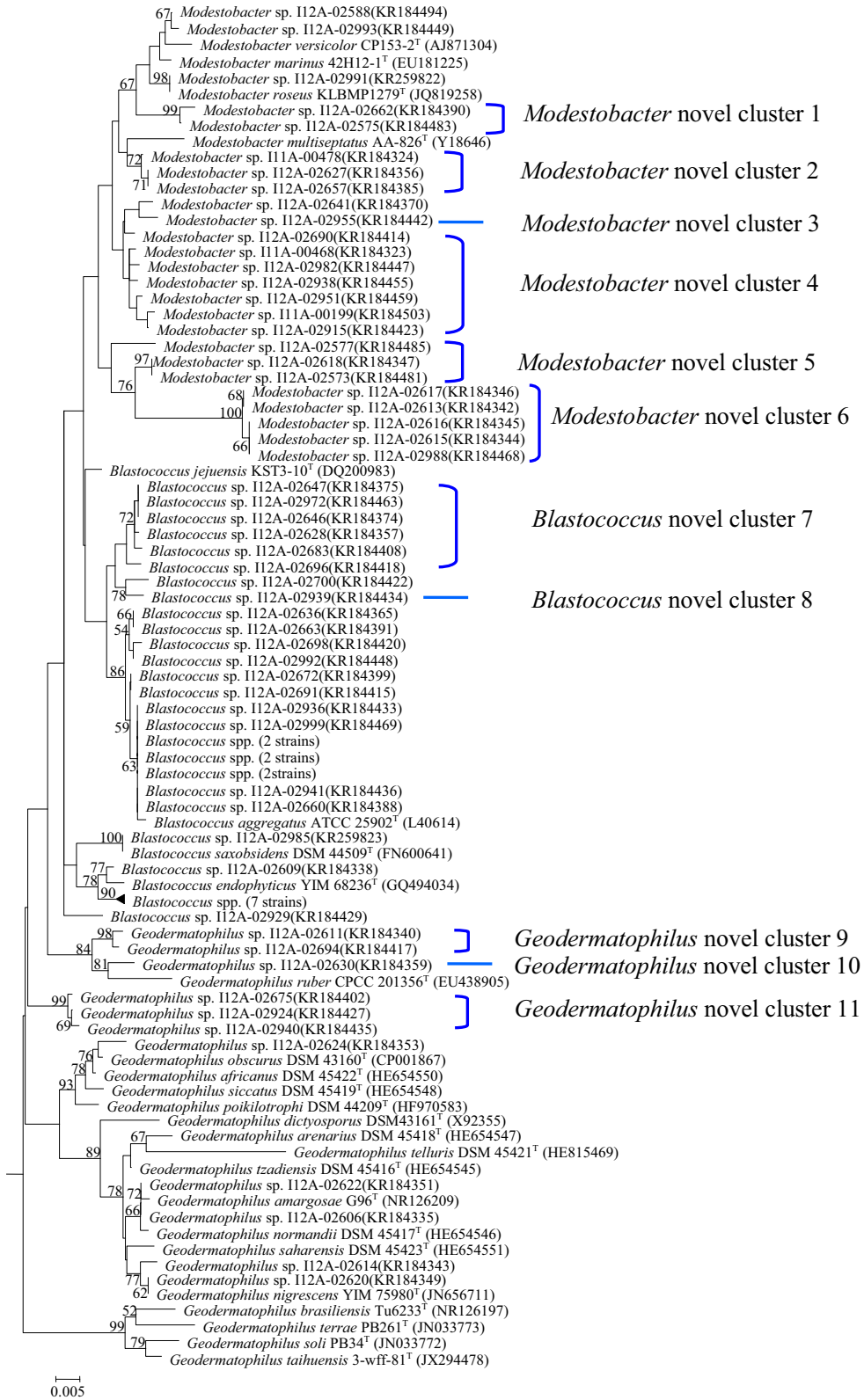

FIGURE 1 | Phylogenetic dendrogram based on the 16S rRNA gene sequences of the newly-isolated strains and 25 type strains of *Geodermatophilaceae*. The sequence of *Kineosporia aurantiaca* 14067T was used as the outgroup. Numbers on branch nodes are bootstrap values. Bar, 1.0% sequence divergence.

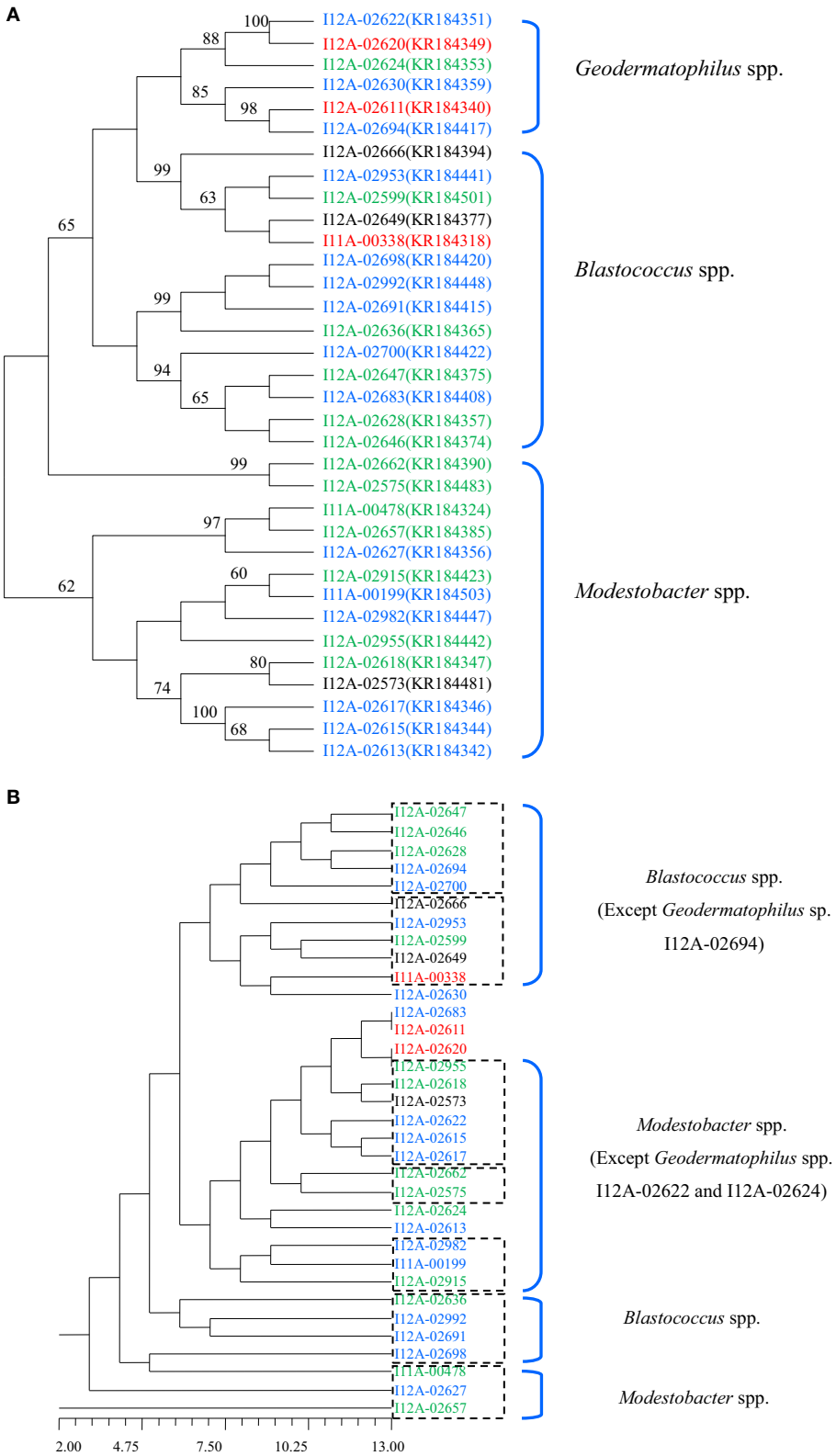

FIGURE 2 | (A) Dendrogram based 16S rRNA gene sequences analysis of the tested strains. **(B)** Dendrogram based on the physiological characteristics profiles of the tested strains. Different colors denote the strains isolated from different types of samples. Blue, Cyanobacteria-dominated soil crusts; Green, Moss-dominated soil crusts; Red, Lichen-dominated soil crusts; Black, Bare sand.

The assayed physiological characteristics of the *Geodermatophilaceae* also showed a probable relationship with the resident microbes of the respective micro-ecological environments. In the dendrogram based on 173 physiological characteristics of the 34 tested *Geodermatophilaceae* strains, strains from the same micro-ecological environment were more likely to gather closely. The clusters shown in the phylogenetic dendrogram based on 16S rRNA gene sequences were interrupted in the dendrogram based on the physiological characteristics profile, which indicated that the micro-ecological environments where the strains were isolated significantly influenced the physiological characteristic profiles of the isolates (**Figure 2**).

Compared to our previous study and other related studies in the literature, we discovered many interesting diverse bioactivities for rare actinobactieria, which may be caused by characteristics of the extreme environments where these strains were found. Isolation and analysis of the bioactive compounds underlying these bioactivities will provide more detailed information on the mechanism of these activities. In this context, the members of the family *Geodermatophilaceae* are found to be the biological pioneers in extreme environments, especially in extreme arid environments. Further study on the cultures in this family will be advantageous to those seeking to understand mechanisms of environmental stress resistance, desertification control, and environmental remediation. In addition, studying these organisms will aid in the discovery of novel metabolic compounds.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01059

Supplementary Figure S1 | Phylogenetic dendrogram based on 16S rRNA gene sequences analysis of the isolates. Bootstrap values >50% (based on 1000 resampled datasets) are shown at branch nodes. Bar, 0.02 substitutions per site.

Supplementary Figure S2 | The number of actinobacteria in the different samples. Sample numbers were ordered from left to right (x-axis) according to the sample number in **Table 1**, and numbers of actinobacterial colonies from bottom to top (y-axis) were estimated and ordered according to the colony numbers that appeared on isolation medium of M4, M3, M2, and M1.

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Actinomycetes from the South China Sea sponges: isolation, diversity, and potential for aromatic polyketides discovery

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Marine sponges often harbor dense and diverse microbial communities including actinobacteria. To date no comprehensive investigation has been performed on the culturable diversity of the actinomycetes associated with South China Sea sponges. Structurally novel aromatic polyketides were recently discovered from marine sponge-derived *Streptomyces* and *Saccharopolyspora* strains, suggesting that sponge-associated actinomycetes can serve as a new source of aromatic polyketides. In this study, a total of 77 actinomycete strains were isolated from 15 South China Sea sponge species. Phylogenetic characterization of the isolates based on 16S rRNA gene sequencing supported their assignment to 12 families and 20 genera, among which three rare genera (*Marihabitans*, *Polymorphospora,* and *Streptomonospora*) were isolated from marine sponges for the first time. Subsequently, β-ketoacyl synthase (KS_α) gene was used as marker for evaluating the potential of the actinomycete strains to produce aromatic polyketides. As a result, KS_α gene was detected in 35 isolates related to seven genera (*Kocuria*, *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Saccharopolyspora*, *Salinispora*, and *Streptomyces*). Finally, 10 strains were selected for small-scale fermentation, and one angucycline compound was detected from the culture extract of *Streptomyces anulatus* strain S71. This study advanced our knowledge of the sponge-associated actinomycetes regarding their diversity and potential in producing aromatic polyketides.

Keywords: marine sponges, actinomycetes, diversity, aromatic polyketides, KS_α gene

Introduction

As one of the oldest multicellular animals (Love et al., 2009), marine sponges (phylum Porifera) often harbor dense and diverse microbial communities, and the sponge-microbe associations represent one of the most complex symbioses on earth (Taylor et al., 2007). Actinobacteria are commonly found in association with sponges (Simister et al., 2012). In the past decade, extensive efforts have been made in isolating actinomycetes from sponges (Zhang et al., 2006; Abdelmohsen et al., 2010, 2014b; Vicente et al., 2013). To date, at least 60 actinobacterial genera have been set apart from marine sponges (Abdelmohsen et al., 2014a). The investigations on the culturable diversity of sponge-associated actinomycetes not only advanced our knowledge of those actinomycetes in

special habitats but also provided new opportunities for natural product search and discovery (Abdelmohsen et al., 2014a). In China oceans, the largest group of sponges inhabits the South China Sea (Zhang et al., 2003). To our knowledge, in previous studies 15 actinobacterial genera have been isolated from South China Sea sponges (Jiang et al., 2007, 2008; Sun et al., 2010; Li et al., 2011; Xi et al., 2012). Nevertheless, previous cultivation attempts were set to a few South China Sea sponge species out of thousands of South China Sea sponges, which probably underestimated the culturable diversity of sponge-associated actinomycetes. Thus, collecting as many sponges as possible from the South China Sea is significant to comprehensively explore their associated actinomycetes.

Previous surveys have demonstrated that sponges are chemically defended from predation and marine pathogens either by the compounds they produce or those produced by symbionts or associated microorganisms (Puglisi et al., 2014). Actinomycetes are known to produce aromatic polyketides by type II polyketide pathway (Schneider, 2005). Actinomycete-derived aromatic polyketide compounds have exhibited a wide range of bioactivities and clinical importance (Hertweck et al., 2007). Notably, a few anthracyclines and tetracyclines have emerged as clinical drugs for decades, such as doxorubicin (antineoplastic) and tetracycline (antibiotic). Furthermore, many of these compounds are promising drug candidates (Hertweck et al., 2007). Therefore, sponge-associated actinomycetes may provide chemical defense for their hosts by producing aromatic polyketides. Recently, in exploring new sources of aromatic polyketides the sponge-associated actinomycetes warranted particular attention. Particularly, a few structurally novel aromatic polyketides were discovered from sponge-associated actinomycetes such as *Saccharopolyspora* and *Streptomyces* strains (Perez et al., 2009; Motohashi et al., 2010; Schneemann et al., 2010a). In view of the remarkable diversity of sponge-associated actinomycetes, the producers of aromatic polyketides are not merely limited to *Saccharopolyspora* and *Streptomyces*. Thus, we opine that the potential of sponge-associated actinomycetes in producing aromatic polyketides is underexplored and it is worth investigating in depth.

Over the past decade, sequence-guided genetic screening strategy has been used in the discovery of certain compound classes from actinomycetes, such as halometabolites (Hornung et al., 2007), type I polyketides (Gontang et al., 2010), and phenazines (Karuppiah et al., 2015), indicating that a small amount of sequence from appropriate genetic loci can be used to predict secondary-metabolite production in cases where the sequences have high identity level to experimentally characterized biosynthetic pathways. The gene-compound route has become a feasible approach for natural product search and discovery. Therefore, genetic screening strategy together with small-scale fermentation and chemical analyses was used in this study to specifically search for aromatic polyketides.

In this work, we aimed to investigate the culturable diversity of sponge-associated actinomycetes from the South China Sea and explore the potential use of the sponge-associated actinomycetes as a novel source of aromatic polyketides. As a result, we cultivated as many as 20 actinomycete genera, screened seven genera as potential producers of aromatic polyketides and identified one angucycline compound from a *Streptomyces* strain. This study advanced our knowledge of South China Sea sponge-associated actinomycetes in respect to their diversity and metabolic potential of aromatic polyketides.

Materials and Methods

Sample Collection

A total of 15 sponge species were collected by scuba diving from the South China Sea, including six at a depth of 5–10 m from coastal waters, respectively Sanya Bay (18°13′N; 109°29′E), Xinying Harbor (19°90′N; 109°52′E), and Xincun Harbor (18°40′N; 110°00′E) and nine at a depth of 10–20 m from a remote island, Yongxing Island (16°50′N; 112°20′E) (**Table 1**). The sponges were identified based on their morphology or 18S rRNA gene/internal transcribed spacer (ITS) sequences (**Table 1**). The samples were placed into plastic bags and transported to the laboratory using ice box, then stored at $z-20$°C until analysis.

Isolation of Actinomycetes

Five media were used for the isolation of sponge-associated actinomycetes (Table S1), four of which were chosen based on previous studies on the culturable diversity of marine sediment-derived and sponge-associated actinomycetes (Mincer et al., 2002; Zhang et al., 2006; Abdelmohsen et al., 2010) and one was designed in this study. All media were supplemented with $K_2Cr_2O_7$ (50 μg ml^{-1}) to inhibit fungi and nalidixic acid (15 μg ml^{-1}) to inhibit Gram-negative bacteria. Sponge samples were rinsed with sterile artificial seawater (26.52 g NaCl, 5.228 g $MgCl_26H_2O$, 3.305 g $MgSO_4$, 1.141 g $CaCl_2$, 0.725 g KCl, 0.202 g $NaHCO_3$, 0.083 g NaBr, 1 L distilled water) to remove the microbes loosely attached on the surface. Subsequently, a few tissue cubes were excised from different sections (including cortex and endosome) of the sponge samples. They were cut into pieces and aseptically ground using sterilized pestles and mortars. Actinomycetes were isolated by means of serial dilution and plating techniques. The inoculated plates were incubated at 28°C for 3–6 weeks. The colonies bearing distinct morphological characteristics were picked up and transferred onto freshly prepared media until pure cultures were obtained.

Genomic DNA Extraction

To prepare cultures for the extraction of genomic DNA from the isolates, a single colony was transferred to a 5 ml microtube with 1 ml of liquid medium from which the isolate was originally picked up. The cultures were incubated for 3–5 days at 28°C with shaking at 180 rpm. Bacterial cells from these cultures were collected by centrifugation and genomic DNA was extracted as described by Sun et al. (2010).

PCR Amplification and Sequencing of 16S rDNA

The *Actinobacteria*-specific primers S-C-Act-0235-a-S-20 (5′-CGCGGCCTATCAGCTTGTTG-3′) and S-C-Act-0878-a-A-19

TABLE 1 | Sponge samples collected from the South China Sea and their actinomycete isolates.

Sponge species	Identification method (NCBI accession no.)	Geographical location	Collection month	No. of isolates	No. of genera
Haliclona sp.	morphology	Sanya Bay	2009.07	11	6
Trachycladus laevispirulifer	morphology	Xinying Harbor	2010.06	3	3
Amphimedon queenslandica	ITS sequence (KC762728)	Xincun Harbor	2011.05	6	3
Haliclona mediterranea	18S rRNA gene sequence (KC762723)	Xincun Harbor	2011.05	3	2
Lamellodysidea sp.	ITS sequence (KC762730)	Xincun Harbor	2011.05	8	5
Cliona sp.	ITS sequence (KC762729)	Xincun Harbor	2011.05	5	2
Phyllospongia foliascens	morphology	Yongxing Island	2011.05	1	1
Agelas clathrodes	18S rRNA gene sequence (KC762715)	Yongxing Island	2011.05	2	2
Ircinia felix	18S rRNA gene sequence (KC762716)	Yongxing Island	2011.05	2	2
Hippospongia lachne	18S rRNA gene sequence (KC762719)	Yongxing Island	2011.05	2	1
Cinachyrella sp.	18S rRNA gene sequence (KC762720)	Yongxing Island	2011.05	5	4
Aplysina fistularis	18S rRNA gene sequence (KC762723)	Yongxing Island	2011.05	8	2
Arenosclera heroni	18S rRNA gene sequence (KJ675584)	Yongxing Island	2013.07	5	4
Plakortis simplex	morphology	Yongxing Island	2013.07	12	4
Phakellia fusca	morphology	Yongxing Island	2013.07	4	3

(5′-CCGTACTCCCCAGGCGGGG-3′) were used for the amplification of actinobacterial 16S rRNA gene fragment (Stach et al., 2003). Cycling conditions were as follows: initial denaturation at 95°C for 4 min, 30 cycles of 95°C for 45 s, 68°C for 45 s, and 72°C for 1 min, and a final extension of 5 min at 72°C. Subsequently, the universal bacterial primers 27F (5′-GAG TTTGATCCTGGCTCAG-3′) and 1500R (5′-AGAAAGGAG GTGATCCAGCC-3′) were used to amplify nearly complete 16S rRNA gene of the actinomycete candidates (Woese et al., 1983). Cycling conditions were as follows: initial denaturation at 95°C for 3 min, 30 cycles of 94°C for 30 s, 54°C for 40 s, and 72°C for 2 min, and a final extension of 10 min at 72°C. The PCR products were purified and sequenced on the ABI 3730 automated sequencer at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai).

PCR Amplification, Cloning, and Sequencing of KS Gene

To screen aromatic polyketide producers from all the isolates, the degenerate primers IIPF6 (5′-TSGCSTGCTTCGAYGCSATC-3′) and IIPR6 (5′-TGGAANCCGCCGAABCCGCT-3′) were used to amplify type II polyketide KS_α gene fragment (Metsä-Ketelä et al., 1999). This primer pair was reported to be favorable for the majority of known KS_α gene and previously used in the investigation on marine sponge-associated actinobacteria (Schneemann et al., 2010b). Cycling conditions were as follows: initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 35 s, 55°C for 40 s, and 72°C for 1 min, and a final extension of 10 min at 72°C. The amplified products of approximately 600 bp were recovered and purified using Agarose Gel DNA Purification Kit (Takara, Dalian). Purified PCR products were cloned into pMD18-T vector (Takara, Dalian) and transformed into $CaCl_2$-competent *Escherichia coli* DH5α. The positive recombinants were screened on X-Gal-IPTG-ampicillin plates. Respectively five positive clones were randomly selected from each library and sequenced using M13F primer on the ABI 3730 automated sequencer at Sangon Biotech Co. Ltd. (Shanghai).

Sequence Analysis

All the sequence data were proofread using Chromas, version 1.62 (Technelysium). The 16S rRNA gene sequences were compared with those from the type strains available in NCBI (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). For KS_α gene analysis, the nucleotide sequences were translated to amino acid sequences using the web tool ORF Finder in NCBI (http://www.ncbi.nlm.nih.gov/projects/gorf/). The deduced amino acid sequences were compared with the KS_α sequences in PKMiner database (http://www.webcitation.org/6C9a5WoFY) using the type II PKS domain classifiers (Kim and Yi, 2012). The top matches were derived from the KS_α sequences associated with 42 experimentally characterized pathways. For phylogenetic analysis, multiple sequence alignment was performed using CLUSTALX, version 1.81. Phylogenetic tree was constructed using Mega 4.1 (Tamura et al., 2007). The consistency of the trees was verified by bootstrapping (1000 replicates) for parsimony.

Small-scale Fermentation

To test the production of aromatic polyketides, small-scale fermentation studies were performed targeting 10 representative strains, which were selected based on KS_α sequence analyses. They were grown in 250 ml Erlenmeyer flasks each containing 100 ml of medium GYM4 (10 g glucose, 4 g yeast extract, 4 g malt extract, 1 liter water, pH 7.2) for 5 days at 28°C with shaking (at 120 rpm) in the dark. Each culture was inoculated separately with a 1 cm^2 piece from a culture grown on a GYM4 agar plate for 2 weeks at 28°C in the dark.

Chemical Analysis of Culture Extracts

After mycelium was removed by vacuum filtration, the fermentation broth was extracted with 100 ml of acetic ether

(EtOAc) and taken to dryness by rotary evaporation. EtOAc extract was dissolved in methanol for HPLC-DAD analysis on an Agilent 1200 series (Agilent Technologies, USA) with an Diode Array Detector (DAD) and a C18 RP-column (Eclipse XDB-C18 5 μm, 4.6 × 150 mm), with a gradient from 5% acetonitrile in water to 100% acetonitrile over 20 min. Ultraviolet-visible (UV-vis) absorption spectra ranging from 200 to 600 nm of the components in each crude extract were examined. Compounds owning characteristic UV-vis absorption of aromatic polyketides were searched and designated as putative candidates. Prior to LC/MS analysis, the compound candidates were preliminarily separated from the crude extracts by semi-preparative HPLC with methanol gradient elution. This procedure was conducted on an Agilent 1200 series (Agilent Technologies, USA) with a variable wavelength detector (VWD) and a C18 RP-column (Unitary C18 5 μm, 10 × 250 mm).

Collected fractions were dried in vacuo and dissolved in methanol for LC/MS analysis. The fractions were detected on an ultra-performance liquid chromatography and quadrupole time of flight mass spectroscopy (UPLC-QTOF-MS Premier, Waters Corporation, USA). The analytes were separated on a C18 RP-column (ACQUITY BEH-C18 1.7 μm, 2.1 × 100 mm, Waters Co.) with methanol gradient elution. High-resolution mass spectrum (HR-MS) of target ion was acquired in positive electro-spray ionization mass spectrum (ESI-MS) mode.

MS data was analyzed using the software MassLynx. The major ion peaks with a mass range of 300–1000 Da were preferentially selected. Corresponding to each peak ($[M+H]^+$ or $[M+Na]^+$), a few suggested molecular formula were obtained. After those not matching aromatic polyketide compounds were excluded, the remaining ones were used as queries (subtracting one H or Na) to match reported aromatic polyketides in SciFinder database (https://scifinder.cas.org/scifinder/). For those retrieved compounds, their UV-vis absorption spectra were compared with our target substance.

Nucleotide Sequence Accession Numbers

The sequences obtained in this study were deposited to GenBank with the 16S rRNA gene sequences under the accession numbers: JX007945–JX008000, KJ094386–KJ094406 and the KS_α gene sequences under the numbers: JX008002–JX008015, KJ094407–KJ094410.

Results

Culture-dependent Diversity of Sponge-associated Actinomycetes

In this study, a total of 77 isolates were identified as actinomycetes, which were assigned to 12 families and 20 genera (**Table 2**). Among the 20 genera, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Nocardiopsis*, *Pseudonocardia*, *Rhodococcus*, *Salinispora*, and *Streptomyces* were previously isolated from South China Sea sponges (Jiang et al., 2007, 2008; Sun et al., 2010; Li et al., 2011; Xi et al., 2012), the other 12 genera marked in **Table 2** were cultivated from South China Sea sponges first time. Based on the latest reviews (Abdelmohsen et al., 2014a; Valliappan et al., 2014) and our retrievals of sponge-derived 16S rRNA gene sequences in GenBank, we found this was the first report of three rare genera, i.e., *Marihabitans*, *Polymorphospora*, and *Streptomonospora*, isolated from marine sponges.

The highest number of the isolates was affiliated with *Salinispora*, followed by *Streptomyces*, *Kocuria*, *Serinicoccus*, *Micromonospora*, *Nocardiopsis*, *Polymorphospora*, and other genera (**Figure 1A**). The number of the isolates differed considerably among different marine sponges. *Plakortis simplex* yielded the highest number of isolates, followed by *Haliclona* sp., *Lamellodysidea* sp., *Aplysina fistularis*, *Amphimedon queenslandica*, and other sponges (**Table 1**). Similarly, the actinobacterial diversity at the genus level also varied as sponge species. The highest diversity was observed in *Haliclona* sp. with six genera cultivated, followed by *Lamellodysidea* sp. and other sponges (**Table 1**). The 77 isolates were assigned to 40 operational taxonomic units (OTUs) based on 99.5% sequence identity, representing 40 species. The most diverse group was *Streptomyces* with 14 OTUs obtained, followed by *Kocuria* and other genera (**Figure 1B**).

On the whole, *Streptomyces* and *Salinispora* were most common groups in the South China Sea sponges. The former was isolated from nine sponges and the latter from six sponges. *Streptomyces* was widespread in the sponges from distinct geographical locations whereas *Salinispora* was mainly distributed in the open sea sponges. Additionally, *Kocuria* was derived from four sponges inhabiting the same site, Xincun Harbor, indicating its distribution specificity.

Structure Diversity Evaluation of Putative Aromatic Polyketide Products

PCR fragments of KS_α gene were amplified from 35 out of 77 isolates (**Table 2**). The 35 isolates were assigned to 17 OTUs. In total, 17 PCR fragments from 17 OTUs were selected for KS_α gene cloning and sequencing, and 18 unique sequences were obtained. Based on homology comparison (**Table 3**) and phylogenetic analysis (**Figure 2**), high structural diversity of putative aromatic polyketide products was observed, concerning different subtypes. Homology-based searches on the amino acid level indicated that the putative KS_α sequences, respectively displayed 85.2–100% maximum similarity to those KSs associated with experimentally characterized biosynthetic pathways (**Table 3**). By comparing those known KS_α sequences in PKMiner database, it was observed that most sequences grouped in the same subtype share ≥93.6% amino acid similarity with each other. Thus, this similarity was used as sequence clustering criterion in this work. Of the obtained 18 KS_α sequences, eight shared ≥93.6% similarity with their top matches, which were derived from six *Streptomyces* strains, one *Micromonospora*, and one *Nocardia* strain. The matches for these eight sequences were to KSs responsible for the biosynthesis of three subgroups, respectively benzoisochromanequinones, angucyclines, and pentangular polyphenols. Specifically, one strain (S97) corresponded to benzoisochromanequinone subtype, three strains (S41, S71, and S107) were linked with angucycline subclass and four

TABLE 2 | Molecular identification of the actinomycetes from South China Sea sponges based on 16S rRNA gene and KS_{α} gene detection.

Family	Genus	OUT no.	Strain (NCBI accession no.)	Nearest type strain (NCBI accession no.)	Identity (%)	PKS II
Brevibacteriaceae	*Brevibacterium**	1	S49 (JX007974)	*B. linens* (NR_026166)	99.3	–
Dermabacteraceae	*Brachybacterium**	2	S26 (JX007960)	*B. squillarum* (GQ339911)	99.5	–
Intrasporangiaceae	*Marihabitans**	3	S53 (JX007977)	*M. asiaticum* (NR_041559)	100	–
	*Serinicoccus**	4	S11 (JX007953)	*S. chungangensis* (HM068886)	98.7	–
		4	S24 (JX007958)	*S. chungangensis* (HM068886)	98.7	–
		4	S38 (JX007966)	*S. chungangensis* (HM068886)	99.0	–
		4	S69 (JX007986)	*S. chungangensis* (HM068886)	98.6	–
Microbacteriaceae	*Microbacterium**	5	S15 (JX007956)	*M. chocolatum* (AM181503)	99.8	–
		5	S25 (JX007959)	*M. chocolatum* (AM181503)	99.9	–
Micrococcaceae	*Arthrobacter**	6	S70 (JX007987)	*A. protophormiae* (NR_026195)	99.8	–
	*Kocuria**	7	S12# (JX007954)	*K. gwangalliensis* (EU286964)	96.8	+
		8	S14 (JX007955)	*K. turfanensis* (NR_043899)	98.8	–
		8	S42 (JX007970)	*K. turfanensis* (NR_043899)	98.6	–
		8	S50 (JX007975)	*K. turfanensis* (NR_043899)	98.7	–
		8	S61 (JX007984)	*K. turfanensis* (NR_043899)	98.7	–
		9	S43 (JX007971)	*K. flava* (NR_044308)	99.7	–
		10	S45 (JX007972)	*K. palustris* (NR_026451)	100	–
		10	S62 (JX007985)	*K. palustris* (NR_026451)	99.9	–
		11	S48 (JX007973)	*K. marina* (NR_025723)	99.7	–
	*Micrococcus**	12	S23 (JX007957)	*M. endophyticus* (NR_044365)	99.8	–
Micromonosporaceae	*Micromonospora*	13	S60 (JX007983)	*M. aurantiaca* (NR_074415)	99.6	–
		13	S80 (JX007997)	*M. aurantiaca* (NR_074415)	99.5	–
		13	S97# (KJ094396)	*M. aurantiaca* (NR_074415)	99.4	+
	*Polymorphospora**	14	S07 (JX007949)	*Polymorphospora* sp. (NR_044592)	98.7	–
		14	S09 (JX007951)	*Polymorphospora* sp. (NR_044592)	98.8	–
		15	S85 (KJ094387)	*P. rubra*(NR_041314)	100	–
	Salinispora	16	S06 (JX007948)	*S. arenicola* (NR_074612)	99.9	+
		16	S08 (JX007950)	*S. arenicola* (NR_074612)	100	+
		16	S32 (JX007962)	*S. arenicola* (NR_074612)	99.8	+
		16	S33# (JX007963)	*S. arenicola* (NR_074612)	99.7	+
		16	S55 (JX007979)	*S. arenicola* (NR_074612)	99.9	+
		16	S56 (JX007980)	*S. arenicola* (NR_074612)	99.9	+
		16	S58 (JX007981)	*S. arenicola* (NR_074612)	99.9	+
		16	S83 (JX008000)	*S. arenicola* (NR_074612)	100	+
		16	S84 (KJ094386)	*S. arenicola* (NR_074612)	100	+
		16	S87 (KJ094389)	*S. arenicola* (NR_074612)	99.9	+
		16	S93 (KJ094392)	*S. arenicola* (NR_074612)	99.8	+
		16	S94 (KJ094393)	*S. arenicola* (NR_074612)	100	+
		16	S99 (KJ094398)	*S. arenicola* (NR_074612)	99.9	+
		16	S100 (KJ094399)	*S. arenicola* (NR_074612)	100	+
		16	S102 (KJ094401)	*S. arenicola* (NR_074612)	99.9	+
		16	S108 (KJ094405)	*S. arenicola* (NR_074612)	100	+
		17	S34# (JX007964)	*S. tropica* (NR_074502)	99.5	+
		17	S54 (JX007978)	*S. tropica* (NR_074502)	99.5	–
		17	S96 (KJ094395)	*S. tropica* (NR_074502)	99.5	–
		17	S98 (KJ094397)	*S. tropica* (NR_074502)	99.4	–
		17	S101 (KJ094400)	*S. tropica* (NR_074502)	99.5	–
		17	S103 (KJ094402)	*S. tropica* (NR_074502)	99.4	–

(Continued)

TABLE 2 | Continued

Family	Genus	OUT no.	Strain (NCBI accession no.)	Nearest type strain (NCBI accession no.)	Identity (%)	PKS II
Mycobacteriaceae	*Mycobacterium*	18	S01 (JX007945)	*M. poriferae* (NR_025235)	98.9	–
		18	S02 (JX007946)	*M. poriferae* (NR_025235)	98.9	–
Nocardiaceae	*Nocardia*	19	S107# (KJ094404)	*N. araoensis* (NR_028652)	98.6	+
	Rhodococcus	20	S106 (KJ094403)	*R. opacus*(NR_074632)	98.2	–
		20	S109 (KJ094406)	*R. opacus*(NR_074632)	98.0	–
Nocardiopsaceae	*Nocardiopsis*	21	S77 (JX007994)	*N. alba* (NR_026340)	100	+
		21	S78# (JX007995)	*N. alba* (NR_026340)	99.9	+
		22	S92# (KJ094391)	*N. halotolerans* (NR_025422)	99.1	+
	*Streptomonospora**	23	S05 (JX007947)	*S. halophila* (NR_044207)	97.7	–
Pseudonocardiaceae	*Pseudonocardia*	24	S76 (JX007993)	*P. carboxydivorans* (NR_044092)	99.2	–
	*Saccharopolyspora**	25	S36# (JX007965)	*S. gloriosae* (EU005371)	99.2	+
		25	S79 (JX007996)	*S. gloriosae* (EU005371)	99.4	+
Streptomycetaceae	*Streptomyces*	26	S10# (JX007952)	*S. parvulus* (NR_041119)	99.8	+
		27	S31# (JX007961)	*S. carnosus* (AB184263)	100	+
		28	S39# (JX007967)	*S. djakartensis* (NR_041178)	99.5	+
		29	S40# (JX007968)	*S. luteosporeus* (AB184607)	97.6	+
		30	S41# (JX007969)	*S. rochei* (NR_041091)	100	+
		31	S52 (JX007976)	*S. flavofuscus* (DQ026648)	98.2	–
		32	S59 (JX007982)	*S. resistomycificus* (NR_042100)	99.8	–
		33	S71# (JX007988)	*S. anulatus* (NR_041062)	99.8	+
		34	S72# (JX007989)	*S. xiamenensis* (NR_044035)	99.4	+
		35	S73 (JX007990)	*S. sclerotialus* (NR_025620)	98.3	–
		35	S74 (JX007991)	*S. sclerotialus* (NR_025620)	98.2	–
		36	S75 (JX007992)	*S. albidoflavus* (NR_041095)	99.3	–
		37	S81# (JX007998)	*S. diastaticus* (NR_043486)	99.6	+
		38	S82 (JX007999)	*S. marinus* (AB473554)	97.8	–
		39	S86# (KJ094388)	*S. griseorubens* (NR_041066)	100	+
		39	S95 (KJ094394)	*S. griseorubens* (NR_041066)	100	+
Streptosporangiaceae	*Nonomuraea**	40	S88 (KJ094390)	*N. ferruginea* (NR_025996)	98.7	–

*The 12 genera marked with *were cultivated from South China Sea sponges for the first time and the 17 strains marked with # were selected for KS_α gene analysis.*

strains (S31, S40, S81, and S86) with spore pigment group. In addition, 10 sequences displayed < 93.6% similarity with their top matches, whose products could not be correlated with specific subtypes. Subsequent phylogenetic analysis also supported our clustering patterns based on maximum similarity.

Small-scale Fermentation and Aromatic Polyketide Discovery

Based on KS_α sequence analysis, 10 strains were selected for small-scale fermentation (**Table 3**), among which one strain (*Micromonospora aurantiaca* S97) was used to test the production of putative benzoisochromanequinone, three strains (*Streptomyces rochei* S41, *Streptomyces anulatus* S71 and *Nocardia araoensis* S107) for putative angucyclines and other six strains (*Streptomyces parvulus* S10, *Saccharopolyspora gloriosa* S36, *Streptomyces djakartensis* S39, *Streptomyces xiamenensis* S72, *Nocardiopsis alba* S78, and *Nocardiopsis halotolerans* S92) for putative other subtypes. Expected products were preliminarily distinguished from the metabolite profiles according to their UV/vis absorption characteristics. Finally, one major metabolite present in the extract of *Streptomyces anulatus* strain S71 (**Figure 3A**) showed its UV-vis absorption (**Figure 4**) similar to that of typical angucyclines such as landomycin, which was absent in the control (**Figure 3B**). Subsequently, by using LC-MS, both HR ESI-MS ($[M+H]^+ m/z = 467.1326$) (**Figure 5**) and UV data (λ_{max}: 252, 434 nm) (**Figure 4**) of the target substance almost corresponded to the data reported for one angucycline amycomycin B (HRESIMS: *m/z* 489.1154 $[M+Na]^+$; UV λ_{max}: 249, 427 nm) (**Figure 6**) (Guo et al., 2012), indicating that the detected compound was either amycomycin B itself or its analog. This finding indicated that *S. anulatus* S71 produced angucycline compound under the lab culture condition. Unfortunately, we did not detect any expected aromatic polyketide from other strains under lab fermentation condition.

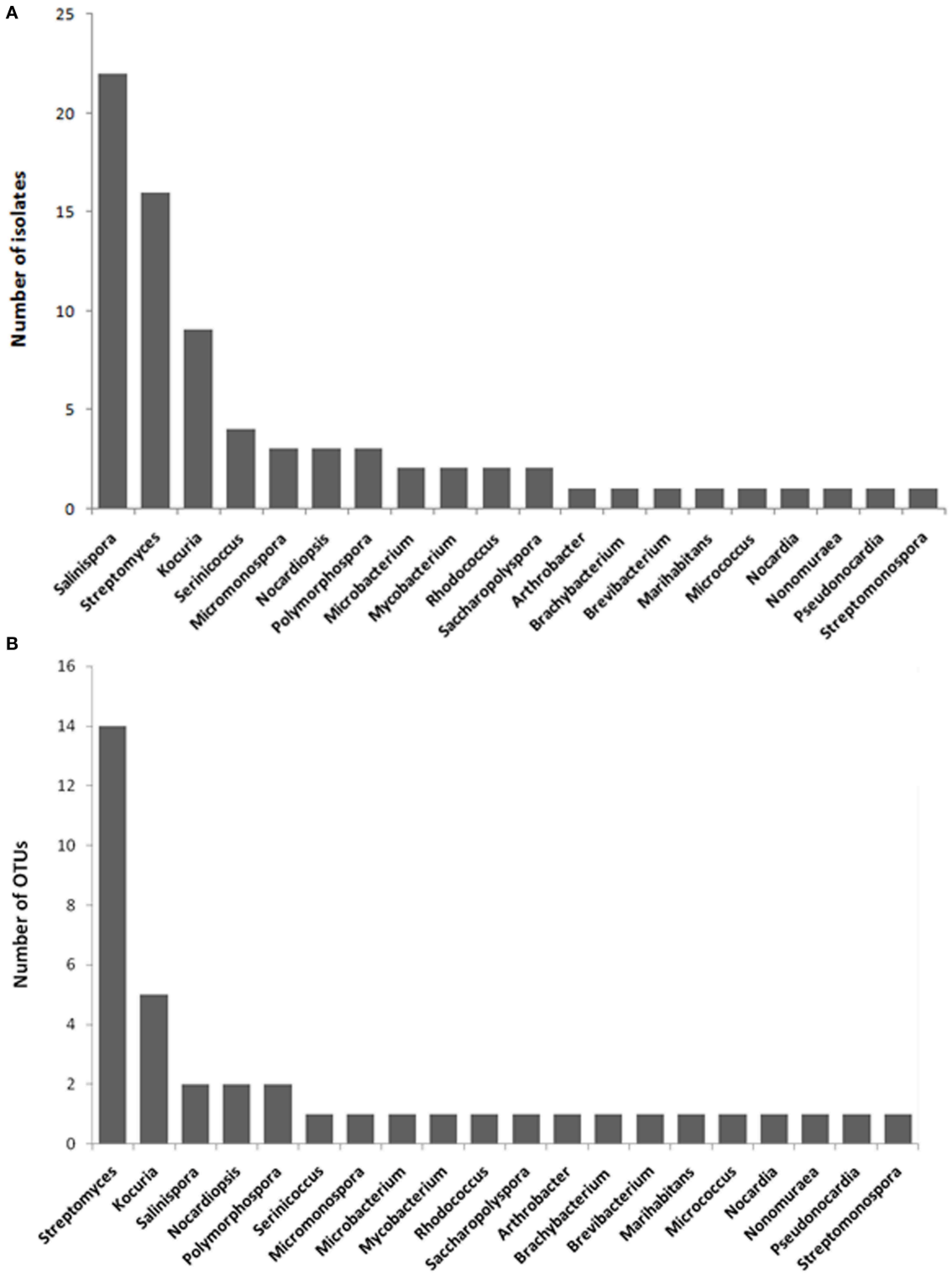

FIGURE 1 | Number of isolates (A) and OTUs per actinobacterial genus (B).

TABLE 3 | KS_α amino acid sequences.

Strain	Nearest type strain	No. of unique clones	NCBI accession no.	Top BLAST match[a](source organism)	BLAST match pathway product[a] (chemotype[b])	Similarity (%)
S12	*Kocuria gwangalliensis*	1	JX008015 AFO70129	ketosynthase(*Streptomyces cyaneus*)	Cur pigment (Pen)	85.2
S97*	*Micromonospora aurantiaca*	1	KJ094408 AHN91973	ketosynthase(*Streptomyces violaceoruber*)	Granaticin (Ben)	95.6
S107*	*Nocardia araoensis*	1	KJ094407 AHN91972	ketosynthase(*Streptomyces* sp.)	Unknown (Ang)	95.1
S78*	*Nocardiopsis alba*	2	JX008012 AFO70126	ketosynthase(*Streptomyces halstedii*)	sch pigment (Pen)	88.2
			JX008013 AFO70127	ketosynthase(*Streptomyces* sp.)	Benastatin (Pen)	89.2
S92*	*Nocardiopsis halotolerans*	1	KJ094409 AHN91974	ketosynthase(*Streptomyces tendae*)	Lysolipin (Pen)	88.7
S36*	*Saccharopolyspora gloriosa*	1	JX008003 AFO70117	ketosynthase(*Streptomyces antibioticus*)	Simocyclinone (Ang)	93.1
S33	*Salinispora arenicola*	1	JX008009 AFO70123	ketosynthase(*Streptomyces griseus*)	fredericamycin (Pen)	91.1
S34	*Salinispora tropica*	1	JX008010 AFO70124	ketosynthase(*Streptomyces griseus*)	fredericamycin (Pen)	90.1
S10*	*Streptomyces parvulus*	1	JX008008 AFO70122	ketosynthase(*Streptomyces antibioticus*)	simocyclinone (Ang)	92.1
S31	*Streptomyces carnosus*	1	JX008007 AFO70121	ketosynthase(*Streptomyces coelicolor*)	whiE pigment (Pen)	100
S39*	*Streptomyces djakartensis*	1	JX008002 AFO70116	ketosynthase(*Streptomyces* sp.)	sch 47554 (Ang)	93.1
S40	*Streptomyces luteosporeus*	1	JX008004 AFO70118	ketosynthase(*Streptomyces halstedii*)	sch pigment (Pen)	96.6
S41*	*Streptomyces rochei*	1	JX008005 AFO70119	ketosynthase(*Streptomyces ambofaciens*)	Unknown (Ang)	99.0
S71*	*Streptomyces anulatus*	1	JX008006 AFO70120	ketosynthase(*Streptomyces* sp.)	sch 47554 (Ang)	94.6
S72*	*Streptomyces xiamenensis*	1	JX008011 AFO70125	ketosynthase(*Actinomadura hibisca*)	Pradimicin (Ant)	88.2
S81	*Streptomyces diastaticus*	1	JX008014 AFO70128	ketosynthase(*Streptomyces coelicolor*)	whiE pigment (Pen)	98.0
S86	*Streptomyces griseorubens*	1	KJ094410 AHN91975	ketosynthase(*Streptomyces cyaneus*)	Cur pigment (Pen)	97.0

[a] *Top BLAST matches are to the KSα domains associated with experimentally characterized biosynthetic pathways of aromatic polyketides.*
[b] *Pen-Pentangular polyphenols, Ben-Benzoisochromanequinones, Ang-Angucyclines, Ant-Anthracyclines.*
*The 10 strains marked with *were selected for small-scale fermentation.*

Discussion

In this study, comprehensive investigation of 15 sponge species and combination of five culture media led to the isolation of 20 actinobacterial genera. The isolation of indigenous marine genera (*Marihabitans*, *Salinispora*, and *Serinicoccus*) showed the marine characteristic of the actinomycetes from the South China Sea sponges. Actinobacteria are widely dispersed throughout the marine environments, including water column, marine organisms, marine snow, and sediments (Ward and Bora, 2006). Here, we respectively compare the culturable diversity of the South China Sea sponge-associated actinomycetes with that of marine sediment-derived, coral-associated, and seawater-derived actinomycetes (**Table 4**). It is apparent that the actinobacterial diversity in any individual habitat cannot cover the diversity revealed in present study. Specifically, among the 20 genera from the South China Sea sponges, one genus (*Marihabitans*) has not been found from marine sediments, four genera (*Marihabitans*, *Nonomuraea*, *Polymorphospora*, and *Streptomonospora*) not isolated from corals, and six genera (*Nonomuraea*, *Polymorphospora*, *Pseudonocardia*, *Saccharopolyspora*, *Salinispora*, and *Streptomonospora*) not cultured from seawater. Consequently, South China Sea sponges displayed their advantage as a prolific source of culturable actinomycetes compared with other marine habitats.

Prior to our study, 15 actinomycete genera have been cultivated from South China Sea sponges, including

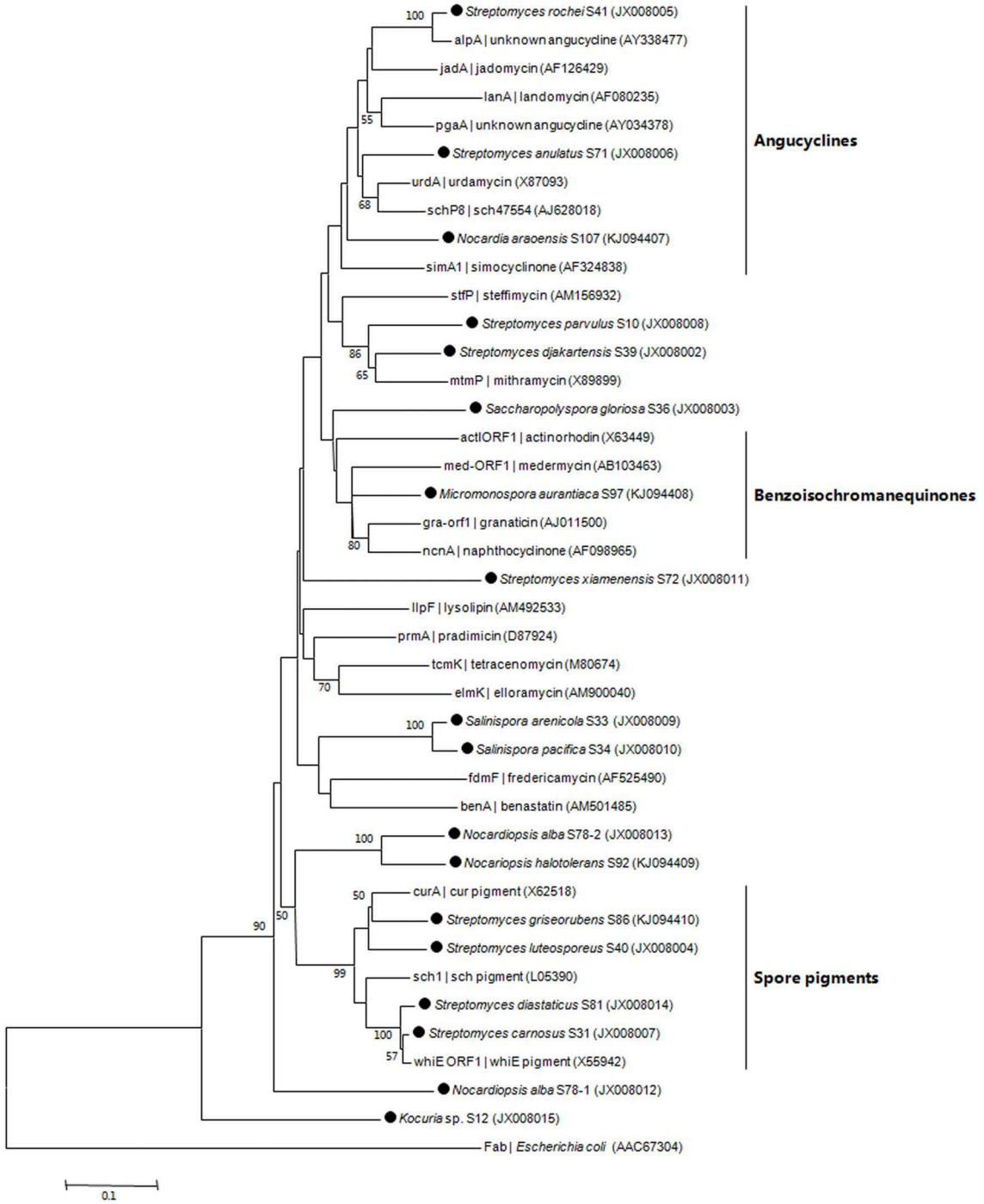

FIGURE 2 | Neighbor-joining tree constructed using aligned KS_{α} domain amino acid sequences (203 amino acid positions). The sequences obtained in this work are marked by black dot. Next to the KS_{α} gene name, the identified, or predicted compounds and GenBank accession number of the gene cluster are indicated. Boot strap values calculated from 1000 resamplings using neighborjoining are shown at the respective nodes when the calculated values were 50% or greater. The scale bar represents 0.1 substitutions per amino acid position.

Actinomadura, *Catenuloplanes*, *Cellulosimicrobium*, *Gordonia*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Nocardiopsis*, *Pseudonocardia*, *Rhodococcus*, *Saccharomonospora*, *Salinispora*, *Sphaerisporangium*, *Streptomyces*, and *Verrucosispora*. By investigating as many as 15 previously unexplored South China Sea sponges, the known diversity of sponge-associated actinomycetes was significantly extended, with a total of 27 genera successfully cultivated (including previously

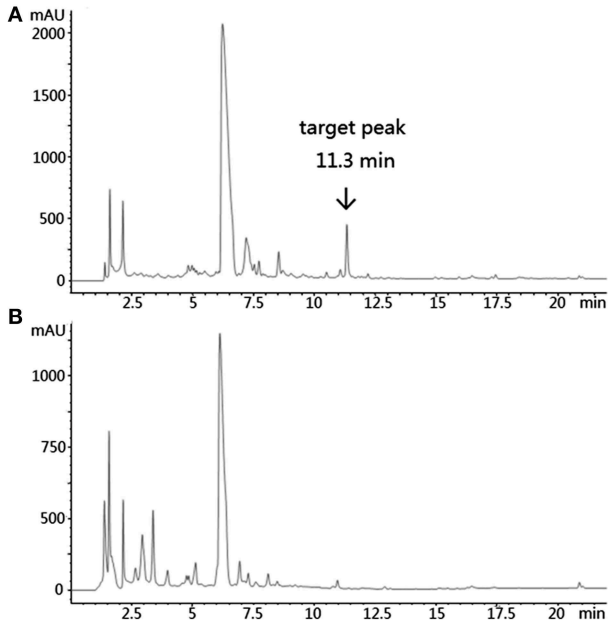

FIGURE 3 | HPLC of the ethyl acetate extract of *S. anulatus* S71 fermentation broth. Target peak was eluted at 11.3 min **(A)**. HPLC of the ethyl acetate extract of broth medium as a negative control **(B)**. Detection wavelength: 210 nm.

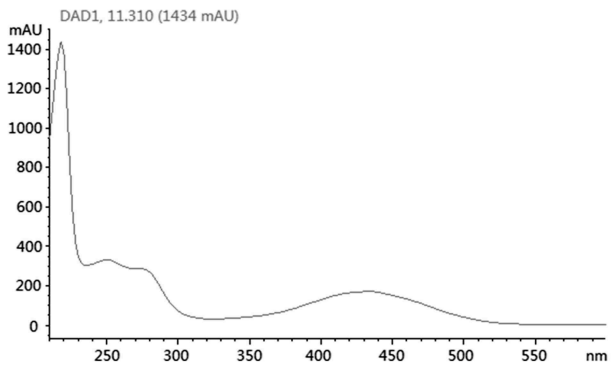

FIGURE 4 | UV/vis spectra of selected peak at t_R 11.3 min.

reported 15 genera and newly cultivated 12 genera in this study). Excitingly, three rare genera (*Streptomonospora*, *Polymorphospora*, and *Marihabitans*) were isolated from marine sponges for the first time. *Streptomonospora* is a group of strictly halophilic filamentous actinomycetes in Nocardiopsaceae. *Streptomonospora* strains were previously derived from hypersaline soil (Cai et al., 2008) and salt lake (Cai et al., 2009). Until recently, two *Streptomonospora* strains were found from marine sediments, indicating its existence in the marine environment (Zhang et al., 2013a). *Polymorphospora* is a genus in *Micromonosporaceae*, and *Polymorphospora* strains were mainly isolated from soil surrounding mangrove roots (Tamura et al., 2006). *Marihabitans* is a genus in *Intrasporangiaceae* (Kageyama et al., 2008). Notably, the genus is quite rare and only one strain was previously cultured from surface seawater (Kageyama et al., 2008).

Over the past decade, actinomycetes have been intensively isolated from sponges inhabiting the Yellow Sea, the Caribbean Sea, the Red Sea, and the Mediterranean Sea as well (Abdelmohsen et al., 2014a). By comparing the diversity of the sponge-associated actinomycetes from the separate geographical locations, we found that different region generally harbored distinct sponge-associated actinomycetes, including both common actinomycete genera (*Micromonospora*, *Nocardiopsis*, *Rhodococcus*, and *Streptomyces*) and respective different actinomycete groups (**Table 5**). Notably, seven genera (*Catenuloplanes*, *Marihabitans*, *Polymorphospora*, *Saccharopolyspora*, *Serinicoccus*, *Sphaerisporangium*, and *Streptomonospora*) not found from the sponges in other oceans were cultivated from the South China Sea sponges, indicating the biogeographic variability in the South China Sea sponge-associated actinobacterial communities.

The use of molecular approaches for describing microbial diversity has greatly enhanced the knowledge of population structure in sponge-associated bacterial communities. Diverse actinobacterial groups belonging to Actinobacteridae have been detected from various sponges (Simister et al., 2012). To our knowledge, at least 22 sponge-associated actinomycete genera have been revealed by molecular techniques, including *Actinomyces*, *Agromyces*, *Amycolatopsis*, *Arthrobacter*, *Brevibacterium*, *Cellulosimicrobium*, *Corynebacterium*, *Kocuria*, *Microbacterium*, *Micrococcus*, *Microlunatus*, *Micromonospora*, *Mycobacterium*, *Nocardioides*, *Nocardiopsis*, *Propionibacterium*, *Pseudonocardia*, *Rhodococcus*, *Ruania*, *Saccharopolyspora*, *Streptomyces*, and *Verrucosispora*. This number is much lower than that of the cultivated genera (60 genera) (Abdelmohsen et al., 2014a). Two factors are thought to lead to this result. First, the majority of the amplicon libraries were constructed using bacterial universal primers, thus it is difficult to detect those low-abundance actinobacterial groups. Second, environmental surveys based on 16S rRNA gene sequencing preferred to describe the bacterial community structure at the phylum level but not genus level. Therefore, the diversity of sponge-associated actinomycetes was mainly revealed by culture-based methods. Notably, to date several genera (*Actinomyces*, *Amycolatopsis*, *Microlunatus*, *Propionibacterium*, *Ruania*) detected by molecular techniques have not been isolated from sponges, suggesting that the diversity is still worth exploring in future.

Sponges contain diverse actinobacterial groups, however, the ecological functions of the actinobacteria are hardly known. Sponge-associated actinomycetes produce bioactive small molecules like their terrestrial counterparts do. The possibility cannot be excluded that some compounds play an important role in the chemical ecology of sponge hosts. Considering actinomycete-derived secondary metabolites commonly occur in a very low concentration, the compounds are difficult to be extracted directly from sponges. Consequently, exploring the metabolic potential of the sponge-associated actinomycete strains facilitates the discovery of novel bioactive molecules.

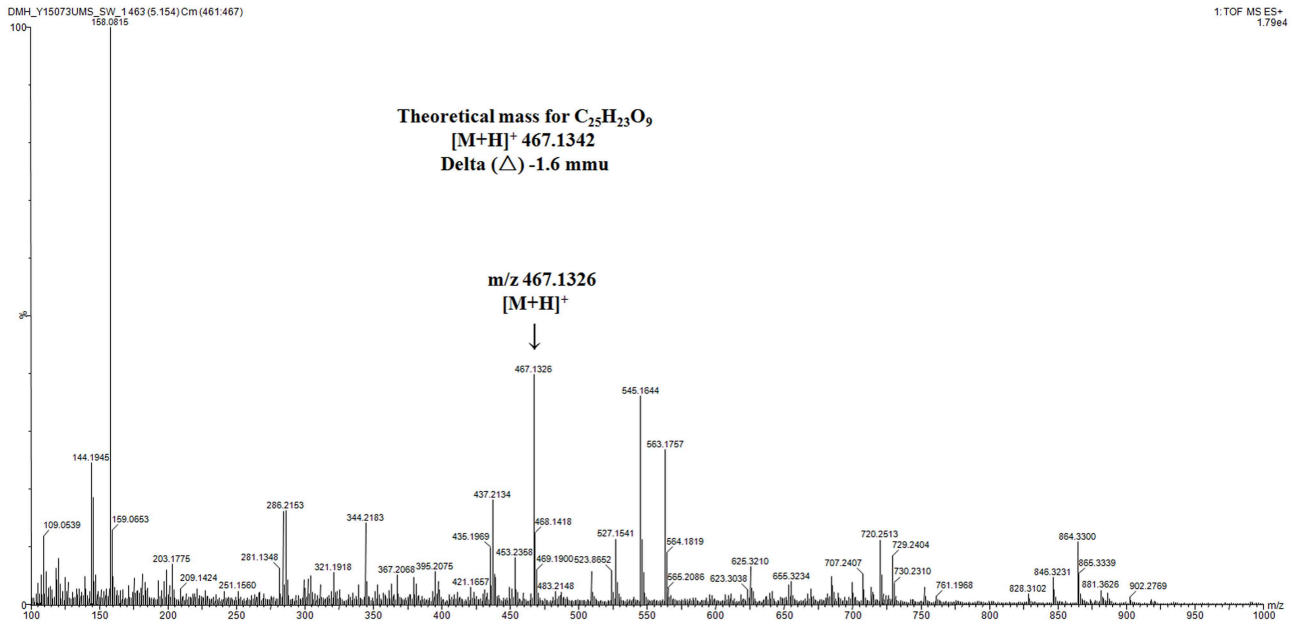

FIGURE 5 | High-resolution mass spectrum of selected ion at t_R 5.15 min in TIC. mmu, milli-mass units.

Aromatic polyketides are known to be produced by a few taxa among diverse actinomycetes. Thus, knowing their taxonomic distribution facilitates the prioritization of strains for aromatic polyketide search and discovery. In this work, seven genera (*Kocuria*, *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Saccharopolyspora*, *Salinispora*, and *Streptomyces*) were screened out as potential producers of aromatic polyketides, including both recognized and previously not recognized producers. Notably, strains related to *Streptomyces*, *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Saccharopolyspora*, and *Salinispora* were known producers of aromatic polyketides (Sun et al., 2007; Perez et al., 2009; Ding et al., 2012; Sousa et al., 2012; Xie et al., 2012; Jensen et al., 2015). However, one genus (*Kocuria*) not traditionally associated with aromatic polyketide production was detected as well, suggesting that poorly studied genera may be potential producers of aromatic polyketides. To date, aromatic polyketides have not been isolated from strains related to *Kocuria*, therefore, their potential in aromatic polyketide biosynthesis deserves further exploration.

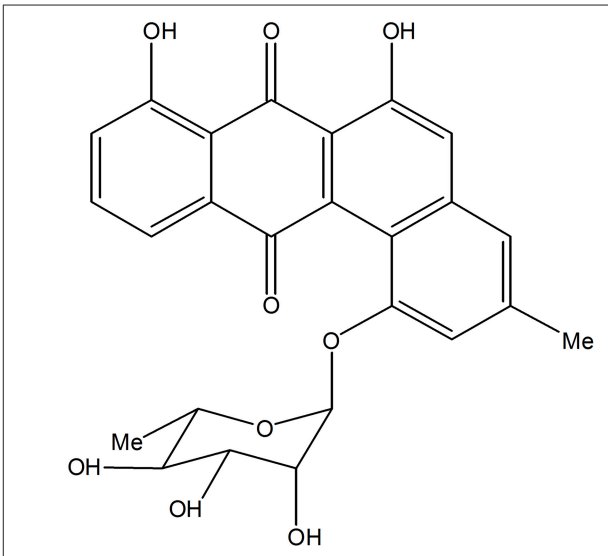

FIGURE 6 | Structure of amycomycin B.

In recent years, phylogenetic prediction has been successfully applied in the discovery of type I polyketides (Gontang et al., 2010). By bioinformatic analyses of KS sequence the prediction was preliminarily made, and test for the production of target compounds was subsequently preformed to confirm the sequence-based analyses. Considering diverse tailoring enzymes involved in the aromatic polyketide biosynthesis (Schneider, 2005), we think it is not feasible to accurately predict target substance merely based on KS_α sequence analysis. However, due to the conserved property of KS_α domain, it is possible to correlate one KS_α sequence (one strain) with one specific subtype (Metsä-Ketelä et al., 2002). Among 17 representative strains, eight were specifically related to three subgroups, respectively angucyclines, benzoisochromanequinones, and spore pigments (**Figure 2**). The angucycline group is the largest group of aromatic polyketides, rich in chemical scaffolds and biological activities (Kharel et al., 2012). The benzoisochromanequinone group comprises fewer compounds than angucyclines but its members show a wide range of biological activities as well (Brimble et al., 1999). Additionally, other nine strains cannot be correlated with specific chemotypes (**Figure 2**). However, these strains should not be neglected

TABLE 4 | Comparison of the culturable diversity of South China Sea sponge-associated actinomycetes with that of marine sediment-derived, coral-associated, and seawater-derived actinomycetes.

South China Sea sponge-associated actinomycetes	Marine sediment-derived actinomycetes	Coral-associated actinomycetes	Seawater-derived actinomycetes	References
Arthrobacter	+	+	+	Wietz et al., 2012; Yang et al., 2013; Zhang et al., 2014
Brachybacterium	+	+	+	Wang et al., 2010; Yang et al., 2013; Zhang et al., 2014
Brevibacterium	+	+	+	Wang et al., 2010; Yang et al., 2013; Zhang et al., 2014
Kocuria	+	+	+	Wang et al., 2010; Yang et al., 2013; Zhang et al., 2014
Marihabitans	−	−	+	Kageyama et al., 2008
Microbacterium	+	+	+	Wang et al., 2010; Yang et al., 2013; Zhang et al., 2014
Micrococcus	+	+	+	Harwati et al., 2007; Yang et al., 2013; Zhang et al., 2014
Micromonospora	+	+	+*	Chen et al., 2011; Yang et al., 2013
Mycobacterium	+	+	+	Al-Awadhi et al., 2012; Yang et al., 2013; Zhang et al., 2014
Nocardia	+	+	+*	Chen et al., 2011; Zhang et al., 2013b
Nocardiopsis	+	+	+*	Maldonado et al., 2005; Zhang et al., 2013b
Nonomuraea	+	−	−	Maldonado et al., 2005
Polymorphospora	+	−	−	Tamura et al., 2006
Pseudonocardia	+	+	−	Maldonado et al., 2005; Zhang et al., 2013b
Rhodococcus	+	+	+	Chen et al., 2011; Al-Awadhi et al., 2012; Yang et al., 2013
Saccharopolyspora	+	+	−	Maldonado et al., 2005; Zhang et al., 2013b
Salinispora	+	+*	−	Gontang et al., 2007
Serinicoccus	+	+*	+	Yi et al., 2004; Gontang et al., 2007
Streptomonospora	+	−	−	Zhang et al., 2013a
Streptomyces	+	+	+	Chen et al., 2011; Zhu et al., 2011; Yang et al., 2013

+, The actinomycete genera are also cultivated from other marine habitats.

−, The actinomycete genera have not been cultivated from other marine habitats.

**16S rRNA gene sequences were submitted to GenBank but paper is unpublished.*

because they potentially have the capacity to produce novel subtypes.

For the rapid identification of aromatic polyketides from crude culture extracts, it is critical to develop an efficient approach. At present, it is feasible to determine the elemental composition of compounds in mixtures and identify natural products using LC/MS and UV/vis spectra (Nielsen et al., 2011; El-Elimat et al., 2013). In the case of aromatic polyketides, UV/vis spectra provided important clues on the presence of unsaturated cyclohexanedione structure and polyphenolic ring system and thus indicated the compound type, and LC/MS analysis gave precise molecular weight and suggested molecular formula of target signal. Subsequently, the molecular formulas were used as queries to match those reported aromatic polyketides in database. If some compounds were retrieved, then their UV-vis absorption maxima are compared with target substance. Only when both UV/vis spectra and high-resolution molecular weight were consistent, the compound was identified as known one or its analog. This method avoided large-scale fermentation and purification processes, thus saved time and resource. It can be used as a dereplication protocol for aromatic polyketides and enhance the efficiency of discovering novel aromatic polyketides.

To our knowledge, actinomycete strains generally contain a number of biosynthetic gene clusters. However, only a few corresponding metabolites have been obtained until now. Apparently, the majority of the biosynthetic gene clusters are unexpressed under standardized laboratory conditions, which leads to a low efficiency in the discovery of their secondary metabolites. Similarly, it is also present in the aromatic polyketide discovery from the South China Sea sponge-associated actinomycetes. Surveying recent advances in microbial natural product discovery, we think two strategies can be considered to exclusively explore the metabolic potential of the strains. One is to try activating silent biosynthetic pathways through external cues, co-cultivation and stress since it has achieved great success in the natural product discovery from fungi and actinomycetes (Scherlach and Hertweck, 2009). The other is to apply genetic manipulation techniques such as gene cluster cloning and heterologous expression because it has shown unique advantage in harvesting rare skeletons of aromatic polyketides (Feng et al., 2011). They should be preferentially attempted in future work.

In summary, a total of 20 actinomycete genera were isolated from the South China Sea sponges, including three rare genera (*Marihabitans*, *Polymorphospora*, and *Streptomonospora*) found from sponges first time. Potential aromatic polyketide producers were distributed in seven genera (*Kocuria*, *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Saccharopolyspora*, *Salinispora*, and *Streptomyces*). By small-scale fermentation, one angucycline compound was detected from

TABLE 5 | Comparison of the culturable diversity of the sponge-associated actinomycetes from the South China Sea, Yellow Sea, Caribbean Sea, Red Sea, and Mediterranean Sea.

Actinomycete genera	South China Sea sponge-associated actinomycetes	Yellow Sea sponge-associated actinomycetes	Caribbean sponge-associated actinomycetes	Red Sea sponge-associated actinomycetes	Mediterranean sponge-associated actinomycetes
Actinoalloteichus	−	+	−	−	−
Actinokineospora	−	−	−	+	−
Actinomadura	+	+	−	−	−
Arthrobacter	+	−	−	+	−
Blastococcus	−	+	−	−	−
Brachybacterium	+	−	−	+	−
Brevibacterium	+	−	−	+	−
*Catenuloplanes**	+	−	−	−	−
Cellulosimicrobium	+	−	+	−	−
Corynebacterium	−	−	−	+	+
Curtobacterium	−	−	−	+	−
Dietzia	−	−	−	+	−
Georgenia	−	+	−	−	−
Gordonia	+	+	−	−	+
Kocuria	+	−	−	+	+
*Marihabitans**	+	−	−	−	−
Microbacterium	+	−	+	+	−
Micrococcus	+	−	−	+	−
Micromonospora	+	+	+	+	+
Mycobacterium	+	−	−	+	+
Nocardia	+	+	−	+	−
Nocardiopsis	+	+	−	+	+
Nonomuraea	+	+	−	−	−
*Polymorphospora**	+	−	−	−	−
Pseudonocardia	+	+	−	−	−
Rhodococcus	+	+	−	+	+
Rothia	−	−	−	+	+
Rubrobacter	−	−	−	−	+
Saccharomonospora	+	−	−	+	−
*Saccharopolyspora**	+	−	−	−	−
Salinispora	+	−	+	+	−
*Serinicoccus**	+	−	−	−	−
Solwaraspora	−	−	+	−	−
*Sphaerisporangium**	+	−	−	−	−
*Streptomonospora**	+	−	−	−	−
Streptomyces	+	+	+	−	+
Verrucosispora	+	−	+	−	−

*The genera marked with *were currently limited to South China Sea. The shading on rows highlight the sponge-associated actinomycete genera widely distributed in distinct oceans.*

one *Streptomyces* isolate. This work advanced our knowledge of sponge-associated actinomycetes regarding their diversity and biogeography, and revealed their potential in aromatic polyketide production.

Author Contributions

ZL and WS designed the study. LH identified the sponge samples. WS performed the experiments. WS and FZ analyzed the data. WS, ZL, and KL wrote the manuscript. All authors read and approved the final manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01048

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Diversity and distribution of *Actinobacteria* associated with reef coral *Porites lutea*

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Actinobacteria is a ubiquitous major group in coral holobiont. The diversity and spatial and temporal distribution of actinobacteria have been rarely documented. In this study, diversity of actinobacteria associated with mucus, tissue and skeleton of *Porites lutea* and in the surrounding seawater were examined every 3 months for 1 year on Luhuitou fringing reef. The population structures of the *P. lutea*-associated actinobacteria were analyzed using phylogenetic analysis of 16S rRNA gene clone libraries, which demonstrated highly diverse actinobacteria profiles in *P. lutea*. A total of 25 described families and 10 unnamed families were determined in the populations, and 12 genera were firstly detected in corals. The *Actinobacteria* diversity was significantly different between the *P. lutea* and the surrounding seawater. Only 10 OTUs were shared by the seawater and coral samples. Redundancy and hierarchical cluster analyses were performed to analyze the correlation between the variations of actinobacteria population within the divergent compartments of *P. lutea*, seasonal changes, and environmental factors. The actinobacteria communities in the same coral compartment tended to cluster together. Even so, an extremely small fraction of OTUs was common in all three *P. lutea* compartments. Analysis of the relationship between actinobacteria assemblages and the environmental parameters showed that several genera were closely related to specific environmental factors. This study highlights that coral-associated actinobacteria populations are highly diverse, and spatially structured within *P. lutea*, and they are distinct from which in the ambient seawater.

Keywords: actinobacteria, *Porites lutea*, diversity, temporal and spatial distribution, 16S rRNA gene

Introduction

Coral reef ecosystem is one of the most important tropical marine ecosystems, mainly distributed in the Indo-West Pacific, Eastern Pacific, Western Atlantic, and the Eastern Atlantic (Moberg and Folke, 1999). Corals provide habitats for numerous bacteria in their mucus layer, tissue, and calcium carbonate skeleton, as well as the dinoflagellates, fungi, archaea, and viruses (Rosenberg et al., 2007). Coral-associated bacteria not only take part in carbon, nitrogen, and sulfur biogeochemical cycles and provide necessary nutrient for coral, but also keep corals from being infected by pathogens (Rosenberg et al., 2007; Raina et al., 2009; Bourne and Webster, 2013).

Highly diverse and heterogeneous bacterial communities have been revealed in different coral species at various locations (Rohwer et al., 2002; Li et al., 2013). *Actinobacteria* is generally accepted

as a ubiquitous major group in corals (Bourne and Munn, 2005; Carlos et al., 2013; Li et al., 2013, 2014a). Yang et al. (2013) detected 19 *Actinobacteria* genera in soft coral *Alcyonium gracllimum* and stony coral *Tubastraea coccinea* in the East China Sea through analysis of 16S rRNA gene clone libraries. Some actinobacterial genera were previously detected in corals by using the culture-dependent method (Lampert et al., 2006; Nithyanand and Pandian, 2009; Nithyanand et al., 2011b; Zhang et al., 2013; Li et al., 2014b). Among these culturable actinobacteria, *Streptomyces*, *Verrucosispora*, *Rhodococcus*, *Micromonospora*, *Nocardia*, *Jiangella*, *Nocardiopsis*, *Pseudonocardia*, and *Salinispora* showed antibacterial activities, which were considered to contribute to coral health (Ritchie, 2006; Nithyanand et al., 2011a; Krediet et al., 2013; Zhang et al., 2013; Li et al., 2014b).

Environmental conditions, coral species, colony physiology, and seasonal variation are considerable influencing factors on the coral-associated bacterial community (Hong et al., 2009). Moreover, due to various microhabitats provided by corals' biological structures, the spatial heterogeneity has been proved in bacterial communities associated with a single coral colony (Rohwer et al., 2002; Sweet et al., 2011; Li et al., 2014a). As a major coral-associated bacterial group, how actinobacteria is spatially and temporally organized in corals, and what is the connection between the actinobacteria communities in corals and in seawater remains poorly understood. Comprehensive investigation of the distribution of this ubiquitous group at spatial and temporal scales will help understanding the variation of coral associated bacteria and the potential function of actinobacteria, and will contribute a lot to bioprospect the actinobacteria resources for utilization as novel sources for bioactive natural products.

Coral reefs are widely distributed in the South China Sea (Liu et al., 2009; Wang et al., 2014). *Porites lutea* is the dominant, typical coral species in the Luhuitou fringing reef, which is located in the south end of the Hainan province (Zhao et al., 2008). In this study, the diversity and distribution of actinobacteria were investigated in coral *P. lutea* and in the surrounding seawater every 3 months for 1 year using culture-independent method for the first time. We aimed to reveal the coral-associated actinobacteria community structures in three divergent coral compartments in different months, compare the actinobacterial communities in the coral and in the surrounding seawater, and research the actinobacteria community variation responds to the environmental factors.

Materials and Methods

Sample Collection

The coral and surrounding sea water samples were collected in four different months (February, May, August, and November) in 2012 from the Luhuitou fringing reef (109°28′E, 18°13′N). Coral fragments (approximately 10 × 10 cm) were collected from the side of three healthy *P. lutea* colonies at the depth of 3–5 m each time using punch and hammer. Coral mucus, tissues and skeleton were separated and stored according to the method described previously (Li et al., 2014a). One liter of seawater adjacent to the coral colonies was collected, and filtered through 0.22 μm-pore-size filter membrane (Millipore). The filter membranes were stored at −80°C until DNA extraction. As the samples were collected at the same time, environmental parameters including water temperature, salinity, dissolved oxygen, pH value, ultraviolet radiation intensity, and rainfall were cited from the published data (Li et al., 2014a).

DNA Extraction and PCR Amplification

The coral tissue and skeleton samples were homogenized thoroughly in liquid nitrogen with sterile mortar and pestle before added to the PowerBead Tubes. The filter membranes with adsorbed microbial cells were cut into pieces, and then added to the PowerBead Tubes. Total DNA was extracted using the PowerSoil DNA Isolation Kit (MoBio, Solana Beach, CA, USA) according to the manufacturer's instruction.

16S rRNA genes were nest PCR amplified, the first PCR reactions using the combination of universal bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). PCR amplifications were performed in a Mastercycler pro (Eppendorf, Hamburg, Germany) in a final volume of 50 μL, containing 2 μL (10 μM) each primer, 1 μL (10–20 ng) template DNA and 25 μL premix *Ex Taq* mixture (Takara, Dalian). The PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 90 s; followed by 72°C for 10 min. In the second PCR reactions, the actinobacteria-specific primer pairs, S-C-Ac-0325-a-S-20 (5′-CGCGCCTATCAGCTTGTTG-3′) and S-C-Act-0878-a-A-19 (5′-CCGTATCCCCAGGCGGGG-3′), were used to amplify the V3-V5 regions (about 640 bp) of the actinobacteria 16S rRNA gene (Stach et al., 2003). In the PCR reactions, 5 μL of 1: 10 dilution of the first round PCR product was used as DNA template, the PCR mixture (50 μL) contain 2 μL (10 μM) each primer, 25 μL premix *Ex Taq* mixture, the PCR conditions were as follows: 95°C for 5 min; 30 cycles of 95°C for 45 s, 68°C for 45 s, 72°C for 60 s; followed by 72°C for 10 min. Each genomic DNA sample was amplified in triplicate PCR reactions. Amplicons from the same sample were pooled and purified using the E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek, China).

Gene Library Construction and Sequencing

Sixteen clone libraries of actinobacterial 16S rRNA genes were constructed using the pMD18-T Vector Cloning Kit and *E. coli* DH5α competent cells (Takara, Dalian) following the manufacturer's instructions. The positive clones from each library inoculated on MacConkey agar with ampicillin (100 μg/ml) were randomly picked and sequenced using M13F (−47) primer on ABI 3730xl capillary sequencers (Applied Biosystems, USA).

Libraries Analysis

The vector sequences were screened by the VecScreen tool provided in NCBI (http://www.ncbi.nlm.nih.gov/tools/vecscreen/). Chimeras were checked by running chimera.uchime packaged in Mothur (Schloss et al., 2009), and potential chimeras were removed. All valid sequences were deposited in GenBank

(accession numbers were shown in Data S1). All qualified sequences were identified by using the classify.seqs command in Mothur with Silva reference alignment database (http://www.mothur.org/wiki/Silva_reference_files, Release 119) at a confidence level of 80%. The sequences, which do not belong to *Actinobacteria*, were removed from further analysis. Sequences were clustered into operational taxonomic units (OTUs) with a 97% threshold using the cluster command in Mothur. The relationships among actinobacterial communities associated with different coral compartments and in the ambient seawater in different months were analyzed by hierarchical cluster analysis. Based on Bray-Curtis similarity estimated from the OTU matrix, clustering was generated by using the complete linkage method with the PRIMER 5 software (Clarke, 1993). The shared OTUs were determined by using the online tool venny (Oliveros, 2007–2015, http://bioinfogp.cnb.csic.es/tools/venny/index.html).

The correlations between *Actinobacteria* assemblages of coral samples and the environmental factors were analyzed by using the software package CANOCO 4.5.1 (ter Braak and Šmilauer, 2002). Redundancy analysis (RDA) was carried out to determine the relationship between the actinobacteria community and the environmental factors including temperature, salinity, dissolved oxygen, pH value, rainfall, and UV radiation and in combination with two nominal variables including the coral divergent compartments and the different sampling months. The significance of the relation between the explanatory variables and the actinobacterial community compositions was tested using Monte Carlo permutation tests (9999 unrestricted permutations, $P < 0.05$).

Results

Coral-associated Actinobacteria Diversity

A total of 2403 sequences were obtained from sixteen 16S rRNA gene clone libraries, resulting in 395 OTUs (stringency at 97%). The rarefaction analysis result showed that most of the curves did not flatten to asymptote, but climbed less steeply (**Figure 1**). The coverages ranged from 0.69 to 0.97 in 16 libraries, and the average coverage was 0.83 (**Table 1**). The highest number of OTUs was found in the tissue collected in May, while the lowest OTUs was found in the skeleton collected in November (**Table 1**). The Shannon indices in mucus collected in different months ranged from 2.32 to 3.44, from 2.45 to 3.55 in tissues, from 1.82 to 3.35 in skeleton, and from 1.53 to 2.82 in sea water (**Table 1**), and the diversity in the actinobacterial community associated with *P. lutea* was higher than which in the surrounding sea water ($P = 0.045$).

Coral-associated Actinobacterial Community Composition

At a confidence threshold of 80%, 2403 qualified reads were assigned to four classes, i.e., *Acidimicrobiia*, *Actinobacteria*, *Thermoleophilia*, and KIST-JJY010. Among them, *Acidimicrobiia* and *Actinobacteria* were ubiquitous and dominant in *P. lutea* and in the seawater samples. *Thermoleophilia* was not detected in corals collected in February, in the mucus and seawater in May, and in the mucus in August, while accounted for 0.5–48.8% in all other samples. KIST-JJY010 was detected only in the mucus in November (0.6%), and in the skeleton in August (2.6%).

Twenty-five described families and 10 unnamed families were detected in the 16 libraries (**Figure 2**). OM1_clade and *Propionibacteriaceae* (genera *Friedmanniella* and *Propionibacterium*) were ubiquitous, major groups in *P. lutea*. Meanwhile, OM1_clade was not detected in the seawater in

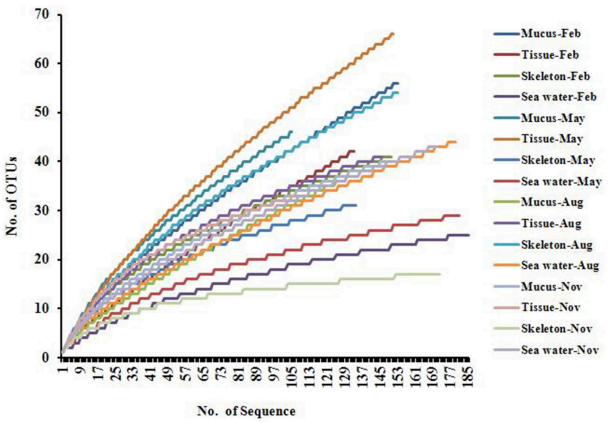

FIGURE 1 | Rarefaction curves of *Actinobacteria* 16S rRNA gene sequences.

TABLE 1 | Number of sequences and OTUs (97%) and diversity estimates of the *Actinobacteria* libraries in *P. lutea* and in the ambient seawater.

Index	A1	A2	A3	A4	B1	B2	B3	B4	C1	C2	C3	C4	D1	D2	D3	D4
No. of Seq.	153	133	150	185	105	151	134	181	132	146	153	179	149	109	172	171
OTUs	56	42	41	25	46	66	31	29	37	41	54	44	40	33	17	43
Chao	343.00	147.60	69.88	34.43	108.14	201.13	44.00	55.25	63.86	64.75	124.13	106.14	55.83	48.17	19.50	66.00
ACE	600.00	756.54	131.72	56.36	194.33	388.96	61.80	63.38	535.51	93.22	182.95	108.04	68.08	46.83	21.10	114.38
Shannon	3.33	2.45	3.08	1.53	3.44	3.55	2.70	1.89	2.32	3.07	3.35	2.68	2.89	3.11	1.82	2.84
Coverage	0.73	0.75	0.85	0.94	0.71	0.69	0.90	0.92	0.79	0.86	0.78	0.83	0.87	0.87	0.97	0.86

A1, mucus in February; A2, tissue in February; A3, skeleton in February; A4, seawater in February; B1, mucus in May; B2, tissue in May; B3, skeleton in May; B4, seawater in May; C1, mucus in August; C2, tissue in August; C3, skeleton in August; C4, seawater in August; D1, mucus in November; D2, tissue in November; D3, skeleton in November; D4, seawater in November.

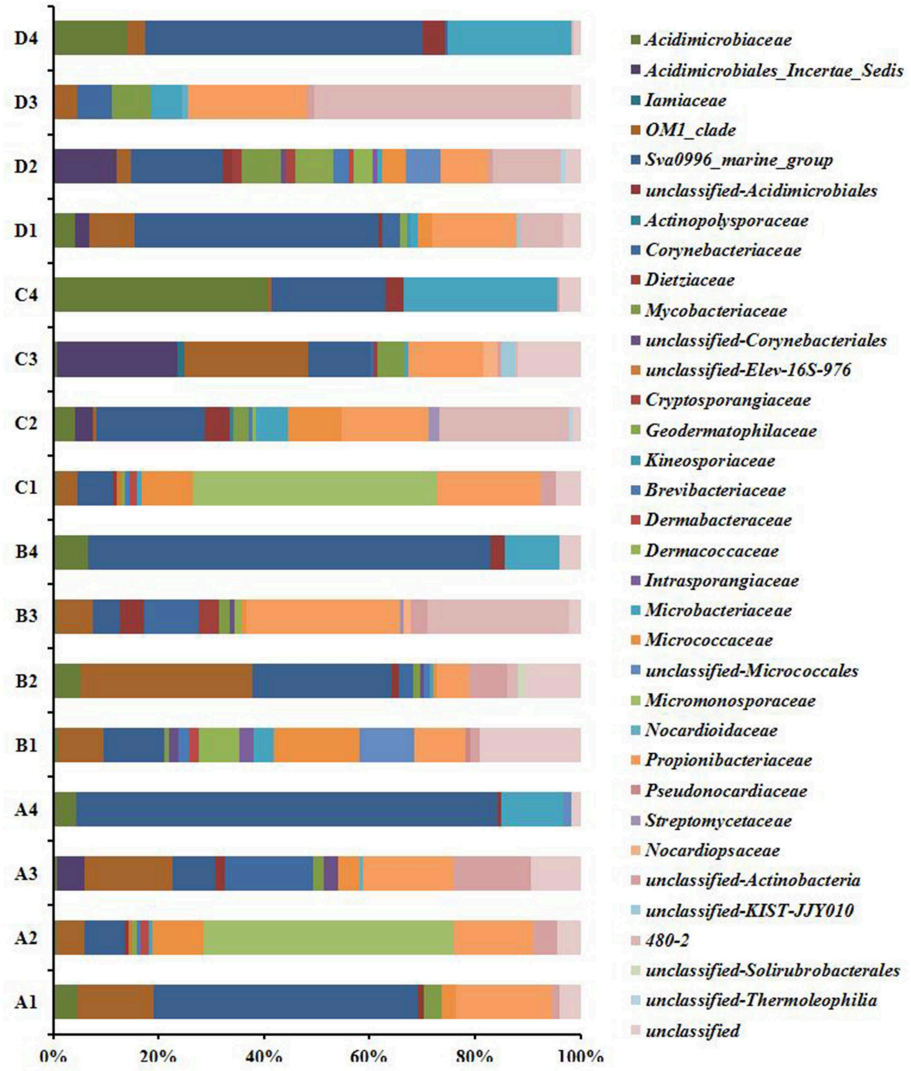

FIGURE 2 | *Actinobacteria* composition profiles. Taxonomic classification of actinobacteria sequences in to family identified by using the classify.seqs command in Mothur using Silva reference alignment database (http://www.mothur.org/wiki/Silva_reference_files, Release 119) with a confidence level of 80% were applied for classification. A1, mucus in February; A2, tissue in February; A3, skeleton in February; A4, seawater in February; B1, mucus in May; B2, tissue in May; B3, skeleton in May; B4, seawater in May; C1, mucus in August; C2, tissue in August; C3, skeleton in August; C4, seawater in August; D1, mucus in November; D2, tissue in November; D3, skeleton in November; D4, seawater in November.

February and May, and rare in the other two seawater libraries, and *Propionibacteriaceae* was absent in all the seawater libraries. *Micromonosporaceae* was the most abundant group in the tissue in February (47.4%) and in the mucus in August (46.2%), in which most of the reads were affiliated with an unclassified group. Nonetheless, *Micromonosporaceae* was absent in all other coral and seawater samples. Sva0996_marine_group was detected in all coral samples (5.2–50%) except in the skeleton collected in November, and which also was abundant in the ambient sea water (21.9–80%). *Micrococcaceae* was absent in the coral skeleton collected in August and in November, and in the sea water samples. Group 480-2 was abundant in the coral tissue in August (24.7%), as well as in the skeleton in May (26.9%) and in November (48.8%), but it was nearly absent in the surrounding seawater. In reverse, *Microbacteriaceae* and *Ilumatobacter* were major groups in sea water, while they were less abundant in *P. lutea*.

Spatial and Temporal Distribution of *P. lutea*-associated Actinobacteria

Results of hierarchical cluster analysis showed that the actinobacteria communities were significantly different between in the coral and in the surrounding seawater samples ($p = 0.01$, $R = 0.993$). The actinobacterial communities associated with the same coral compartments tended to cluster together (**Figure 3**). The season factor did not significantly influence the variation in the actinobacteria communities. The RDA results indicated that 38.9% of the total variance in the coral-associated actinobacterial

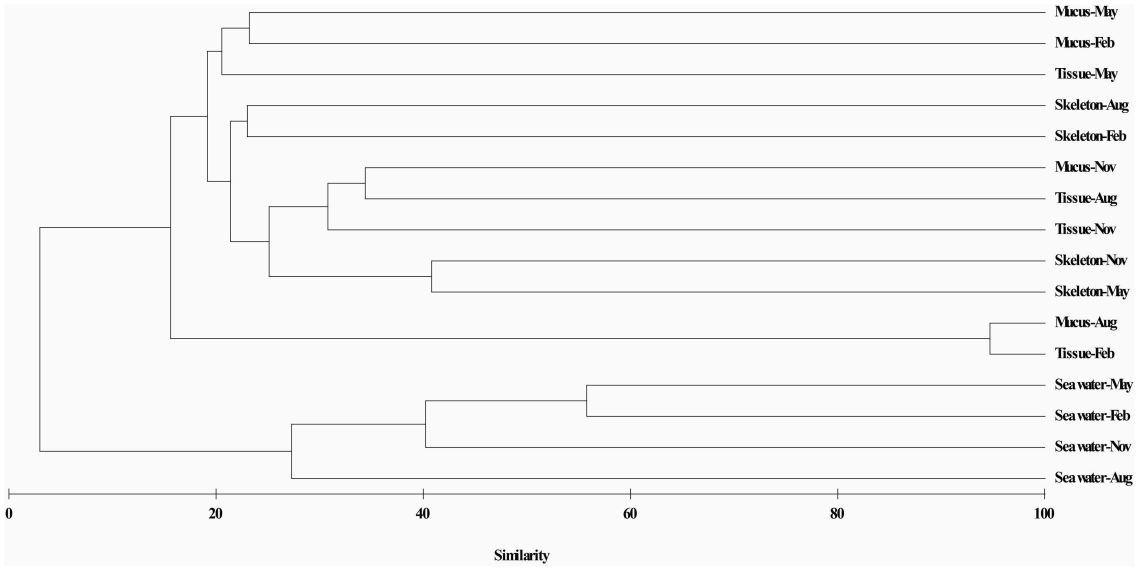

FIGURE 3 | Hierarchical cluster analysis of actinobacteria communities associated with *P. lutea*. Clustering was based on Bray-Curtis similarity estimated from the OTUs matrix by using the complete linkage method.

composition was explained by the environmental, spatial and temporal factors (**Figure 4**). The first and second axes differentiated the actinobacteria assemblages in the distinct coral compartments (**Figure 4**, Table S1). This result was consistent with the hierarchical cluster analysis. None of the environment parameters analyzed in this study was determined as the significant influencing factor in the variation of the *P. lutea* associated actinobacteria communities. A triplot map illustrated the relationship between major actinobacterial groups, with abundance more than 1%, and the environmental parameters (**Figure 4**). *Friedmanniella* and *Micrococcus* were positively related with the salinity. *Microbacterium*, *Propionibacterium*, and group 480-2 were positively correlated with seawater temperature, but negatively correlated with dissolved oxygen.

To investigate the distribution of OTUs in the three divergent coral compartments (mucus, tissue, and skeleton) and in the surrounding seawater, a venn diagram was constructed. The results showed that only 5 OTUs were present in all of *P. lutea* mucus, tissue and skeleton, and in sea water, which were identified as Sva0996_marine_group, *Ilumatobacter*, *Corynebacterium*, OM1_clade and *Microbacterium* (**Table 2**, Figure S1A). Another 17 OTUs, which were identified as *Candidatus*_Microthrix, *Corynebacteriales*, *Friedmanniella*, *Micrococcus*, *Mycobacterium*, OM1_clade, *Propionibacterium*, Sva0996_marine_group, *Yonghaparkia* and 480-2 were common in mucus, tissue, and skeleton (**Table 2**, Figure S1A). Twelve OTUs distributed in *Propionibacterium*, *Friedmanniella*, OM1_clade, Sva0996_marine_group, *Kocuria*, *Mycobacterium*, *Corynebacteriales*, *Brevibacterium,* and *Brachybacterium* were present in coral libraries in all four different months (**Table 3**, Figure S1B). The most abundant OTU0003, which was classified as *Propionibacterium*, was present in all coral samples with a high abundance (128 out of total 1687 reads in the coral libraries, 7.6%). The secondary abundance OTU0004 affiliated with *Friedmanniella* was present in all libraries except in skeleton collected in November.

Discussion

Highly Diverse Actinobacteria Associated with *P. lutea*

In comparison with previously reported results (Lampert et al., 2006, 2008; Bruck et al., 2007; Kageyama et al., 2007; Santiago-Vázquez et al., 2007; Ben-Dov et al., 2009; Nithyanand and Pandian, 2009; Seemann et al., 2009; Shnit-Orland and Kushmaro, 2009; de Castro et al., 2010; Thomas et al., 2010; Nithyanand et al., 2011a,b; Cardenas et al., 2012; Chiu et al., 2012; Sun et al., 2012, 2014; Zhang et al., 2012, 2013; Yang et al., 2013; Chen et al., 2014; Li et al., 2014a,b; EIAhwany et al., 2015; Sarmiento-Vizcaíno et al., 2015), 12 genera including *Actinopolyspora*, *Blastococcus*, *Candidatus*_Aquiluna, *Demetria*, *Fodinicola*, *Friedmanniella*, *Geodermatophilus*, *Iamia*, *Modestobacter*, *Ornithinimicrobium*, *Tersicoccus*, and *Yonghaparkia* were firstly detected in corals in this study (**Table 4**). Furthermore, many unclassified groups were detected in *P. lutea*, including even the group at the class taxon level. These results suggested that highly diverse and abundant known actinobacteria were associated with *P. lutea* as well as unknown groups. It was also noticed that many actinobacterial groups were only detected by the culture-independent method (**Table 4**), and some of them were ubiquitous and abundant, such as *Friedmanniella*, *Ilumatobacter*, and OM1_clade. Their physiological properties and ecological significance are worthy of deep research. For this purpose, the development and innovation of the isolation and cultivation methods in order to obtain pure cultures from the coral holobiont is particularly important.

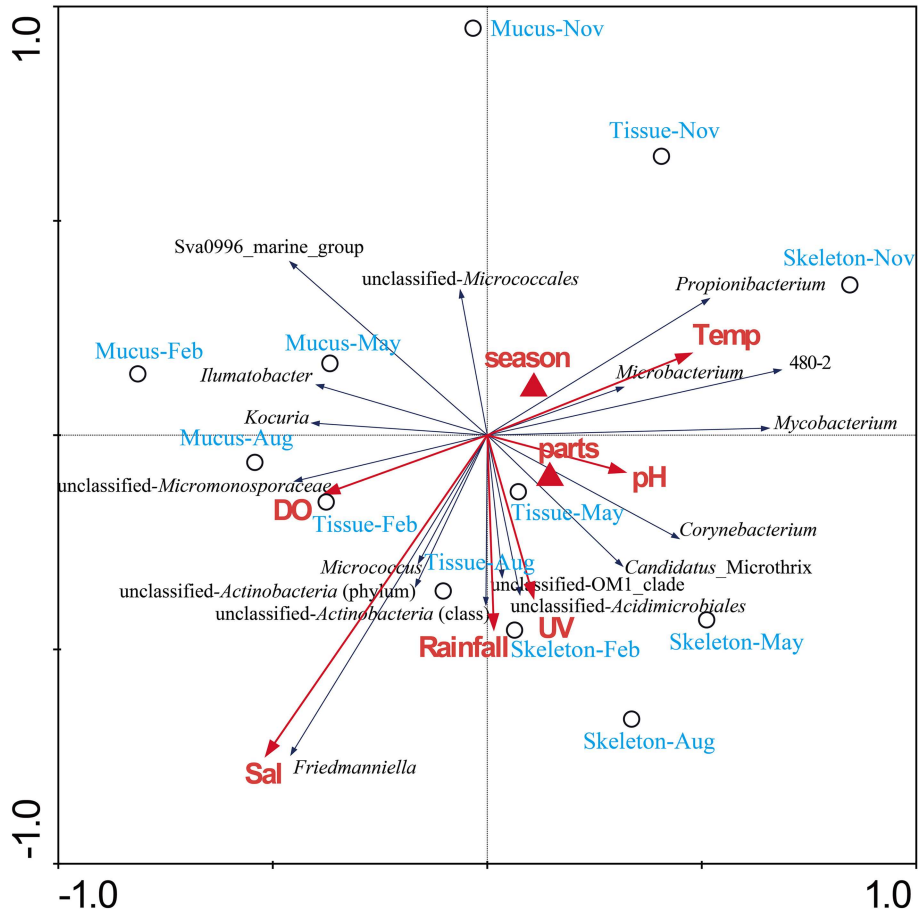

FIGURE 4 | RDA ordination triplot showing the relationship among the environmental variables, coral samples, and actinobacterial components. Correlations between environmental variables and the first two RDA axes are represented by the lengths and angles of the arrows (environmental-factor vectors). Only abundant actinobacterial groups (>1%) were showed in the triplot. UV, ultraviolet radiation intensity; Temp, seawater temperature; DO, dissolved oxygen.

According to our summary (**Table 4**), genera *Agrococcus, Amycolatopsis, Arthrobacter, Brachybacterium, Brevibacterium, Candidatus*_Microthrix, *Corynebacterium, Cellulosimicrobium, Cellulomonas, Dermatophilus, Dietzia, Gordonia, Janibacter, Jiangella, Kocuria, Kytococcus, Microbacterium, Micromonospora, Micrococcus, Mycobacterium, Nocardioides, Nocardiopsis, Propionibacterium, Pseudonocardia, Rhodococcus, Rothia, and Streptomyces* were detected in diverse coral species including scleractinian corals, such as *Acropora digitifera* (Nithyanand and Pandian, 2009; Nithyanand et al., 2011b), *P. lutea* (Li et al., 2014b; Sun et al., 2014) and *Galaxea fascicularis* (Li et al., 2014b), and gorgonian corals, *Siderastrea sidereal* (Cardenas et al., 2012) and *Platygyra carnosus* (Chiu et al., 2012). Most of them were present also in other marine organisms, such as sponges (Kim and Fuerst, 2006; Zhang et al., 2006; Selvin et al., 2009; Abdelmohsen et al., 2010, 2014; Schneemann et al., 2010; Sun et al., 2010; Webster and Taylor, 2012; Vicente et al., 2013), mollusks (Romanenko et al., 2008; Peraud et al., 2009), fishes (Sheeja et al., 2011), seaweeds (Lee, 2008; Singh and Reddy, 2013), seagrasses (Ravikumar et al., 2012), and sea cucumber (Kurahashi et al., 2009). Moreover, some of these widely distributed groups were considered as the bioactive compounds producers (Fiedler et al., 2005; Tabares et al., 2011; Margassery et al., 2012; Vicente et al., 2013; Manivasagan et al., 2014; Valliappan et al., 2014; EIAhwany et al., 2015), and probably take part in nitrogen (Su et al., 2013) and phosphorus (Sabarathnam et al., 2010) biogeochemical cycles. Whether they play these functional roles in corals *in situ* need to be further investigated.

Comparison of Actinobacterial Communities in the Corals and in the Ambient Seawater

Comparing the actinobacteria communities between in *P. lutea* and in the surrounding seawater will help us to understand the source of coral associated actinobacteria, and the interaction between the bacteria in sea water and in corals. Consisted with previous study on bacteria communities (Li et al., 2014a), the *P. lutea* associated actinobacteria communities were significantly different from which in the ambient seawater (**Figure 3**). Groups such as *Propionibacteriaceae*, *Micromonosporaceae,* and *Micrococcaceae*, were specifically associated with the corals rather than in the ambient seawater, where they originated from should be in doubt. Whether the wide distributed groups such

TABLE 2 | OTUs presented in all of the coral and seawater libraries, or presented in all three divergent compartments of *P. lutea*.

OTUs	Observed in samples	Abundance	Phylogenetic affiliation
OTU0001	Mucus, Tissue, Skeleton, Sea water	303	Sva0996_marine_group
OTU0007	Mucus, Tissue, Skeleton, Sea water	63	*Ilumatobacter*
OTU0011	Mucus, Tissue, Skeleton, Sea water	46	*Corynebacterium*
OTU0017	Mucus, Tissue, Skeleton, Sea water	33	OM1_clade
OTU0020	Mucus, Tissue, Skeleton, Sea water	24	*Microbacterium*
OTU0002	Mucus, Tissue, Skeleton	186	480-2
OTU0003	Mucus, Tissue, Skeleton	128	*Propionibacterium*
OTU0004	Mucus, Tissue, Skeleton	122	*Friedmanniella*
OTU0009	Mucus, Tissue, Skeleton	52	*Candidatus*_Microthrix
OTU0012	Mucus, Tissue, Skeleton	43	OM1_clade
OTU0013	Mucus, Tissue, Skeleton	40	OM1_clade
OTU0014	Mucus, Tissue, Skeleton	40	Sva0996_marine_group
OTU0023	Mucus, Tissue, Skeleton	21	*Micrococcus*
OTU0025	Mucus, Tissue, Skeleton	18	OM1_clade
OTU0027	Mucus, Tissue, Skeleton	18	*Mycobacterium*
OTU0028	Mucus, Tissue, Skeleton	17	*Corynebacteriales*
OTU0030	Mucus, Tissue, Skeleton	15	*Propionibacterium*
OTU0032	Mucus, Tissue, Skeleton	13	*Mycobacterium*
OTU0034	Mucus, Tissue, Skeleton	12	Sva0996_marine_group
OTU0035	Mucus, Tissue, Skeleton	12	Sva0996_marine_group
OTU0042	Mucus, Tissue, Skeleton	8	Sva0996_marine_group
OTU0056	Mucus, Tissue, Skeleton	5	*Yonghaparkia*

as Sva0996_marine_group, OM1_clade, *Microbacteriaceae* and *Ilumatobacter* travel between the ambient seawater and the corals need to be investigated.

When researchers make a general observation of the whole bacterial communities, which were observed significantly different in coral mucus, tissue, and skeleton (Rohwer et al., 2002; Bourne and Munn, 2005; Sweet et al., 2011; Lee et al., 2012). However, it is unclear whether actinobacteria has a similar distribution pattern. In this study, both the hierarchical cluster analysis (**Figure 3**) and the RDA analysis (**Figure 4**) showed that the actinobacteria communities from the same compartment tended to cluster together. The distinct physiochemical microenvironments provided by corals probably is one of the causes (Le Tissier, 1990; Brown and Bythell, 2005; Sweet et al., 2011; Tremblay et al., 2011). Only a small fraction of OTUs (22 out of 299 OTUs in the coral libraries) was common in the coral mucus, tissue, and skeleton libraries in this study (**Table 2**). This result suggested that these members might have capabilities to adapt to different micro-environments in divergent compartments of *P. lutea*. A large amount of the OTUs was specifically associated with a certain coral compartment. Whether and how the properties of distinct actinobacteria assemblages in different coral compartments actually contribute to the close relationship constructed between these associates and corals should be addressed from a functional perspective.

TABLE 3 | OTUs presented in *P. lutea* collected in four different months.

OTUs	Coral samples	Abundance	Phylogenetic affiliation
OTU0003[a]	Feb, May, Aug, Nov	128	*Propionibacterium*
OTU0004	Feb, May, Aug, Nov	122	*Friedmanniella*
OTU0013	Feb, May, Aug, Nov	40	OM1_clade
OTU0014	Feb, May, Aug, Nov	40	Sva0996_marine_group
OTU0015	Feb, May, Aug, Nov	39	*Kocuria*
OTU0017	Feb, May, Aug, Nov	33	OM1_clade
OTU0022	Feb, May, Aug, Nov	21	Sva0996_marine_group
OTU0025	Feb, May, Aug, Nov	18	OM1_clade
OTU0027	Feb, May, Aug, Nov	18	*Mycobacterium*
OTU0028	Feb, May, Aug, Nov	17	*Corynebacteriales*
OTU0033	Feb, May, Aug, Nov	13	*Brevibacterium*
OTU0059	Feb, May, Aug, Nov	5	*Brachybacterium*

[a] *OTU0003 was present in all 12 libraries. The other OTUs listed in this table were present in either of the compartment mucus, tissue and skeleton of corals collected in four different months.*

Relationship of environmental factors and the *P. lutea*-associated Actinobacteria

It is different from previous conclusion of the distribution of coral-associated bacteria (Chen et al., 2011; Li et al., 2014a), actinobacteria associated with *P. lutea* did not show the apparent seasonal dynamic variations. We suggest that the actinobacteria compositions are relatively stable in distinct compartments in *P. lutea*. In addition, none of the environmental factors analyzed in this study was determined as the most significant influence on the actinobacteria communities. Even so, some genera were found closely correlated with specific environmental factors. For instance, *Propionibacterium* showed negatively correlation with dissolved oxygen, probably due to its capability of living in the anaerobic conditions (Patrick and McDowell, 2012). Moreover, the OTUs0003 and 0004 affiliated with *Propionibacteriaceae* was present in almost all 12 clone libraries with a very high abundance. Whether they are true symbionts, and what functions they play are worth further research.

Conclusion

The diversity and distribution of coral-associated actinobacteria were first comprehensively investigated in this study. Highly diverse actinobacteria was revealed in the 16S rRNA gene clone libraries of scleractinian coral *P. lutea* in the South China Sea. Twelve *Actinobacteria* genera were detected in corals for the first time as well as a large number of unclassified groups. The actinobacterial community compositions were distinct in *P. lutea* and in the surrounding seawater. Furthermore, the higher similarity of actinobacteria composition was observed in the same compartment (i.e., mucus, tissue, or skeleton) of *P. lutea*. This study will help attracting the attentions on the ecological role of actinobacteria in corals besides the natural products bioprospecting.

TABLE 4 | Summary of the *Actinobacteria* associated with corals.

Family	Genus	Source coral	Isolate/clone	References
Acidimicrobiaceae	*Ilumatobacter*	*Porites lutea*	Clone	Chen et al., 2014
		Porites lutea	Clone	This study
Iamiaceae	***Iamia***	*Porites lutea*	Clone	This study
Actinopolysporaceae	***Actinopolyspora***	*Porites lutea*	Clone	This study
Actinospicaceae	*Actinospica*	Zoanthid *Palythoa australiae*	Clone	Sun et al., 2014
Brevibacteriaceae	*Brevibacterium*	*Acropora digitifera*	Isolate	Nithyanand and Pandian, 2009
		Tubastraea coccinea	Clone	Yang et al., 2013
		Acropora millepora	Isolate	Li et al., 2014b
		Galaxea fascicularis	Isolate	Li et al., 2014b
		Porites lutea	Isolate	Li et al., 2014b
		Porites lutea	Clone	This study
Dermacoccaceae	***Demetria***	*Porites lutea*	Clone	This study
	Dermacoccus	*Tubastraea coccinea*	Clone	Yang et al., 2013
	Kytococcus	*Fungia scutaria*	Isolate	Lampert et al., 2006
		Porites lutea	Clone	This study
Dietziaceae	*Dietzia*	*Leptogorgia minimata*	Isolate	Bruck et al., 2007
		Scleronephthya sp.	Isolate	Sun et al., 2012
		Alcyonium gracllimum	Clone	Yang et al., 2013
		Tubastraea coccinea	Clone	Yang et al., 2013
		Zoanthid *Palythoa australiae*	Clone	Sun et al., 2014
		Porites lutea	Clone	This study
Geodermatophilaceae	***Blastococcus***	*Porites lutea*	Clone	This study
	Geodermatophilus	*Porites lutea*	Clone	This study
	Modestobacter	*Porites lutea*	Clone	This study
Intrasporangiaceae	*Janibacter*	*Acropora gemmifera*	Isolate	Kageyama et al., 2007
		Alcyoniu gracllimum	Clone	Yang et al., 2013
		Acropora gemmifera	Isolate	Valliappan et al., 2014
		Porites lutea	Clone	This study
	Ornithinimicrobium	*Porites lutea*	Clone	This study
	Serinicoccus	*Tubastraea coccinea*	Clone	Yang et al., 2013
Mycobacteriaceae	*Mycobacterium*	*Sinularia* sp.	Isolate	Thomas et al., 2010
		Scleronephthya sp.	Isolate	Sun et al., 2012
		Alcyoniu gracllimum	Clone	Yang et al., 2013
		Tubastraea coccinea	Clone	Yang et al., 2013
		Porites lutea	Isolate	Li et al., 2014b
		Porites lutea	Clone	This study
Nocardiaceae	*Rhodococcus*	*Iciligorgia schrammi*	Isolate	Bruck et al., 2007
		Scleronephthya sp.	Isolate	Sun et al., 2012
		Tubastraea coccinea	Clone	Yang et al., 2013
Nocardioidaceae	*Nocardioides*	*Palythoa caribaeorum*	Isolate	Seemann et al., 2009
		Scleronephthya sp.	Isolate	Sun et al., 2012
		Tubastraea coccinea	Clone	Yang et al., 2013
		Porites lutea	Clone	This study
Nocardiopsaceae	*Nocardiopsis*	*Platygyra lamellina*	Clone	Lampert et al., 2008
		Acropora millepora	Isolate	Li et al., 2014b
		Galaxea fascicularis	Isolate	Li et al., 2014b
		Porites lutea	Isolate	Li et al., 2014b
		Porites lutea	Clone	This study

(Continued)

TABLE 4 | Continued

Family	Genus	Source coral	Isolate/clone	References
Propionibacteriaceae	***Friedmanniella***	*Porites lutea*	Clone	This study
	Propionibacterium	*Cirrhipiathes lutkeni*	Isolate	Santiago-Vázquez et al., 2007
		Mussimilia hispida	Isolate	de Castro et al., 2010
		Acropora digitifera	Isolate	Nithyanand et al., 2011b
		Zoanthid *Palythoa australiae*	Clone	Sun et al., 2014
		Porites lutea	Clone	This study
	Tessaracoccus	*Porites lutea*	Clone	Chen et al., 2014
Pseudonocardiaceae	*Pseudonocardia*	*Acropora millepora*	Isolate	Li et al., 2014b
		Galaxea fascicularis	Isolate	Li et al., 2014b
		Zoanthid *Palythoa australiae*	Clone	Sun et al., 2014
		Porites lutea	Clone	This study
	Amycolatopsis	*Galaxea fascicularis*	Isolate	Li et al., 2014b
		Zoanthid *Palythoa australiae*	Clone	Sun et al., 2014
	Prauserella	*Galaxea fascicularis*	Isolate	Li et al., 2014b
	Saccharomonospora	*Antipathes dichotoma*	Isolate	Seemann et al., 2009
Streptomycetaceae	*Streptomyces*	*Iciligorgia schrammi*	Isolate	Bruck et al., 2007
		Acropora digitifera	Isolate	Nithyanand et al., 2011b
		Antipathes dichotoma	Isolate	Zhang et al., 2012
		Scleronephthya sp.	Isolate	Sun et al., 2012
		Alcyonium gracllimum	Clone	Yang et al., 2013
		Tubastraea coccinea	Clone	Yang et al., 2013
		Zoanthid *Palythoa australiae*	Clone	Sun et al., 2014
		Acropora millepora	Isolate	Li et al., 2014b
		Galaxea fascicularis	Isolate	Li et al., 2014b
		Porites lutea	Isolate	Li et al., 2014b
		Sarcophyton glaucum	Isolate	ElAhwany et al., 2015
		Porites lutea	Clone	This study
Cellulomonadaceae	*Cellulomonas*	*Scleronephthya* sp.	Isolate	Sun et al., 2012
		Alcyomum gracllimum	Clone	Yang et al., 2013
		Zoanthid *Palythoa australiae*	Clone	Sun et al., 2014
Dermatophilaceae	*Dermatophilus*	*Fungia scutaria*	Isolate	Lampert et al., 2006
		Alcyonium gracllimum	Clone	Yang et al., 2013
		Zoanthid *Palythoa australiae*	Clone	Sun et al., 2014
Micromonosporaceae	*Micromonospora*	*Fungia scutaria*	Clone	Lampert et al., 2008
		Platygyra lamellina	Clone	Lampert et al., 2008
		Antipathes dichotoma	Isolate	Zhang et al., 2012
		Tubastraea coccinea	Clone	Yang et al., 2013
		Acropora millepora	Isolate	Li et al., 2014b
		Galaxea fascicularis	Isolate	Li et al., 2014b
		Porites lutea	Isolate	Li et al., 2014b
		Scleronephthya sp.	Isolate	Sun et al., 2012
		Porites lutea	Clone	This study
	Verrucosispora	gorgonian corals	Isolate	Zhang et al., 2013
	Salinispora	*Nephthea* sp.	Isolate	Ma et al., 2013
Acidimicrobiales_Incertae_Sedis	*Candidatus*_Microthrix	*Alcyonium gracllimum*	Clone	Yang et al., 2013
		Tubastraea coccinea	Clone	Yang et al., 2013
		Porites lutea	Clone	This study

(Continued)

TABLE 4 | Continued

Family	Genus	Source coral	Isolate/clone	References
Corynebacteriaceae	*Corynebacterium*	*Fungia granulose*	Isolate	Ben-Dov et al., 2009
		Alcyonium gracllimum	Clone	Yang et al., 2013
		Tubastraea coccinea	Clone	Yang et al., 2013
		Zoanthid *Palythoa australiae*	Clone	Sun et al., 2014
		Porites lutea	Clone	This study
Cryptosporangiaceae	***Fodinicola***	*Porites lutea*	Clone	This study
Dermabacteraceae	*Brachybacterium*	*Acropora digitifera*	Isolate	Nithyanand and Pandian, 2009
		Galaxea fascicularis	Isolate	Li et al., 2014b
		Porites lutea	Isolate	Li et al., 2014b
		Porites lutea	Clone	This study
Microbacteriaceae	*Agrococcus*	gorgonian corals	Isolate	Zhang et al., 2013
		Porites lutea	Clone	This study
	***Candidatus*_Aquiluna**	*Porites lutea*	Clone	This study
	Curtobacterium	*Acropora digitifera*	Isolate	Nithyanand et al., 2011b
	Leucobacter	*Siderastrea sidereal*	Isolate	Cardenas et al., 2012
	Microbacterium	*Siderastrea sidereal*	Isolate	Cardenas et al., 2012
		Tubastraea coccinea	Clone	Yang et al., 2013
		Porites lutea	Isolate	Chen et al., 2014
		Acropora millepora	Isolate	Li et al., 2014b
		Galaxea fascicularis	Isolate	Li et al., 2014b
		Porites lutea	Clone	This study
	Yonghaparkia	*Porites lutea*	Clone	This study
Micrococcaceae	*Arthrobacter*	Stony coral	Isolate	Shnit-Orland and Kushmaro, 2009
		Porites lutea	Clone	This study
	Kocuria	*Acropora digitifera*	Isolate	Nithyanand et al., 2011b
		Porites lutea	Isolate	Chen et al., 2014
		Zoanthid *Palythoa Australia*	Clone	Sun et al., 2014
		Porites lutea	Clone	This study
	Micrococcus	*Acropora digitifera*	Isolate	Nithyanand et al., 2011b
		Galaxea fascicularis	Isolate	Li et al., 2014b
		Porites lutea	Clone	This study
	Rothia	*Platygyra carnosus*	Isolate	Chiu et al., 2012
		Porites lutea	Clone	This study
	Tersicoccus	*Porites lutea*	Clone	This study
Gordoniaceae	*Gordonia*	*Scleronephthya* sp.	Isolate	Sun et al., 2012
		Alcyonium gracllimum	Clone	Yang et al., 2013
		Tubastraea coccinea	Clone	Yang et al., 2013
		Galaxea fascicularis	Isolate	Li et al., 2014b
		Acropora millepora	Isolate	Li et al., 2014b
		Porites lutea	Isolate	Li et al., 2014b
Jiangellaceae	*Jiangella*	*Acropora millepora*	Isolate	Li et al., 2014b
		Galaxea fascicularis	Isolate	Li et al., 2014b
Promicromonosporaceae	*Cellulosimicrobium*	*Acropora millepora*	Isolate	Li et al., 2014b
		Porites lutea	Isolate	Li et al., 2014b
	Myceligenerans	*Fam. Caryophillidae*	Isolate	Sarmiento-Vizcaíno et al., 2015
Tsukamurellaceae	*Tsukamurella*	*Galaxea fascicularis*	Isolate	Li et al., 2014b

The genera firstly reported in this study were shown in bold.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01094

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Actinobacterial Diversity in the Sediments of Five Cold Springs on the Qinghai-Tibet Plateau

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The actinobacterial diversity was investigated in the sediments of five cold springs in Wuli region on the Qinghai-Tibet Plateau using 16S rRNA gene phylogenetic analysis. The actinobacterial communities of the studied cold springs were diverse and the obtained actinobacterial operational taxonomic units were classified into 12 actinobacterial orders (e.g., *Acidimicrobiales*, *Corynebacteriales*, *Gaiellales*, *Geodermatophilales*, *Jiangellales*, *Kineosporiales*, *Micromonosporales*, *Micrococcales*, *Nakamurellales*, *Propionibacteriales*, *Pseudonocardiales*, *Streptomycetales*) and unclassified *Actinobacteria*. The actinobacterial composition varied among the investigated cold springs and were significantly correlated ($r = 0.748$, $P = 0.021$) to environmental variables. The actinobacterial communities in the cold springs were more diverse than other cold habitats on the Tibetan Plateau, and their compositions showed unique geographical distribution characteristics. Statistical analyses showed that biogeographical isolation and unique environmental conditions might be major factors influencing actinobacterial distribution among the investigated cold springs.

Keywords: *Actinobacteria*, diversity, 16S rRNA gene, cold springs, Qinghai-Tibet Plateau

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INTRODUCTION

A large portion of the Qinghai-Tibet Plateau (QTP) is underlain by permafrost, which is suitable for gas hydrate development (Wang and French, 1995; Zhou et al., 2000). Recent evidence indicates that gas hydrate is present in the permafrost zone of Qilian Mountains in the northern margin of QTP (Lu et al., 2009; Zhu et al., 2010). Large numbers of factures and faults are present in the identified hydrate-containing permafrost zone (Lu et al., 2009; Wang, 2010; He et al., 2012), along which cold springs are commonly distributed (Lu et al., 2007; Li et al., 2012).

The environmental condition of the cold springs in the hydrate-containing permafrost zone is similar to marine cold seeps in terms of geochemistry. Cold seeps occur in geologically active and passive continental margins, where continuous methane is advected upward through sediments by forced gradients, supporting abundant microbial populations (Levin, 2005). The methane-fueled communities in marine cold seeps possess high metabolic rates, and they play important roles in carbon and nitrogen cycling (Hinrichs and Boetius, 2002; Boetius and Suess, 2004; Nakagawa et al., 2007; Reeburgh, 2007; Dang et al., 2010). Because of their potentially important role in global climate change, microbial communities in marine cold seeps have received much attention (Sibuet and Olu-Le Roy, 2002; Reeburgh, 2007).

As one of the largest taxonomic units within the *Bacteria* domain, *Actinobacteria* are drawing increasing interests from microbiologists because their biotechnological and commercial

value (Goodfellow et al., 1988; Demain, 1995). The characterized actinobacterial strains can be grouped into six known classes: *Acidimicrobiia*, *Actinobacteria*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria*, and *Thermoleophilia* (Goodfellow et al., 2012). The actinobacterial diversity and community structures have been investigated in various environments, including marine environments (Goodfellow and Haynes, 1984; Stach et al., 2003; Maldonado et al., 2005; Stach and Bull, 2005; Ward and Bora, 2006), soils (Gremion et al., 2003; Cho et al., 2006; Wu et al., 2009), terrestiral aquatic ecosystems (e.g., freshwater rivers, saline/hypersaline lakes, hot springs, glacial meltwater; Mohagheghi et al., 1986; Mevs et al., 2000; Zwart et al., 2002; Hahn et al., 2003; Warnecke et al., 2004; Mancinelli, 2005; Stach and Bull, 2005; Allgaier and Grossart, 2006; Newton et al., 2007; Hahn, 2009; Holmfeldt et al., 2009; Liu et al., 2009a,b; Song et al., 2009; Wu et al., 2009; Jiang et al., 2010a, 2012a; Ghai et al., 2012, 2014; Goodfellow et al., 2012). These previous studies show that *Actinobacteria* are ubiquitous and actinobacterial community diversity is variable among samples from different ecosystems. The actinobacterial community in marine sediments was mainly composed of the orders of *Acidimicrobiales*, *Actinomycetales*, *Corynebacteriales*, *Frankiales*, *Micrococcales*, *Micromonosporales*, *Pseudonocardiales*, *Streptomycetales,* and unclassified *Actinobacteria* (Stach et al., 2003; Goodfellow et al., 2012), while the *Actinobacteria* in freshwater ecosystems consisted of acI, acII, acIII, acIV, acSTL, soilII+III, acTH1, and Luna (Hahn et al., 2003; Warnecke et al., 2004; Ghai et al., 2012). In contrast, limited is known about microbial communities in terrestrial cold springs up to date. Previously, one 16S rRNA gene-based microbial study showed the presence of *Actinobacteria* in the cold springs of Wuli, QTP (Li et al., 2012). However, the actinobacterial diversity in these cold springs might be under-represented due to the use of universal bacterial primers (Cottrell and Kirchman, 2000; Jiang et al., 2010a).

The objective of this study was to investigate the actinobacterial diversity and community structure in five Tibetan cold springs based on 16S rRNA gene phylogenetic analyses. We also compared the actinobacterial diversity in the sampled Tibetan cold springs with that in other habitats.

MATERIALS AND METHODS

Site Description and Sample Collection

In July 2010, five cold springs were sampled in Wuli Area (**Figure 1**), Qinghai Province, China, that is adjacent to the Daha coal mine (Zhou, 2004) and located in the Fenghuo Mountain-Wuli gas hydrate zone (Zhu et al., 2011). The Wuli area is located at the elevation of ∼4600 m. Water pH and temperature were measured in the field using a digital soil pH meter (Ferrymorse-Seed Company) and a mercury thermometer, respectively. During sample collection (around noon), the ambient temperature was 15–17°C, whereas the water temperature of the sampled cold springs was around 1–3°C. Sediments from five cold springs (named as QCS1, QCS3, QCS4, QCS5, and QCS6, respectively) were collected into 50 mL sterile Falcon tubes using a sterile spatula. The collected samples were stored at −20°C in the field as well as during transportation and subsequently at −80°C in the laboratory until further analyses.

Porewater Chemistry and Sediment Mineralogy

Cation composition of pore water was analyzed by using inductively coupled plasma-optical emission spectrometry (ICP-OES; Varian Vista MPX, Varian, Palo Alto, CA, USA). Anion composition was analyzed using ionic chromatography (IC) on a Dionex ISC90 equipped with a conductivity detector and an AS14A column (eluent, 10 μM Na_2CO_3/$NaHCO_3$; flow rate, 1.0 mL/min; Jiang et al., 2010a). The sediment mineralogy was analyzed by using powder X-ray diffraction (XRD) on a Rigaku D/Max 2550/PC X-ray diffractometer with Cu Ka radiation (40 kV; 100 mA; Zhang et al., 2009).

DNA Extraction, PCR, and Phylogenetic Analyses

DNA of the sediment samples was extracted using FastDNA® SPIN Kit for Soil (MP Biomedicals, LLC, Solon, OH, USA) according to the manufacturer's protocols. The actinobacterial 16S rRNA gene from the extracted DNA samples was amplified using the actinobacterial 16S rRNA gene-specific forward primer S-C-Act-0235-a-S-20 (5′-CGC GGC CTA TCA GCT TGT TG-3′) and reverse primer S-C-Act-0878-a-A-19 (5′-CCG TAC TCC CCA GGC GGG G-3′; Stach et al., 2003) with the same PCR conditions as described previously (Wu et al., 2009). PCR products were purified using Agarose Gel DNA Fragment Recovery Kit Ver. 2.0 (TaKaRa, Dalian, China) according to the manufacturer's instructions. 16S rRNA gene clone libraries were constructed by ligating the purified PCR products into pGEM®-T Easy Vector system (Promega, Madison, WI, USA) and transformed into competent *Escherichia coli* JM109 cells according to the manufacturer's protocols. Positive clones were randomly picked for sequencing with an ABI 3730 XL DNA Sequencer (Applied BioSystems, Foster City, CA, USA). Rarefaction analysis was performed to evaluate the saturation of the sampled clones using the PAST software package[1] (see Supplementary Figure S1).

All the obtained clone sequences were assembled and edited by using Sequencher v.4.1 (GeneCodes, Ann Arbor, MI, USA) and then checked by BLAST function in NCBI (National Center of Biotechnology Information[2]). Potential chimeric sequences were removed from further analyses. Operational taxonomic units (OTUs) were identified at a 97% cutoff by using Mothur v1.36.1 with furthest neighbor method (Schloss et al., 2009). One sequence from each OTU was selected and the closest references were picked up from the GenBank database for phylogenetic analyses (see Supplementary Table S1). The representative sequences of OTUs and references were combined and aligned using ClustalW in MEGA (molecular evolutionary genetics analysis) program, version 6.06. Maximum likelihood

[1]http://folk.uio.no/ohammer/past/

[2]http://blast.ncbi.nlm.nih.gov/Blast.cgi

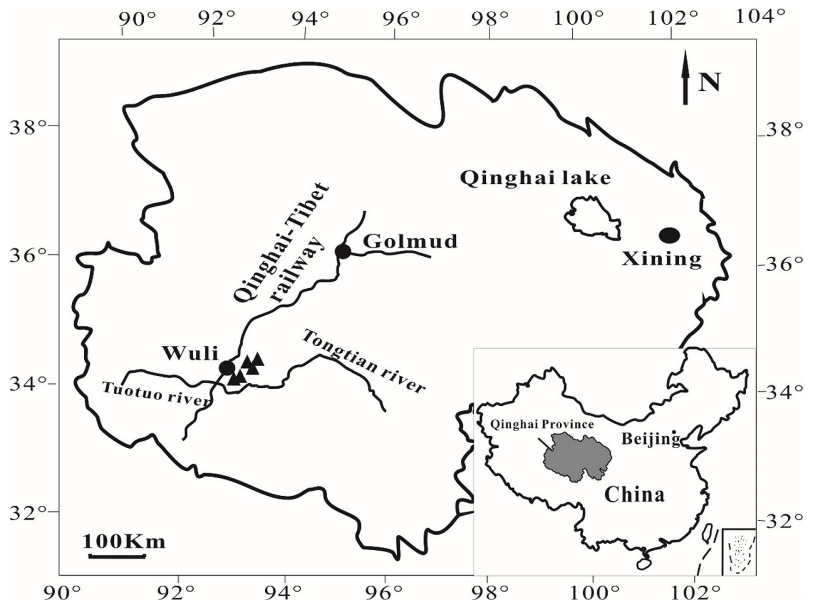

FIGURE 1 | A geographic map showing the locations of sampling sites in Wuli County, Qinghai-Tibet Plateau, China.

phylogenetic trees were constructed using the above aligned sequences. Bootstrap replications of 1000 were assessed. The unique clone sequences determined in this study were deposited in the GenBank database under accession numbers JX667788–JX667977, JF712624–JF712648, and KU052203–KU052216.

Statistical Analysis

Alpha-diversity indices, such as Simpson, Shannon, Equitability and Chao 1, were calculated by using the PAST software package (Hammer et al., 2001). Coverage values of the clone libraries were calculated with the equation $C = 1\text{-}n/N$, where n was the number of phylotypes that occurred only once in the clone library and N was the total number of sequenced clones (Jiang et al., 2010b). All obtained environmental variables were normalized (values ranged between 1 and 100) to improve normality and homoscedasticity for statistical analyses. Clustering analysis were performed by using PAST software package with unweighted pair group method with arithmatic mean. Mantel tests were performed to assess the correlation between actinobacterial community composition and environmental variables by using the PAST software package. Briefly, the biotic matrices were constructed on the basis of Bray-Curtis dissimilarity of actinobacterial community compositions. The abiotic matrices were constructed on the basis of the Euclidean distances of normalized environmental variables.

In order to compare the actinobacterial community composition difference between the QTP cold springs and other related habitats, reference actinobacterial clone sequences from Tibetan hot springs (Jiang et al., 2012a), Tibetan (hyper-)saline lakes (Jiang et al., 2010a), freshwater sample of Daotang river (Jiang et al., 2010a), Atlantic ocean deep-sea sediment (Stach et al., 2003), the Three Gorges Dam of the Yangtze River (Jiang et al., 2012b) and Tengchong hot springs (Song et al., 2009) were downloaded from the GenBank database and combined with the ones obtained in this study. In order to avoid any bias resulting from different primers, only actinobacterial 16S rRNA sequences amplified from the same primer set and PCR protocol as this study were included in subsequent analysis. The combined actinobacterial 16S rRNA sequences were aligned using ClustalW in MEGA and then were subjected to OTU identification at the 97% cutoff using Mothur v1.36.1 with furthest neighbor method (Schloss et al., 2009). Clustering analysis was performed to discern the difference of actinobacterial community composition among habitats based on Jaccard similarity using the PAST software package.

RESULTS

Porewater Chemistry and Mineralogy

The pH of the sampled cold springs were neutral, and the temperature ranged 1.5–2.5° (**Table 1**). The concentration of Si^{4+} and total Fe were 0.6–5.1 and 0.0–6.9 mg/L, respectively. Heavy metals Mn and Sr only occurred in the QCS1 sample. The sediment samples were mainly composed of quartz, plagioclase, calcite, montmorillonite, illite, and kaolinite.

Phylogenetic Diversity of *Actinobacteria*

Five clone libraries (QCS1, QCS3, QCS4, QCS5, and QCS6) were constructed. A total of 484 actinobacterial 16S rRNA gene clone sequences were obtained: 117, 85, 76, 103, and 103 clone sequences for QCS1, QCS3, QCS4, QCS5, and QCS6, respectively. The number of sequenced clones represented 76–91% coverage for each clone library (**Table 2**). Out of these clone sequences, one hundred and twenty OTUs (29,

TABLE 1 | Geographic and geochemical parameters of the studied cold springs on the Qinghai-Tibet Plateau.

Sample ID	QCS1	QCS3	QCS4	QCS5	QCS6
GPS location (N/E)	34°20′/ 94°38′	34°20′36.7″/ 92°44′51.6″	34°20′42.5″/ 92°45′1.5″	34°20′53.8″/ 92°45′29.3″	34°21′19.7″/ 92°45′29.7″
Elevation (m)	4610	4611	4609	4637	4612
Temperature (°C)	2.5	1.5	2.5	2	2
pH	7	6.8	7	7.2	7
Mg^{2+} (mg/L)	40.8	57.9	6	41	56.7
Si^{4+} (mg/L)	2.7	3.2	0.6	3.5	5.1
Ca^{2+} (mg/L)	50.1	84.2	12.7	90.4	185.2
K^{+} (mg/L)	5.19	5.4	4.6	9.2	96
Na^{+} (mg/L)	104.2	97.5	8.7	55.8	69.1
F^{-} (mg/L)	0	4.8	2	5.1	13
Cl^{-} (mg/L)	133	883.1	75.1	390.7	372
NO_3^{-} (mg/L)	3.6	98.3	40.4	165.5	26
PO_4^{2-} (mg/L)	0.4	0.0	0.0	0.0	0.0
SO_4^{2-} (mg/L)	159.1	717.5	146.2	1609.2	3057
Total Fe (mg/L)	0.0	0.2	1.8	0.1	6.9
Total Mn (mg/L)	0.1	0.0	0.0	0.0	0.0
Total Sr (mg/L)	1.0	0.0	0.0	0.0	0.0

TABLE 2 | Ecological estimates and major group affiliation of clone sequences retrieved from the five cold springs on the Qinghai-Tibet Plateau.

Clone libraries	QCS1	QCS3	QCS4	QCS5	QCS6
Library sizes (No. of clones)	117	85	76	103	103
Coverage (%)	91	85	76	88	86
No. of observed OTUs	29	27	32	27	31
Simpson	0.9	0.9	0.9	0.9	0.9
Shannon	2.9	2.8	2.9	2.6	3.0
Equitability	0.9	0.8	0.8	0.8	0.9
Chao 1	34.6	36.8	47.3	34.3	46.2

27, 32, 27, 31 for QCS1, QCS3, QCS4, QCS5, and QCS6, respectively) were identified (**Table 2**). These identified OTUs could be classified into *Acidimicrobiales*, *Corynebacteriales*, *Gaiellales*, *Geodermatophilales*, *Jiangellales*, *Kineosporiales*, *Micromonosporales*, *Micrococcales*, *Nakamurellales*, *Propionibacteriales*, *Pseudonocardiales*, *Streptomycetales,* and unclassified *Actinobacteria* (**Figure 2**). The diversity indices such as Shannon (2.6–3.0), Chao 1 (34.3–46.2) varied among the studied cold springs (**Table 2**). *Acidimicrobiales*, *Geodermatophilales*, *Micrococcales*, *Propionibacteriales,* and *Pseudonocardiales* were dominant actinobacterial groups (**Figure 3C**). Among the studied samples, *Acidimicrobiales*, *Micrococcales*, *Pseudonocardiales,* and unclassified *Actinobacteria* were dominant (relative abundance > 10%) in the QCS1 sample; *Acidimicrobiales*, *Micrococcales*, *Pseudonocardiales,* and *Propionibacteriales* dominated in the QCS3 sample; *Acidimicrobiales*, *Geodermatophilales*, *Micrococcales,* and *Propionibacteriales* were dominant in the QCS4 and QCS5 samples; and *Acidimicrobiales*, *Corynebacteriales*, *Kineosporiales*, *Micrococcales,* and *Propionibacteriales* dominated in the QCS6 sample (**Figure 3C**).

The order of *Micrococcales* was the most dominant (average abundance 25.6%) group in the studied cold spring samples, and a large portion of clones affiliated with *Micrococcales* were closely related (identity: 95–99%) to cultured psychrophilic *Actinobacteria*, such as *Arthrobacter* sp. (Reddy et al., 2000; Fong et al., 2001; Wang et al., 2009) and *Demequina* sp. (Finster et al., 2009; **Figure 2** and Supplementary Table S1). Furthermore, many clone sequences obtained in this study were affiliated with *Acidimicrobiales*, and they were related to clone sequences retrieved from cold habitats such as arctic soil exposed by glacier retreat (Quince et al., 2011), cold spring sediment in Shawan, Xinjiang, China (Zeng et al., 2010), and Shule River permafrost soils on the Tibetan Plateau (**Figure 2**). The remaining 5.9% (32 out of 484) of the clone sequences retrieved in this study belonged to unclassified *Actinobacteria* (**Figure 2**).

Relationships between Actinobacterial Community Composition and Environmental Variables

Cluster analysis showed that the cold spring geochemistry (**Figure 3A**) presented similar grouping patterns to actinobacterial community composition (**Figure 3B**) among the studied samples. Mantel tests showed that actinobacterial community composition of the studied cold springs was significantly correlated ($r = 0.748$, $P = 0.021$) with the combined environmental variables but not significantly ($P > 0.05$) with any single environmental variable measured in this study. Furthermore, cluster analysis showed that the actinobacterial communities in the QTP samples (including clod springs, hot springs and lakes) were grouped into one cluster, which has little similarity (Jaccard similarity < 0.05) with that of marine sediments from Atlantic ocean and Tengchong hot springs (**Figure 4**).

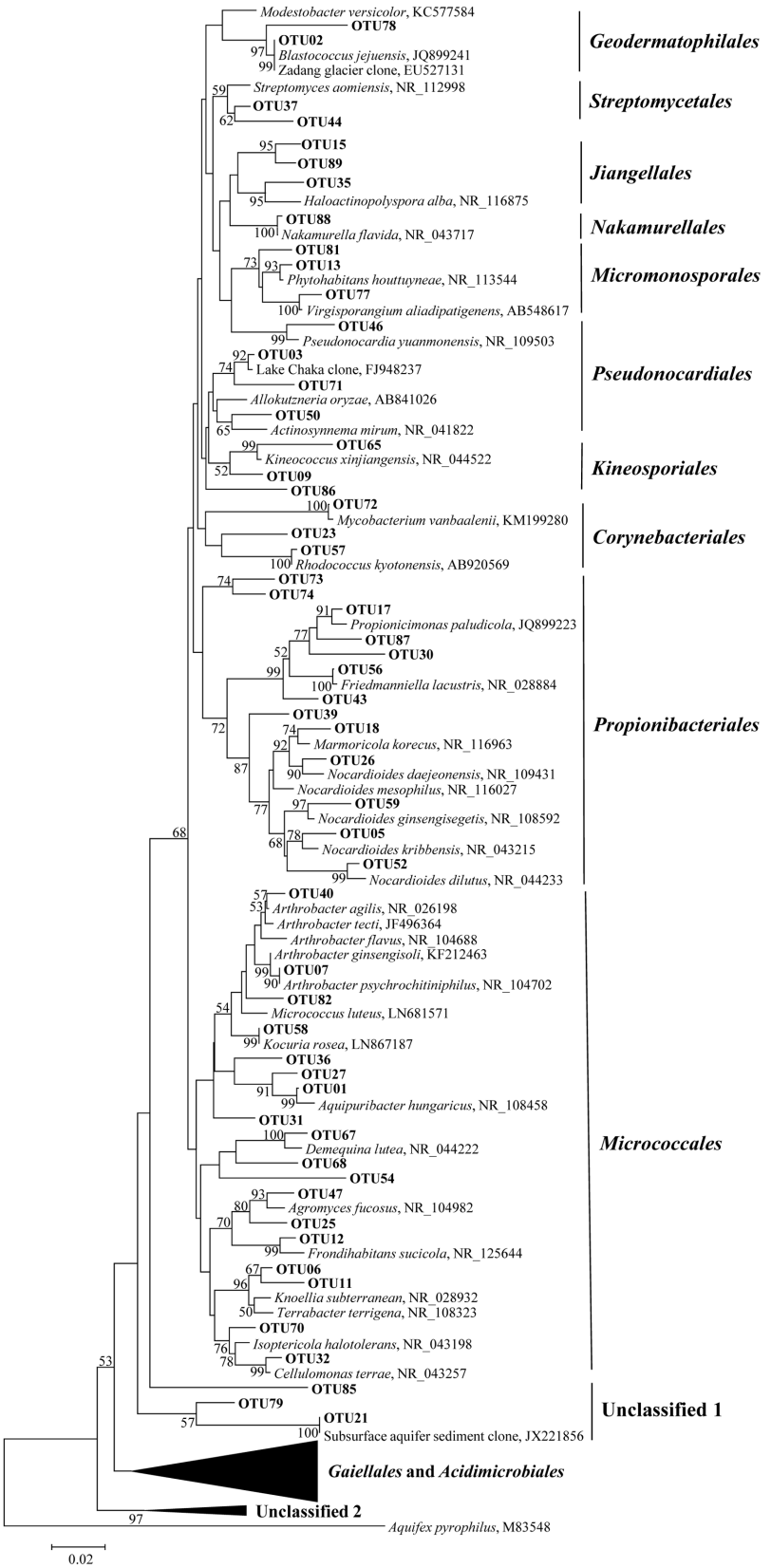

FIGURE 2 | Continued

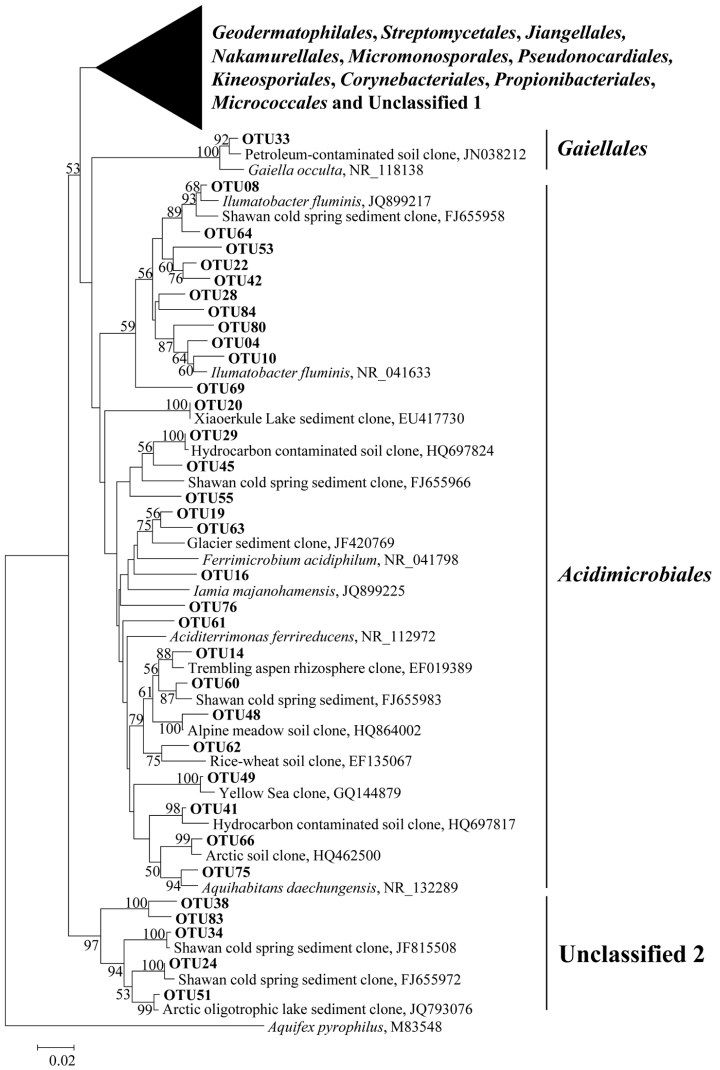

FIGURE 2 | Continued

Maximum-likelihood tree (partial sequences, ~640 bp) showing the phylogenetic relationships of the actinobacterial 16S rRNA gene sequences cloned from the studied samples to closely related sequences from the GenBank database. One representative clone sequence within each OTU was shown. Bootstrap values of >50% (for 1000 iterations) were shown.

DISCUSSION

Actinobacterial Communities in the QTP Cold Springs

The actinobacterial community composition in cold springs on the QTP was similar to that of cold habitats in other locations. The actinobacterial communities of the studied QTP cold springs were composed of major groups related to psychrophilic *Actinobacteria* species (e.g., *Arthrobacter psychrochitiniphilus*, *Demequina lutea*) and environmental clone sequences retrieved from cold habitats, such as snow/ice and soils in Qinghai–Tibetan Plateau and Arctic/Antarctic. This indicated that low temperature was a major environmental factor for dominating actinobacterial distribution in cold habitats.

Excluding low-temperature property, actinobacterial community composition in the studied cold springs may be affected by environmental variable composition. For example, samples of QCS3, QCS4, and QCS5 had similar environmental variables composition, and thus possessed similar actinobacterial community compositions (**Figures 3A,B**); the environmental variable composition of QCS1 and QCS6 was different from the other studied samples (**Figure 3A**): QCS1 possess highest concentration of Na^+ and heavy metal Mn and Sr (**Table 1**), and QCS6 sample has highest Ca^{2+} and total Fe (**Table 1**), thus it is reasonable to observe distinct actinobacterial community compositions in QCS1 and QCS6 samples from that in QCS3, QCS4, and QCS5 samples (**Figure 3B**). Previous studies have shown that microbial community composition could be affected by multiple environmental parameters, such as

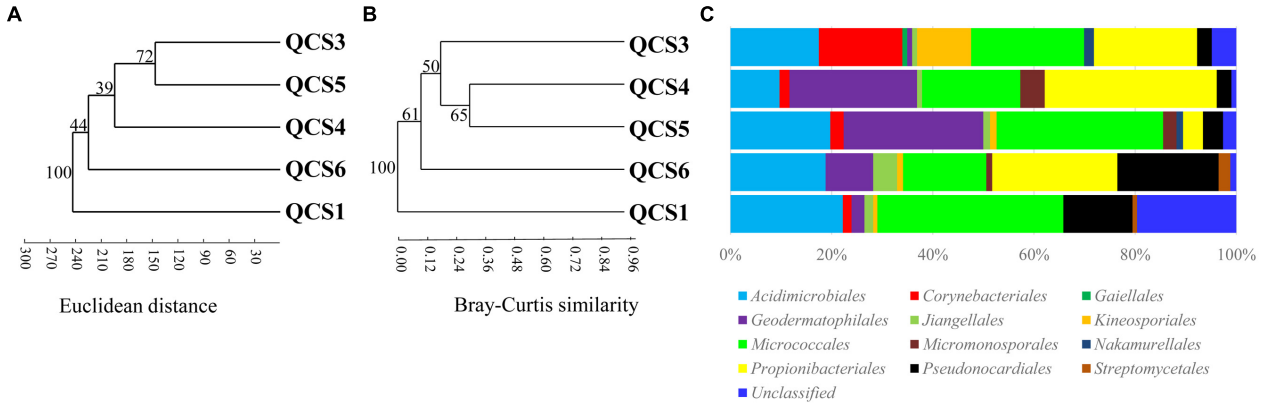

FIGURE 3 | (A) Cluster analysis of environmental variables in the studied samples based on Euclidean distance; **(B)** Cluster analysis of actinobacterial community composition in the studied samples based on Bray-Curtis similarity; **(C)** Schematic figures showing the frequencies of OTUs affiliated with major actinobacerial orders in this study.

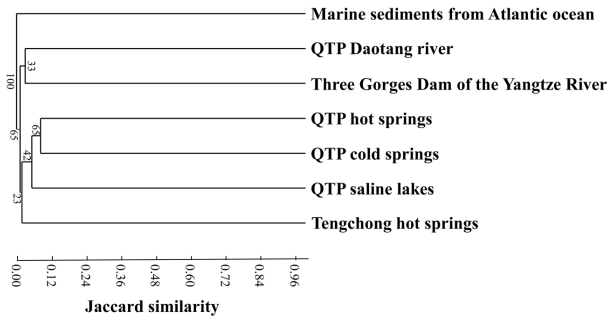

FIGURE 4 | Jaccard similarity-based cluster analysis showing the differences between actinobacterial 16S rRNA gene clone libraries of the QTP cold springs in this study and those from hot springs on the QTP (Jiang et al., 2012a), (hyper-)saline lakes on the QTP (Jiang et al., 2010a), freshwater sample of Daotang river on the QTP (Jiang et al., 2012b), Tengchong hot springs of Yunnan Province, China (Song et al., 2009), Atlantic ocean deep-sea sediment in the edge of the Saharan debris flow near the Canary Islands (Stach et al., 2003), and waters near the Three Gorges Dam in the middle reach of the Yangtze River (Jiang et al., 2012b).

salinity (Lozupone and Knight, 2007), temperature (Lindh et al., 2013), and heavy metals (Gong et al., 2015). Therefore, it is not surprising to observe significant correlation between actinobacterial community composition and environmental variables in the studied cold springs.

It is notable that some of the retrieved actinobacterial clone sequences from the cold springs showed high identity with those obtained from petroleum- or coal-related environments. This observation is expected in that the sampling sites in this study was located in the Wuli-Daha coal-bearing belt (Zhou, 2004) and Fenghuo Mountain-Wuli gas hydrate-bearing belt (Zhu et al., 2010) in southern Qinghai Province. The underlying coal or gas hydrate might provide abundant nutrients, which support diverse actinobacterial communities in the studied cold springs (Santos et al., 2008; Jiang et al., 2010a).

Actinobacterial Difference between the QTP Cold Springs and Other Habitats

The actinobacterial community in the investigated cold springs was more diverse than other cold environments. For example, the *Actinobacteria* sequences obtained in this study were distributed into 12 orders (**Figures 2** and **3C**). In contrast, the *Actinobacteria*-related clones retrieved in the snow of four glaciers on the Tibetan Plateau were mainly affiliated with the order *Micrococcales* and unclassified *Actinobacteria* (Liu et al., 2009b). This suggested Tibetan cold springs might contain more suitable growth conditions for *Actinobacteria* than glaciers.

Actinobacterial communities from different habitats possessed certain geographic characteristics. The actinobacterial clones from the studied cold springs (this study) were closely related to those from the QTP hot springs and saline lakes (**Figure 4**), this indicated that the actinobacterial communities in the studied cold springs were more similar to that in other QTP samples (including hot springs and lakes) than to those in the samples from other locations. For example, the majority of the retrieved actinobacterial 16S rRNA gene clone sequences in the investigated cold springs were affiliated with *Micrococcales*, *Propionibacteriales,* and *Acidimicrobiales*. Actinobacterial clones retrieved from Tibetan saline lakes were mainly classified with *Micrococcales*, *Propionibacteriales,* and *Frankiales* (Jiang et al., 2010a). In contrast, the actinobacterial communities in Tengchong hot springs were mainly affiliated with unclassified *Actinobacteria*, *Rubrobacterales,* and *Frankiales* (Song et al., 2009). Previous studies have shown that *Actinobacteria* in hot springs, soils and oceans possess geographic distributions (Ward and Bora, 2006; Wawrik et al., 2007; Valverde et al., 2012). In addition, the *Actinobacteria* communities in the studied QTP cold spring sediments were different from those in marine sediments (Stach et al., 2003; Goodfellow et al., 2012) and freshwater ecosystems (Hahn et al., 2003; Warnecke et al., 2004; Ghai et al., 2012). The observed geographic distribution of *Actinobacteria* in the QTP samples could be ascribed to the distinct conditions (e.g., dry climate, low pressure, high intensity

of UV radiation) of the cold springs, hot springs, and saline lakes on the QTP from other ecosystems (Jiang et al., 2010a, 2012a). However, the underlying reasons still await further investigation.

In summary, the actinobacterial communities in the studied Tibetan cold springs possessed unique compositional characteristics and were mainly consisted of *Acidimicrobiales*, *Corynebacteriales*, *Gaiellales*, *Geodermatophilales*, *Jiangellales*, *Kineosporiales*, *Micromonosporales*, *Micrococcales*, *Nakamurellales*, *Propionibacteriales*, *Pseudonocardiales*, *Streptomycetales,* and unclassified *Actinobacteria*. Biogeographical isolation and unique environmental conditions might be predominant factors affecting the observed similarities and differences in the actinobacterial communities between the investigated cold springs and other habitats.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01345

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Actinobacterial Diversity in Volcanic Caves and Associated Geomicrobiological Interactions

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Volcanic caves are filled with colorful microbial mats on the walls and ceilings. These volcanic caves are found worldwide, and studies are finding vast bacteria diversity within these caves. One group of bacteria that can be abundant in volcanic caves, as well as other caves, is Actinobacteria. As Actinobacteria are valued for their ability to produce a variety of secondary metabolites, rare and novel Actinobacteria are being sought in underexplored environments. The abundance of novel Actinobacteria in volcanic caves makes this environment an excellent location to study these bacteria. Scanning electron microscopy (SEM) from several volcanic caves worldwide revealed diversity in the morphologies present. Spores, coccoid, and filamentous cells, many with hair-like or knobby extensions, were some of the microbial structures observed within the microbial mat samples. In addition, the SEM study pointed out that these features figure prominently in both constructive and destructive mineral processes. To further investigate this diversity, we conducted both Sanger sequencing and 454 pyrosequencing of the Actinobacteria in volcanic caves from four locations, two islands in the Azores, Portugal, and Hawai`i and New Mexico, USA. This comparison represents one of the largest sequencing efforts of Actinobacteria in volcanic caves to date. The diversity was shown to be dominated by *Actinomycetales*, but also included several newly described orders, such as *Euzebyales,* and *Gaiellales.* Sixty-two percent of the clones from the four locations shared less than 97% similarity to known sequences, and nearly 71% of the clones were singletons, supporting the commonly held belief that volcanic caves are an untapped resource for novel and rare Actinobacteria. The amplicon libraries depicted a wider view of the microbial diversity in Azorean volcanic caves revealing three additional orders, *Rubrobacterales*, *Solirubrobacterales,* and *Coriobacteriales*. Studies of microbial ecology in volcanic caves are still very limited. To rectify this deficiency, the results from our study help fill in the gaps in our knowledge of actinobacterial diversity and their potential roles in the volcanic cave ecosystems.

Keywords: Actinobacteria, volcanic lava caves, microbe-mineral interactions, microbial diversity

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INTRODUCTION

Actinobacteria are an ubiquitous phyla found to thrive in almost any environment, from soil and marine, to less expected environments such as insects, plants, roots, and caves (See Tiwari and Gupta, 2013; Subramani and Aalbersberg, 2013 for reviews). Recent culture independent studies have found Actinobacteria in high abundance in a variety of cave types, including volcanic caves (Pašić et al., 2010; Northup et al., 2011; Cuezva et al., 2012; Niyomyong et al., 2012; Quintana et al., 2013; Barton et al., 2014; Hathaway et al., 2014). Furthermore, many characterized species of Actinobacteria have been described from caves (Groth et al., 1999; Lee et al., 2000, 2001; Jurado et al., 2005a,b; Lee, 2006).

Primary and secondary metabolites from Actinobacteria have been described as important sources of industrial compounds (Miao and Davies, 2010). Rare Actinobacteria, important for novel secondary metabolite production, have been found in many different soil types (Tiwari and Gupta, 2012; Guo et al., 2015), but caves, volcanic caves included, remain an underexploited environment to screen for industrially important compounds. Goodfellow and Fiedler (2010) suggested examining underexploited sources of Actinobacteria and using taxonomic diversity as a surrogate for chemical diversity, based on the assumption that novel species may contain unique compounds, reducing the re-discovery of the same handful of known secondary metabolites.

Cave Actinobacteria are of particular interest because of the unique environment in which they live. The extreme (i.e., low nutrient inputs, low productivity) and often pristine environment would result in bacteria exploiting different metabolic pathways, including the capacity for biomineralization and rock-weathering (Cuezva et al., 2012; Miller et al., 2012a,b). Caves are characterized by microenvironments, which result from several types of reactions, including microbial processes that often involve redox reactions (Barton and Northup, 2007). These mineral microniches control the diversity of subsurface microbial populations (Jones and Bennett, 2014), since microbial colonization of rock surfaces is driven by the rock's chemistry and the organism's metabolic requirements and tolerances, suggesting that subsurface microbial communities have specific associations to specific minerals. In fact, caves on Earth can harbor a wide variety of mineral-utilizing microorganisms that figure prominently in the formation of secondary mineral deposits and unusual mineralized microstructures recognized as biosignatures. Tubular mineralized sheaths (Boston et al., 2001; Northup et al., 2011), bacteria concealed within mineral deposits (Northup et al., 2011), microfossils preserved in minerals (Provencio and Polyak, 2010; Souza-Egipsy et al., 2010), filamentous fabrics (Hofmann et al., 2008) and "cell-sized" etch pits or microborings produced by bacteria (McLoughlin et al., 2007) are some of the proposed models for biosignatures found in subsurface environments.

The main goal of the research presented here is to obtain a better understanding of the actinobacterial diversity in volcanic caves from different parts of the world. Comprehensive studies on microbial community ecology of caves identifying abundant, rare and novel species and their environmental implications are still scarce. In the course of this study, we aim to unravel the diversity and composition of volcanic cave Actinobacteria, some of the biogeochemical role of Actinobacteria in caves and their geomicrobiological interactions. Recently, a rapid expansion of interest in subsurface environments has emerged to better understand biodiversity, origins of life on Earth and on other planets. In fact, the reported early results on liquid water and rather recent volcanic activity yielding volcanic caves on Mars, suggesting that the Martian subsurface can house organic molecules or traces of microbial life (Léveillé and Datta, 2010; Northup et al., 2011), make the search for microbial life on Earth's volcanic caves even more compelling. Overall, this work helps us to understand whether volcanic caves under study present similar levels of diversity and do Actinobacteria found in volcanic caves show diversity across different scales from community level to morphology to microbe-mineral interactions.

MATERIALS AND METHODS

Morphological Characterization of Colored Microbial Mats

Sampling of Azorean, Canadian, Canarian, Hawaiian, and New Mexican Volcanic Caves

Samples of visible white and/or yellow microbial mats on volcanic cave walls and ceilings (**Figure 1**) were collected from: (1) Bird Park Cave and Kipuka Kanohina Cave System, Hawai`i (USA); (2) Helmcken Falls Cave, British Columbia (Canada); (3) Cave 12 from El Malpais National Monument, New Mexico (USA); (4) Gruta de Terra Mole and Gruta dos Montanheiros in Terceira and Pico Islands, Azores (Portugal), and (5) Fuente de la Canaria, Falda de La Horqueta, Llano de los Caños and Honda del Bejenado caves in La Palma Island, Canary islands (Spain). Samples were taken by gently scraping the colored microbial mats with a sterile scalpel, gathering it into sterile vials and stored at 4°C until laboratory procedures.

Scanning Electron Microscopy

Bulk samples with microbial mats from Canarian volcanic caves (Spain) were directly mounted on a sample stub and sputter coated with a thin gold/palladium film. Samples were subsequently examined on a Jeol JSM-7001F field emission scanning electron microscope (FESEM) equipped with an Oxford X-ray energy dispersive spectroscopy (EDS) detector. FESEM examinations were operated in secondary electron (SE) detection mode with an acceleration potential of 15 kV at Instituto Superior Tecnico, University of Lisbon, Portugal. Samples from Helmcken Falls Cave (Canada) were prepared, processed, and observed at the University of British Columbia (UBC) BioImaging Facility (Cheeptham et al., 2013). Rock chips with microbial mats from Azores, New Mexico, and Hawai`i were mounted, processed and observed as described in Hathaway et al. (2014).

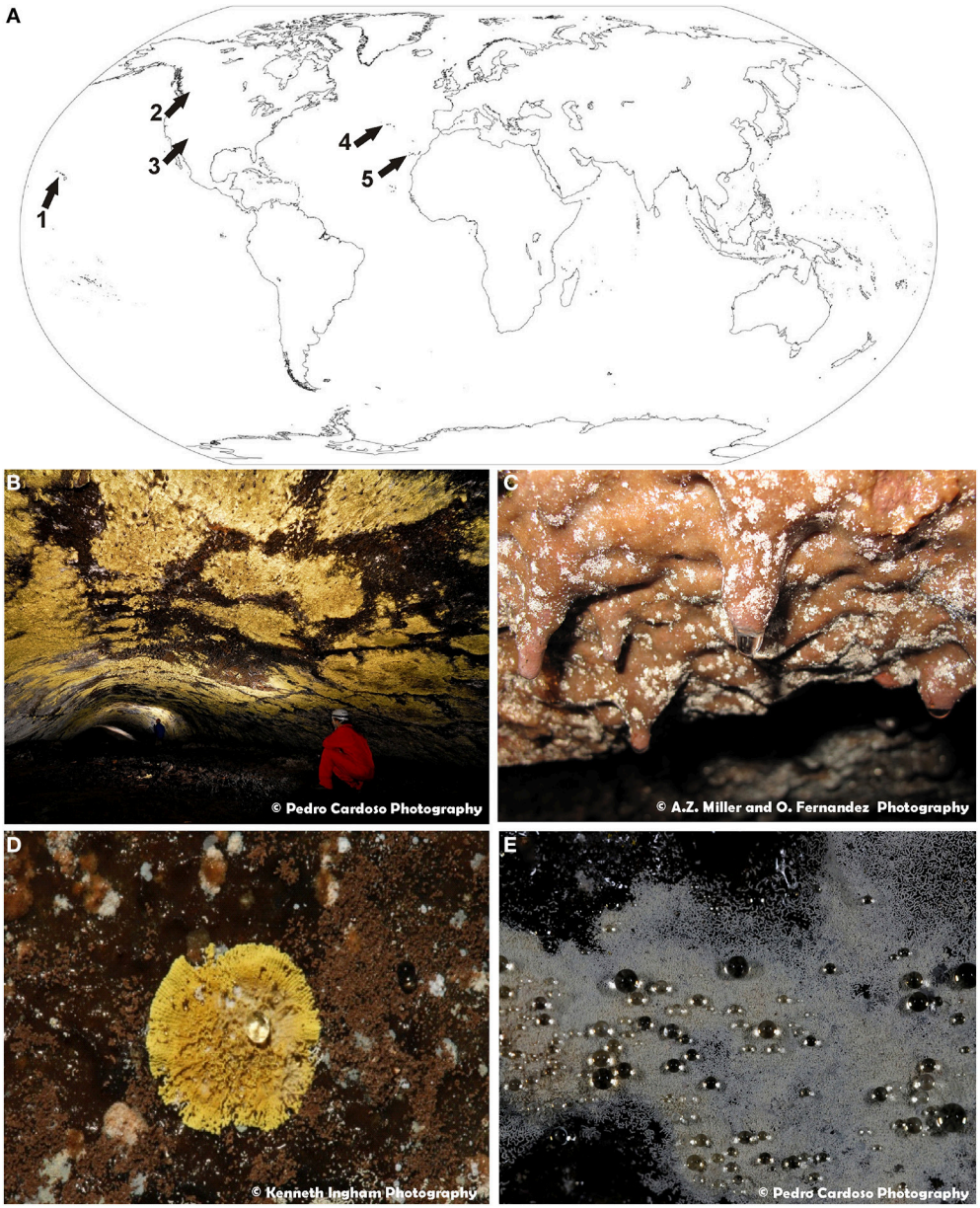

FIGURE 1 | (A) World map of volcanic caves studied in this work: 1—Hawaiian volcanic caves (U.S.A.); 2—Helmcken Falls Cave, British Columbia (Canada); 3—New Mexico volcanic caves (U.S.A.); 4—Azorean lava caves (Portugal); 5—La Palma caves, Canary Islands (Spain). **(B)** General view of extensive yellow microbial mats from Gruta da Terra Mole (Azores, Portugal). **(C)** General view of white colonies forming dendritic branches on basaltic lava from Fuente de la Canaria cave (La Palma Island, Spain). **(D)** Close-up view of a yellow colony from Gruta dos Montanheiros (Azores, Portugal). **(E)** Close-up view of white microbial mat covered with water droplets from Gruta da Terra Mole (Azores, Portugal).

Estimation, Description, and Novelty of Actinobacterial Diversity

Sample Collection and Clone Library Preparation and OTU-based Analysis for New Mexico (USA), Hawai`i (USA), and Azores Islands (Portugal)

Microbial mat samples of various colors were collected from the dark zone of five caves (Cave 12, Cave 255, Cave 266, Cave 261, and Cave 315) from El Malpais National Monument, New Mexico, six caves on the Big Island of Hawai`i (Bird Park, Epperson's, Kaumana, and Thurston Caves and the Maelstrom and Kula Kai Caverns Sections of the Kipuka Kanohina Cave System), four caves on the Azorean island of Pico (Furna do Lemos, Gruta dos Montanheiros, Gruta da Ribeira do Fundo, and Gruta das Torres) and 11 caves on the Azorean island of Terceira (Algar do Carvão, Gruta das Agulhas, Gruta da Achada, Gruta dos Buracos, Gruta dos Balcões, Gruta da Branca Opala, Gruta da Madre de Deus, Gruta do Natal, Gruta da

Terra Mole, Gruta dos Principiantes, and Gruta da Malha), see **Figure 1** and Supplemental Table 1. DNA from microbial mats of various colors was aseptically collected. DNA was extracted and purified using the MoBio PowerSoil™ DNA Isolation Kit using the manufacturer's protocol (MoBio, Carlsbad, CA), with the exception of the substitution of bead beating for 1.5 min (Biospec Products, Bartlesville, OK, USA) instead of vortexing for cell lysis. 16S rDNA sequences were amplified with universal bacterial primers 46 forward (5′ -GCYTAAYACATGCAAGTCG-3′) and 1409 reverse (5′ -GTGACGGGCRGTGTGTRCAA- 3′) (Northup et al., 2010).

Amplification reactions were carried out in a 25-μL volume with 1X PCR buffer with 1.5 mM Mg^{2+}, 0.4 μM of each primer, 0.25 mM of each dNTPs, 5 μg of 50 mg/mL BSA (Ambion, Austin, TX, USA) and 1U AmpliTaq LD (Applied Biosystems, Foster City, CA, USA), and carried out under the following thermocyling conditions on an Eppendforf Mastercycler 5333 (Eppendorf, Hauppauge, NY, USA): 94°C for 5 min, followed by 31 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1.5 min, with a final extension at 72°C for 7 min. Amplicons were cleaned and purified using the Qiagen PCR cleanup kit (Qiagen, Germantown, Maryland) and cloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Sequencing was carried out at the Washington University Genome Sequencing Facility. The subset of Actinobacteria were identified with RDP classifier (Maidak et al., 2001), and used for further analysis.

Alignments of the resulting actinobacterial sequences set were generated using INFERNAL (Nawrocki et al., 2009), trimmed to 104–1403 bp to remove ragged ends, and clustered into Operational Taxonomic Units (OTUs) at 97% similarity with QIIME using uclust (Caporaso et al., 2010). Taxonomy was assigned using uclust against the greengenes 13.8 database (Edgar, 2010; McDonald et al., 2012). Sequences were compared with the GenBank database in March 2015 using the Basic Local Alignment Search Tool (BLAST)[1] to determine closest relatives (Altschul et al., 1997). An identity matrix was generated using Bio Edit[2]. The tree was built using FastTree with the gamma and nt options (Price et al., 2009, 2010). OTUs and location were added to the tree using the phyloseq package in R (McMurdie and Holmes, 2013; R Core Team, 2015).

All other OTU-based approaches were performed with software package mothur 1.34 (Schloss et al., 2009). Rarefaction curves, non-parametric diversity indexes npsShannon (Chao and Shen, 2003), Shannon (Shannon, 1948) and Simpson (Simpson, 1949) and estimator Chao1 (Chao, 1984), as well as the Good's Coverage (Good, 1953) were calculated to infer the richness and evenness of the samples.

16S rRNA Gene Amplicon Library Preparation, Pyrosequencing, Bioinformatics, and OTU-based Analysis in Azorean Volcanic Caves

16S rRNA gene amplicon libraries were prepared from the previously described Azorean microbial mat samples collected from the previously mentioned caves with the exception of Algar do Carvão (Supplemental Table 1). The small subunit rRNA gene was amplified from community DNA targeting the V1 and V3 hypervariable region, with barcoded fusion primers containing the Roche-454 A and B Titanium sequencing adapters, a eight-base barcode sequence, the universal forward primer 5′– AGRGTTTGATCMTGGCTCAG -3′ and the universal reverse primer 5′–GTNTTACNGCGGCKGCTG-3′. Amplicon 454 pyrosequencing, as originally described by Dowd et al. (2008), was performed with PCR amplification as described in Brantner et al. (2014). Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing Roche 454 FLX titanium instruments and reagents and following manufacturer's guidelines.

The raw pyrosequencing reads were processed using version 1.34 of the mothur software package (Schloss et al., 2009). Sequencing reads were assigned to the appropriate samples based on the corresponding barcode and were quality filtered to minimize the effects of random sequencing errors, by eliminating sequence reads <200 bp, sequences that contained more than one undetermined nucleotide (N) and sequences with a maximum homopolymer length of 8 nucleotides. Identification and removal of chimeras was performed with Chimera.uchime (Schloss et al., 2011). Sequences not passing these quality controls were discarded. When preparing the inputs for analysis, the "remove.groups" command was used to discard all sequences not belonging to the phyla Actinobacteria.

OTUs were assigned from the uncorrected pairwise distances between aligned 16S rRNA gene sequences, using the average neighbor clustering (Schloss and Westcott, 2011), considering a cut-off value of 97% similarity. All OTU-based approaches were performed with software package mothur 1.34 (Schloss et al., 2009) as well as the taxonomic assignment of the sequences, performed by the Greengenes-based alignment using default parameters. A list of GenBank accession numbers is provided in Supplemental Table 2.

RESULTS AND DISCUSSIONS

Morphology of Colored Microbial Mats and Associated Microbe-mineral Interactions

One of the important factors influencing the microbial diversity of subsurface environments is the mineral microniches they develop on (Jones and Bennett, 2014). In order to broaden our understanding of the interactions of microorganisms in volcanic caves and their diversity around the world, an extensive SEM study was performed. Colored microbial mats with different morphologies from Azorean, Canadian, Canarian, Hawaiian, and New Mexican volcanic caves were investigated (**Figure 1A**). Abundant white and yellow microbial mats were distinctly visible to the naked eye (**Figures 1B,C**). These colored mats may consist of large, dense expanses of microorganisms with coarse and irregular edges covering extensive areas of volcanic cave walls and

[1]www.ncbi.nlm.nih.gov/BLAST/.
[2]www.mbio.ncsu.edu/BioEdit/bioedit.html.

ceilings (**Figure 1B**) or small colonies spread all over the surface (**Figure 1C**). Some colonies adopted the form of white spots with irregularly radiate pattern (**Figure 1C**) or yellow, round and isolated spots with a symmetrical character (**Figure 1D**). They can grow on the rock surfaces or on secondary mineral deposits, such as ooze-like deposits frequently found in these volcanic caves. In general, the microbial mats have finely granular surface (**Figure 1D**) and act as water condensation points, being covered with water droplets, particularly during the wet seasons (**Figure 1E**).

SEM images revealed the presence of possible Actinobacteria-like structures in most of the volcanic caves from all over the world showing a large variety of microbial morphologies and spore surface ornamentation (**Figure 2**). To confirm this observation, Sanger and pyrosequencing were performed. In general, these microbial mats were formed by a tangled mass of hyphae, spores, filamentous and coccoid cells (**Figures 2A–C**). Coccoid elements, with a diameter of about 0.5 μm, are frequently found in close heaps, intermingled with filamentous forms (**Figures 2B,E**). Most of these masses exhibited characteristic arthrospores of *Streptomyces* or close relatives with hairy (**Figures 2C,F**), smoothly (**Figure 2H**), spiny (**Figure 2I**) surface ornamentations. Spirals at the end of the aerial mycelium were also observed (**Figure 2J**). A notable feature of some of these bacteria is their filamentous growth with true branching, as depicted for instance in **Figure 2H**. Chains of coccoid cells resembling beads-on-a-string (**Figure 2G**) were found within both white and yellow mats. Some other microbial structures were difficult to associate to specific genera or species (**Figures 2D,E,G,K,L**). In addition, large spheres with lumpy surface connected by a network of hairy filaments and EPS (**Figure 2K**) or $CaCO_3$ spheres (EDS microanalysis) coated with a filamentous network (**Figure 2L**) were occasionally observed in the colored microbial mats. Average sizes varied between 10 and 15 μm.

The microbial mats studied in this work were found to be involved in microbe-mineral interactions as revealed by SEM investigations (**Figure 3**). Cell-sized etch pits attributed to dissolution of the substrate under attached cells were noticed (**Figures 3A–C**). Microboring caused by euendolithic growth of coccoid cells was particularly evident on the silicified substrate, leaving imprints of their surface ornamentation on the mineral grains (**Figure 3C**). These microbial mats may also figure prominently in the deposition of minerals due to the presence of filaments, some of which are coated with minerals (**Figures 3D–F**). Among them, reticulated filaments similar to those reported by Melim et al. (2008) and Miller et al. (2012a) were found associated with the white microbial mats from the Kula Kai Caverns of the Kipuka Kanohina Cave Preserve (Hawai`i, U.S.A.) and Falda de La Horqueta cave, in La Palma Island, Spain (**Figures 3E,F**). All these features evidence microbe-mineral interactions and may represent mineralogical signatures of life. Both constructive and destructive mineral features in caves have been recognized as biosignatures valuable for the searching of traces of life on Earth and other planets (Banfield et al., 2001; McLoughlin et al., 2007; Hofmann et al., 2008; Northup et al., 2011).

The role of microorganisms in biomineralization and rock-weathering processes in caves has been discussed in recent years (Cuezva et al., 2012; Porca et al., 2012; Saiz-Jimenez, 2012). Both processes involve destruction and construction of mineral structures. Destructive processes include dissolution, etching or pitting, whereas constructive processes comprise precipitation of secondary minerals, such as calcite, struvite, witherite, and birnessite. In terms of weathering of minerals, the major processes promoted by microorganisms are biochemical and biophysical mechanisms of etching, dissolution and boring occurring via mechanical attachment and secretion of exoenzymes or organic acids (Lee et al., 2012). Extensively etched mineral grains such as calcite and Mg-silicate minerals were found associated with actinobacterial morphologies on coralloid-type speleothems from the Ana Heva volcanic lava tube cave in Chile (Miller et al., 2014). In many cases, it is difficult to determine the exact mechanism by which microorganisms induce mineral dissolution, but the pitting of underlying mineral grains, as shown in **Figure 3**, illustrates that it does occur.

On the other hand, microorganisms may directly precipitate minerals as part of their metabolic activity, and they can also indirectly impact mineral formation by altering the chemical microenvironment such as pH or redox conditions or providing nucleation sites for precipitation through the production of organic polymers (Benzerara et al., 2011). Numerous biogenic minerals have been reported in subterranean environments (Sanchez-Moral et al., 2003, 2004; Spilde et al., 2005; De los Ríos et al., 2011; Miller et al., 2012b, 2014), and some of them have been associated with actinobacterial communities. Laiz et al. (2003) found that 61% of the Actinobacteria isolated from Altamira Cave (Spain) produced mineral crystals on culture media. In general, culture and field sample biominerals were composed of calcite, aragonite, Mg-calcite or vaterite. Groth et al. (2001) also tested the ability of cave-dwelling bacteria from Grotta dei Cervi (Italy) for producing mineral crystals. These authors reported extensive mineral production among Actinobacteria, which induced the precipitation of calcite (e.g., *Brachybacterium* sp.) or vaterite (e.g., *Rhodococcus* sp.). Needle-fiber mats were also related to biomineralization processes by actinomycetes (Cañaveras et al., 1999, 2006). Struvite was formed by Actinobacteria isolated from tuff in Roman catacombs (Sanchez-Moral et al., 2003), and witherite, a naturally occurring barium carbonate, was produced by species of the genera *Agromyces* and *Streptomyces* isolated from tuff (Sanchez-Moral et al., 2004). Calcium carbonate spheres closely related to dense networks of interwoven filaments were observed within the colored microbial mats from Azorean, Canarian and Hawaiian volcanic caves (**Figure 2L**). Similar spherical particles were previously reported by Cuezva et al. (2012) and Diaz-Herraiz et al. (2013), who proposed vaterite as their mineralogical phase. According to Cuezva et al. (2012) the gray colonies found on Altamira cave walls, dominated by Actinobacteria, were able to bioinduce $CaCO_3$ precipitation.

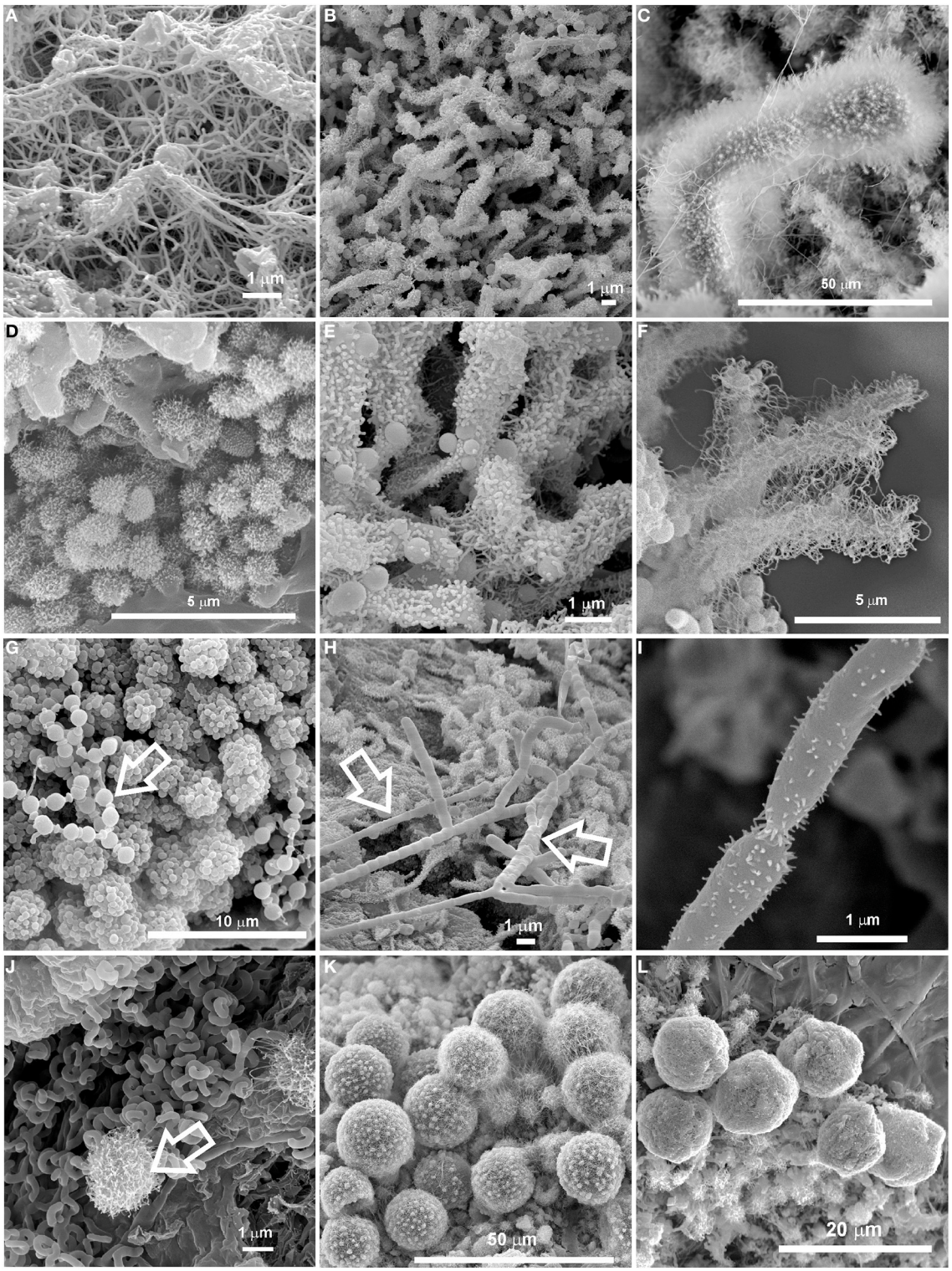

FIGURE 2 | SEM images of colored microbial mats found in Azorean, Canadian, Canarian, Hawaiian and New Mexican volcanic caves showing a large variety of microbial morphologies and spore surface ornamentation. (A,B) Dense network of interwoven filaments in Honda del Bejenado and Fuente de la Canaria caves (La Palma Island, Spain); **(C)** Dense masses of *Streptomyces*-like spore chains with hairy ornamentation from Cave 12 in El Malpais National Monument (New Mexico, U.S.A.); **(D)** Coccoid cells with surface appendages or obtuse protuberances from Gruta da Terra Mole (Terceira Island, Azores, Portugal); **(E)** Detailed view from **(B)** showing coccoid cells and clumps of spore chains with obtuse protuberances and surface appendages; **(F)** Close-up view of clusters of *Streptomyces*-like spore

(Continued)

FIGURE 2 | Continued
chains with extensive hairy ornamentation from Gruta da Terra Mole (Terceira Island, Azores); **(G)** Aggregates of coccoid cells with smooth surface and spherical cells arranged in chains resembling beads-on-a-string (arrow) from Bird Park Cave (Hawai`i, U.S.A.); **(H)** Chain of *Streptomyces*-like arthrospores from Honda del Bejenado Cave (La Palma Island, Spain); **(I)** Spores with spiny ornamentation from Helmcken Falls Cave, (British Columbia, Canada); **(J)** Spiral spore chains of *Streptomyces* and a coccoid cell with obtuse protuberances (arrow) from Falda de La Horqueta Cave (La Palma Island, Spain); **(K)** Large spheres with lumpy surface or protuberances connected by a network of filaments or appendages from Gruta dos Montanheiros (Pico Island, Azores); **(L)** $CaCO_3$ spheres coated with a filamentous network from the Tapa Section of the Kipuka Kanohina Cave Preserve (Hawai`i, U.S.A.).

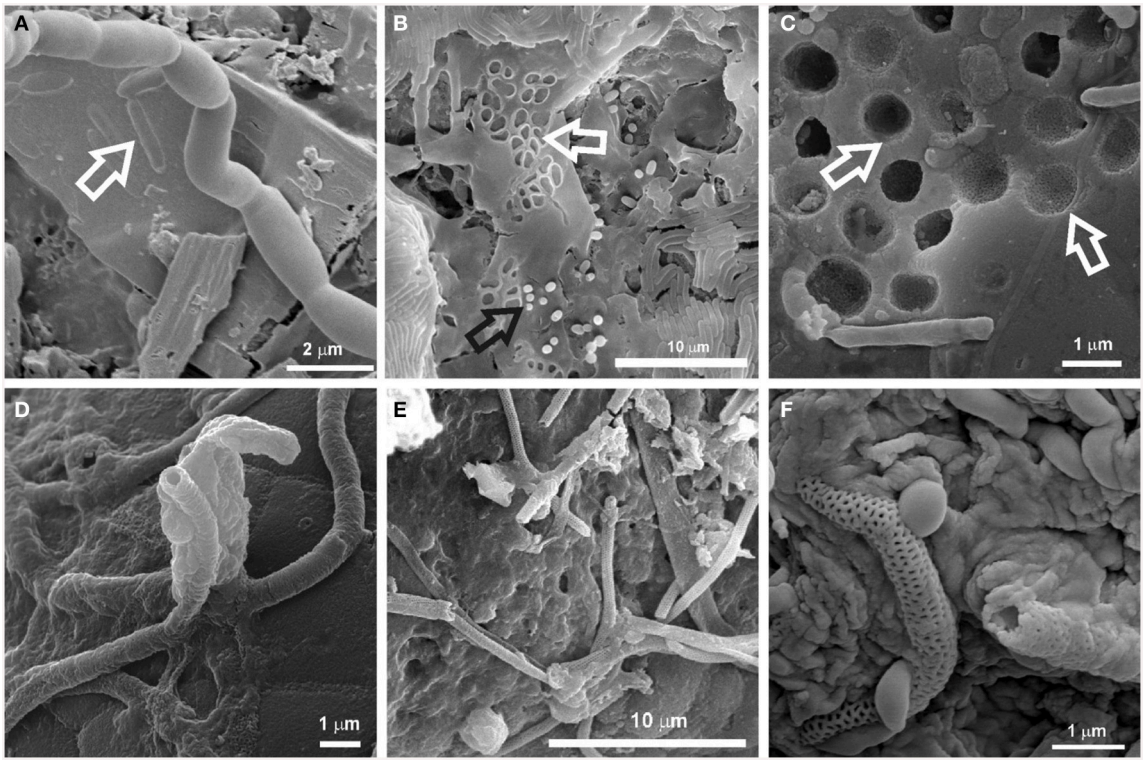

FIGURE 3 | SEM images of biosignatures found associated with microbial mats in Azorean, Canadian, Canarian, and Hawaiian volcanic caves. (A) Cell-shaped etched pits on mineral grain (arrow) from Helmcken Falls Cave (British Columbia Canada); **(B)** Cell imprints (white arrow) and rods on EPS matrix from a white microbial mat in Gruta da Terra Mole (Terceira Island, Azores, Portugal); **(C)** Microborings produced by euendolithic cells on silicified mineral grains from Ana Heva cave in Easter Island, Chile (adapted from Miller et al., 2014); **(D)** Tubular mineralized sheaths embedded in EPS found on black deposits from Cueva del Llano de los Caños cave(La Palma Island, Spain). **(E)** Reticulated filaments found in white microbial mats in the Kula Kai Caverns of the Kipuka Kanohina Cave Preserve (Hawai`i, U.S.A.); **(F)** Close-up view of mineral encrusted filaments with reticulated ornamentation associated with white microbial mats on ooze-like deposits from Fuente de la Canaria cave (La Palma Island, Spain).

Actinobacterial Diversity Found in New Mexico (USA), Hawai`i (USA), and Azores islands (Portugal)

The SEM study revealed notable microbial diversity. In order to confirm the presence of Actinobacteria in these volcanic caves and further investigate their diversity, three geographically distinct locations, New Mexico (USA), Hawai`i (USA) and Azores islands (Portugal), were chosen for clone library analysis. A total of 1176 Actinobacteria sequences generated by clone libraries were determined to be of high quality and used in this analysis (Supplemental Table 1). These sequences clustered into 164 OTUs across all locations, belonging to seven orders. *Actinomycetales* (sequences = 76.7%, OTUs = 52.4%), *Euzebyales* (9.9%, 8.5%) and *Acidimicrobiales* (9.6%, 17.7%) represented the majority of the OTUs (**Figures 4A,B**, upper panel). *Bifidobacteriales* (0.8%, 3.0%), *Gaiellales* (0.9%, 5.5%), *Rubrobacterales* (0.5%, 3.0%), and candidate *0319-7L1* (0.5%, 3.0%) represented less than 1% of the sequences (**Figures 4A,B**, lower panel). Sequences that could not be assigned to taxonomic affiliations were labeled as "unclassified" (1.1%, 6.7%). Singletons and doubletons were the most common OTU type over all (116 singletons, 23 doubletons). Of the doubletons, 14 had two sequences from the same cave, and 20 had sequences from the same location.

Five of the OTUs (3.05%) represented 74.1% of the total number of sequences found. The most predominant OTU (OTU 025) belonged to the *Pseudonocardiaceae* family, with 593

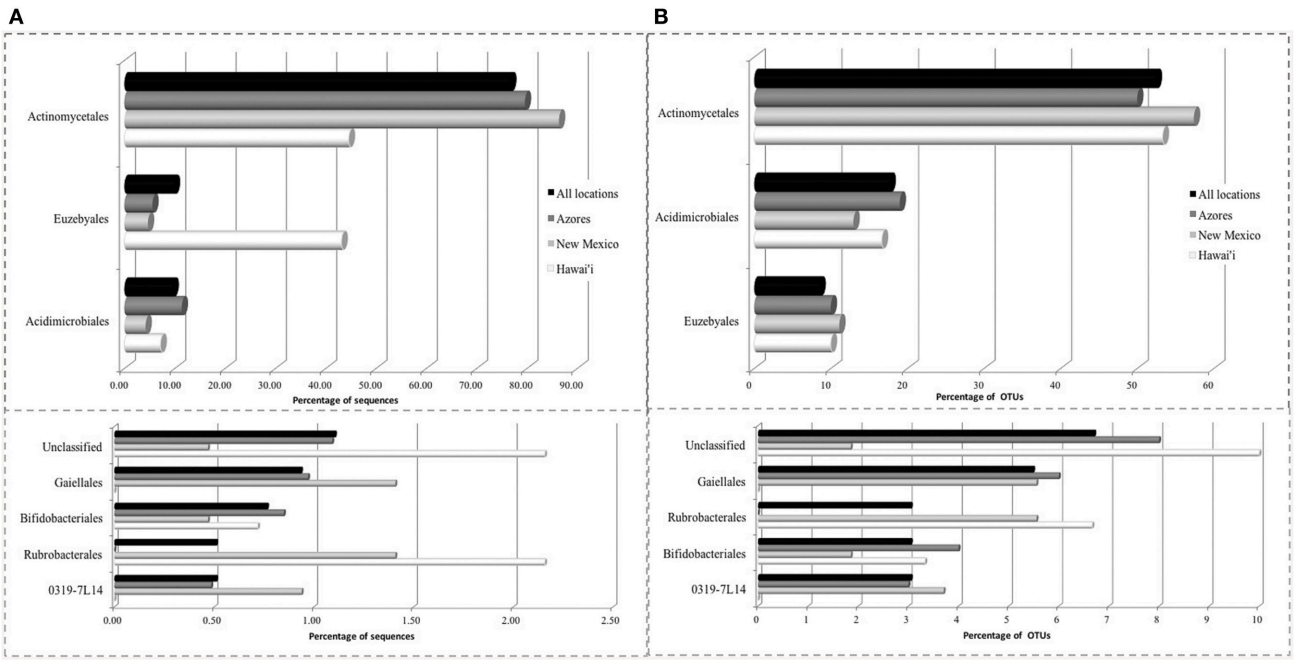

FIGURE 4 | Order-Level delineation of the 16S rRNA gene sequences (A) and OTUs (B) in Azores, New Mexico and Hawai`i lava caves.

sequences (50.4%) in 59 of the 82 samples. The second most common OTU (OTU 089), also a *Pseudonocardiaceae,* had 98 sequences (8.3%) in 29 samples, but was not found in Hawai`i. *Pseudonocardiaceae* was the most commonly found sequence and OTU in each location. This finding is consistent with other cave studies, which found *Pseudonocardiaceae* to comprise 52% of actinobacterial sequences in Carlsbad Cavern (Barton et al., 2007), 30–50% in three Slovenian limestone caves (Porca et al., 2012), and the most abundant OTU in a limestone cave in China (Wu et al., 2015).

OTUs belonging to the orders *Actinomycetales*, *Euzebyales*, *Acidomicrobiales,* and *Bifidobacteriales* were shared by at least two of the three locations under study (**Figure 5**, Supplemental Figure 1A). These ubiquitous OTUs may represent a core subsurface microbiota, a hypothesis that we will test in the future with more extensive sequencing. Furthermore, caves are not homogeneous habitats: they are characterized by zonal environments according to the distance to entrances (Poulson and White, 1969; Howarth, 1983, 1993), passage geometry, and microenvironments, which result from several types of reactions, including microbial processes that often involve redox reactions (Barton and Northup, 2007).

The number of shared OTUs in the three locations was relatively low; three out of five belonged to *Pseudonocardiaceae* and two were *Euzebyales* (Supplemental Figure 1A). Azores and New Mexico shared six other OTUs, four *Pseudonocardiaceae*, one *Euzebyales* and one *Bifidobacteriales*. Both archipelagos shared two *Acidomicrobiales*, one *Pseudonocardiaceae* and one unclassified OTU. Chao 1 estimator suggests that even though a more comprehensive sampling is required to provide a more complete assessment of these microbial communities, our sampling effort was probably enough to describe the cosmopolitan OTUs (Supplemental Figure 1B).

None of the sequences recovered were classified as *Streptomyces*, which was odd, given that *Streptomyces* are present in almost every other environment studied (i.e., soil, marine, etc., Schrempf, 2006), and were found in cultured isolates from the Azores (Riquelme and Dapkevicius personal communication). We believe this anomaly is due to primer bias. Farris and Olson (2007) showed that many Actinobacteria were not amplified in PCR despite being 100% identical to the universal primers used. While this does not conclusively establish that our sequencing missed *Streptomyces* that are present, it is cause for concern. Future sequencing efforts will utilize Actinobacteria-specific primers to test our hypothesis that *Streptomyces* are being missed and to better characterize the diversity of the Actinobacteria in caves.

Euzebyales emerged as the second most abundant order (number of sequences) in New Mexico and Hawai`i; however, *Acidomicrobiales* had the second most OTUs in New Mexico and Hawai`i, and was second for both sequences and OTUs in the Azores (**Figure 6**). *Euzebyales* was recently described and has two known genera (Kurahashi et al., 2010), and highly similar sequences have been identified from numerous environments (sea cucumbers, saline soils and caves) suggesting this order may be widespread in numerous habitats (Cuezva et al., 2012; Ludwig et al., 2012; Ma and Gong, 2013; Velikonja et al., 2014). The *Acidimicrobiales* order was described by Stackebrandt et al. (1997) and comprises members that are obligate acidophiles, oxidize ferrous iron or reduce ferric iron. It has already been

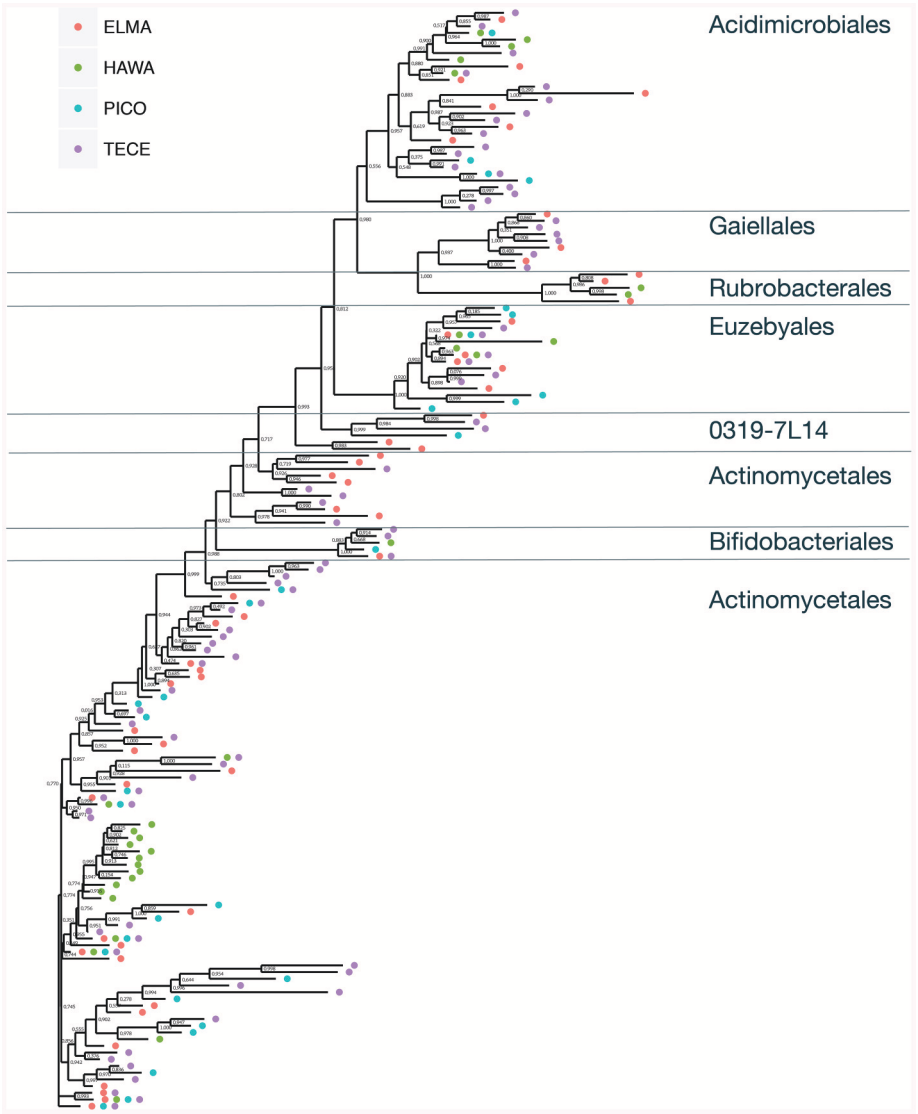

FIGURE 5 | Unrooted approximate maximum likelihood tree showing the relationship and occurrences of Actinobacteria OTUs across all four sample locations. Bootstrap values are indicated.

described in caves (Macalady et al., 2007; Ortiz et al., 2013; De Mandal et al., 2014), other volcanic environments (Cockell et al., 2013) and Fe-rich environments (Sánchez-Andrea et al., 2011; Grasby et al., 2013).

Evaluation of Diversity Coverage and Richness of the Clone Libraries

The coverage average estimated for the different locations ranged from 78 to 86%. Due to some variation in sampling effort in each case, a re-sampling analysis was performed, randomly selecting the smallest number of sequences across the different groups (139), 1000 times per each sample, to standardize the values. Diversity indices and estimators are summarized in **Table 1A**. Non-parametric Shannon and Shannon suggested more diverse communities within New Mexico caves compared to Hawai`i and Azores. Simpson diversity indices suggest the highest diversity values for Hawai`i. All indices agree with the less diverse communities being in Azores. The Shannon index gives more weight to the rare species and Simpson to the dominant ones. Considering the Simpson indexes of the three locations, the community composition in Azores caves would include more cosmopolitan species with high abundance and Hawai`i caves would be composed of phylotypes with narrower distribution. In islands, population size and genetic diversity tend to be limited due to the smaller extension of the habitats. Comparable taxa–area relationships (Bell et al., 2005) and distance–decay relationships for microbes and larger organisms were found to be significant although with variations in the rates of the processes (reviewed by Green and Bohannan, 2006; Soininen, 2012). However, we found differences between the diversity indices for

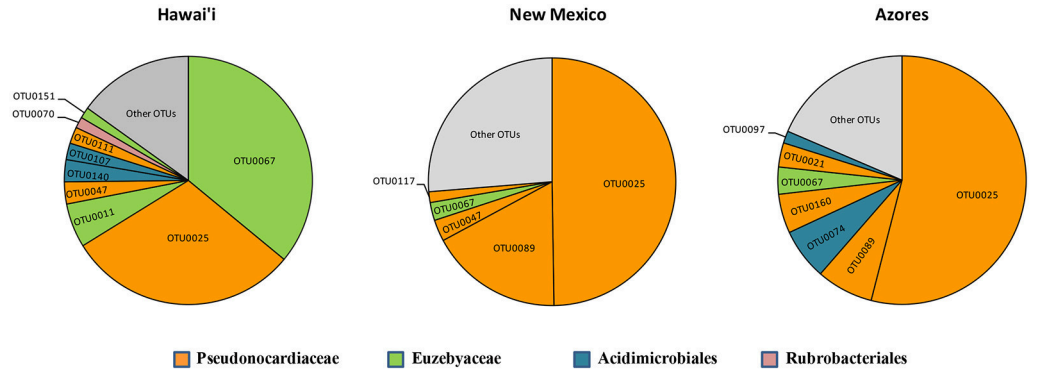

FIGURE 6 | Most abundant OTUs for Hawai`i, New Mexico, and Azores. OTUs were clustered at 97% similarity and represent more than 1% of the total sequences recovered in each geographical location.

TABLE 1A | Summary of the observed richness, diversity indices, coverage, and Chao 1 richness estimator at 97% similarity level at the three locations under study.

	Azores	New Mexico	Hawai`i
Richness	29.19	38.52	30
Shannon	2.01 (1.73–2.29)	2.16 (1.86–2.46)	2.13 (1.87–2.39)
Npshannon	2.35	2.69	2.48
Simpson	0.31 (0.22–0.39)	0.28 (0.20–0.35)	0.22 (0.17–0.27)
Invsimpson	3.31 (2.60–4.56)	3.62 (2.86–4.93)	4.49 (3.68–5.78)
Chao	97.79 (51.97–240.21)	158.98 (82.10–374.89)	100 (51.51–257.75)
coverage	0.86	0.78	0.85

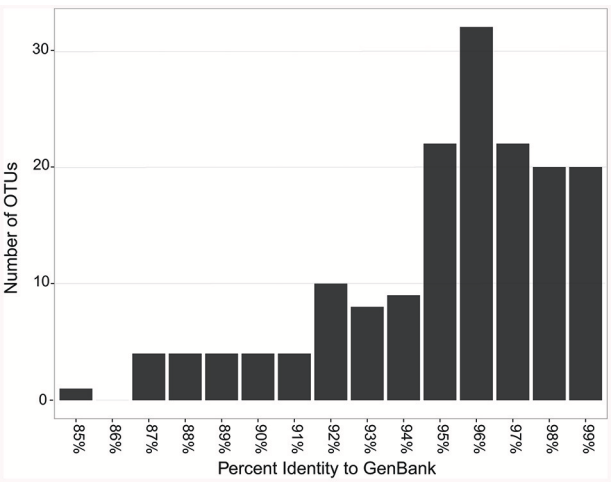

FIGURE 7 | Sequence identity based on BLAST comparisons to GenBank.

Azores and Hawai`i, which could be related to differences in island size, isolation and age of lava flows. We should be aware that the amount of data available is still small and that further studies may still reveal different trends.

Phylogenetic Analysis

When the representative sequences from each OTU were compared to known sequences in GenBank, 17 out of 164 OTUs (10%) shared ≤90% identity with known sequences in GenBank (**Figure 7**). Fifty two percent of the OTUs shared between 91 and 96% identity and 38% shared over 97% identity with known sequences. The most novel OTUs were mostly singletons, and were classified as *Pseudonocardiaceae* (four OTUs), *Rubrobacteraceae* (one OTU), *Bifidobacteriales* (five OTUs), and unclassified (seven OTUs). They were found in all four locations, however, more of the OTUs were found in the Azorean islands (13 out of 100) than in Hawai`i (2 out of 30) or New Mexico (3 out of 54). Physical isolation is an important driver of microbial evolution (Papke and Ward, 2004); thus, island isolation would promote unique evolutionary forces that result in the development of a novel genetic reservoir. However, in our results we did not observe significant differences between continental and island territories according to genetic novelty.

An approximate maximum likelihood tree shows the relationship between the sequences and occurrence of OTUs (**Figure 5**). For this analysis Pico and Terceira were considered separate locations. *Gaiellaceae*-like sequences were found in New Mexico and the Azores, but not Hawai`i. All but one of the sequences were singletons. *Gaiellaceae*, another recently described family, was originally found in a water borehole, and sequences from this family have subsequently been found in soil, volcanic soil, thermal springs and marine ascidians (Albuquerque et al., 2011; Kim et al., 2014; Rozanov et al., 2014; Steinert et al., 2015). *Rubrobacterales* occurred in the New Mexico and Hawai`i samples. The order *Actinomycetales* has many polytomies with most of them occurring in the samples from Hawai`i, Pico, and Terceira. These samples are either unresolved parts of the tree due to missing data or represent rapid speciation in the *Actinomycetales*. Representatives of *Euzebyales* were found in all four locations (**Figure 5**). The different clades suggest there is significant diversity within the sequences found.

While we acknowledge the limitation of our study to capture the full range of diversity in these sites, the high number of singletons found in this study suggests that there are Actinobacteria belonging to the rare biosphere in caves. The rare biosphere has been shown to influence both alpha and beta diversity, exhibiting unique geographic patterns (Lynch and Neufeld, 2015).

With over two thirds of our OTUs being singletons and most of the doubletons from one location, there is evidence to suggest endemism in cave Actinobacteria. Endemism in caves has been documented for obligate cave fauna in the United States and the Azores (Culver et al., 2003; Reboleira et al., 2012). Furthermore, studies of Actinobacteria in other environments have been shown to display endemism (Wawrik et al., 2007; Valverde et al., 2012). The combination of rare and endemic Actinobacteria, together with their abundance in caves, support the idea that caves are a good location to further test hypotheses regarding bacterial biogeography as well as to look for novel actinobacterial metabolites. Rigorous testing will require that future studies be conducted with next generation sequencing to comprehensively sample the diversity present in these habitats.

16S rRNA Gene Amplicon Library Preparation, Sequencing, Bioinformatics, and OTU-based Analysis in Azorean Volcanic Caves

The observed structure of the microbial communities in volcanic caves in the three locations is consistent with bacterial communities composed of consortia of few cosmopolitan members and a high number of low abundant phylotypes. To test whether this structure could be biased by the fact of having a limited number of sequences, a pyrosequencing approach was performed with the same sample points considered for clone libraries in Azores.

Actinobacterial sequences amplified using the universal primers were identified and after quality control and filtering of the crude pyrotags, 19,476 sequences with good quality were retained, consisting of 906 unique sequences. The average sequence length was 247.5 bp (range 233–275; median 247.1; sd 4.1). After clustering, a total of 382 OTUs were obtained.

Nine orders were found in Azorean caves with pyrosequencing, the seven previously found, i.e., candidate *0319-7L1* (sequences = 0.4%, OTU = 2.9%), *Acidimicrobiales* (1.2%, 1.6%), *Actinomycetales* (92.6%, 62.8%), *Bifidobacteriales* (0.7%, 4.5%), *Euzebyales* (2.7%, 4.5%), *Gaiellales* (1.1%, 8.4%), *Rubrobacterales* (0.04%, 0.3%), plus *Coriobacteriales* (0.3%, 3.4%), and *Solirubrobacterales* (0.3%, 4.5%) (**Figure 8**). While *Rubrobacterales* was found in the clone libraries, it was only found in New Mexico and Hawai`i (**Figure 5**). Amplicon sequencing revealed this order to be present in the Azores as well, highlighting the importance of pyrosequencing to capture the full range of diversity in these samples. *Actinomycetales* and *Gaiellales* orders showed an increase in the percentage of sequences and OTUs recovered; *Bifidobacteriales* had a higher percentage of OTUs. All other orders displayed lower percentages both for sequences and OTUs. Unclassified sequences represented 0.7 and 7.3%, respectively.

The amplicon libraries approach showed a more complete picture of the subterranean diversity in Azorean volcanic caves. *Rubrobacterales* comprised a group of novel OTUs, with all sequences sharing no more than 92% similarity with known sequences in GenBank, as well as *Solirubrobacterales,* with all of the sequences ranging between 90 and 95% similarity (Stackebrandt et al., 1997; Reddy and Garcia-Pichel, 2009). *Rubrobacterales* was first described in cave environments in Niu Cave (Zhou et al., 2007), and were also recovered from speleothems in Kartchner Caverns. This order includes members with heat, cold, dryness and high radiation resistance, found in high number in biodeteriorated monuments (Gurtner et al., 2000; Jurado et al., 2012) and volcanic environments (Cockell et al., 2013). *Solirubrobacterales* have also been described in caves (Paterson, 2012; De Mandal et al., 2014) and in other volcanic environments (Gomez-Alvarez et al., 2007; Cockell et al., 2013). *Coriobacteriales* (Stackebrandt et al., 1997; Gupta et al., 2013) showed a high percentage of sequences, 89.1%, with more than 97% similarity. This order was previously described in cave habitats in speleothem formations in Kartchner Caverns (Ortiz et al., 2013), and in Lower Kane cave (Paterson, 2012).

Evaluation of Diversity Coverage and Richness of the Amplicon Libraries

Sampling completeness assessed by Good's coverage estimator for each data set returned values above 98% (**Table 1B**). Diversity indices revealed a higher diversity at Pico Island compared to Terceira Island as well as chao richness estimator (**Table 1B**).

The dominance of the *Pseudonocardiaceae* family compared to any other member of the microbial community is remarkable, in accordance with results from both clone and amplicon libraries. *Pseudonocardiaceae* encompases a wide array of rare *Actinomycetes*, many of which can produce secondary metabolites (Tiwari and Gupta, 2013). While we acknowledge that this finding may be in part the result of primer bias, the prevalence of this family is not uncommon in caves (Barton et al., 2007; Porca et al., 2012; Wu et al., 2015). Little is known of role these bacteria play in most ecosystems, however the family encompases a wide variety of metabolic pathways and physiologies (Huang and Goodfellow, 2011). Most of our sequences were unable to be classified at the genus level, leaving some doubt as to the true role of this group of bacteria in volcanic caves. However, the ubiquity of this family in cave studies emphasizes the need for further molecular studies with improved primers to capture Actinobacteria diversity and cultivation of members of this family found in subterranean bacterial biofilms. An examination of the communities *in situ* combined with metatranscriptome analysis would shed light on the question of this group's role in volcanic cave ecosystems.

CONCLUSIONS

Our collective attempt to better understand actinobacterial diversity and functions in volcanic caves led us to observe

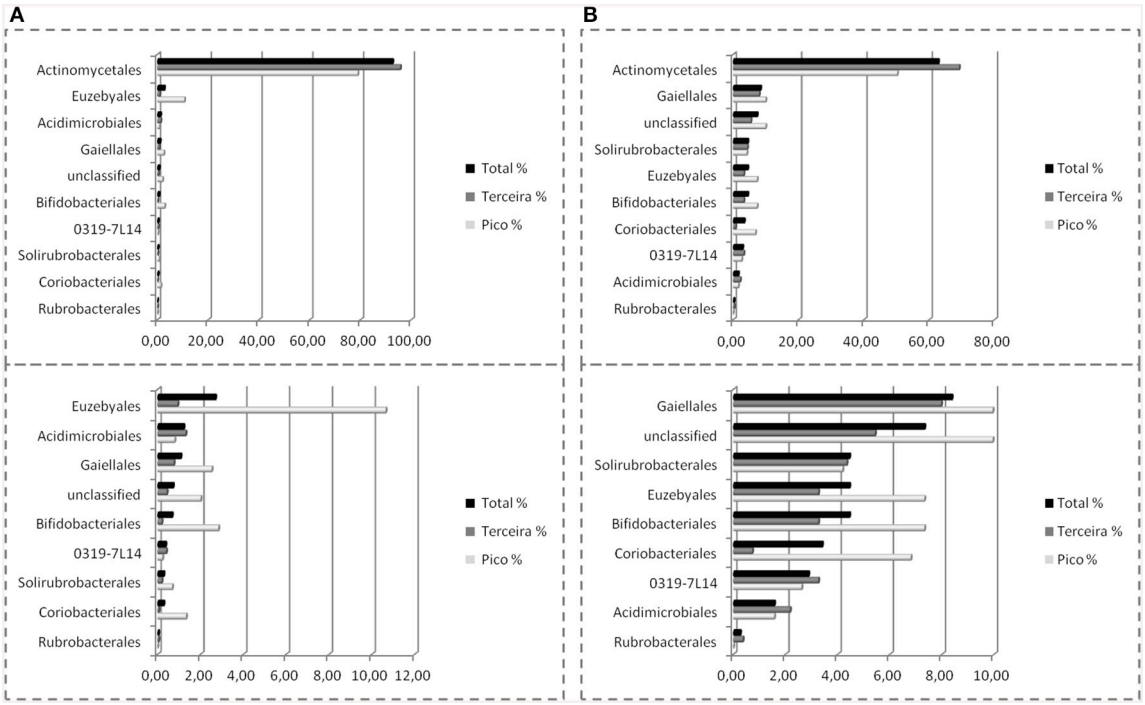

FIGURE 8 | Order-Level delineation of the 16S rRNA gene sequences (A) and OTUs (B) in Azorean volcanic caves obtained by amplicon library.

TABLE 1B | Summary of the characteristics of the pyrosequencing data.

	Azores	Pico	Terceira
Richness	382	191	141.57
Shannon	1.99 (1.96–2.02)	2. 67 (2.60–2.75)	1.68 (1.61–1.75)
Npshannon	2.04	2.76	1.79
Simpson	0.40 (0.40–0.41)	0.24 (0.23–0.26)	0.45 (0.43–0.46)
Invsimpson	2.48 (2.43–2.53)	4.11 (3.88–4.37)	2.25 (2.15–2.35)
Chao	529.70 (477.26–611.02)	262.19 (229.43–322.89)	256.86 (203.65–355.78)
Coverage	0.99	0.98	0.98

I.e., observed richness, diversity indices, coverage and Chao 1 richness estimator at 97% similarity level at the two islands from the Azorean archipelago under study.

patterns of diversity and novelness through a range of data obtained from 454 pyrosequencing to cloning. To date, within the realm of actinobacterial community study, our work is one of the largest sampling efforts in volcanic caves from different parts of the world including Spain, Portugal, USA and Canada. The sequencing effort, both in clone and amplicon libraries, represents one of the most comprehensive studies of Actinobacteria in volcanic caves around the world. The clone libraries illustrate the novelness and phylogenetic relationship of Actinobacteria in volcanic caves from three geographically distant locations. The amplicon libraries of the Azorean sequences gave a more in-depth view of the Actinobacteria communities and revealed more diversity than has previously been described. Both methods showed large numbers of newly described orders, and a dominance of *Actinomycetales*. Together they provide an outline of the community structure of Actinobacteria in caves, and highlight the importance of caves as a source of rare and novel Actinobacteria.

Through scanning electron microscopy examinations, we learned about bacterial morphology, their relationships and possible contribution of the Actinobacteria to cave environment. The identification of Ca-rich elements coated within some of the filamentous networks in the colored microbial mats suggests a possible role of Actinobacteria in calcium deposition. Both constructive and destructive mineral features, such as biominerals, cell imprints, microboring and mineralized filaments were some of the biosignatures found associated with samples studied herein. We can thus consider that volcanic caves on Earth are plausible repositories of terrestrial biosignatures where we can look for evidence of early life.

Beyond contributing to understanding cave microbial ecology, community and microbial roles and related function in such extreme subsurface habitats, our study hopes to initiate more study in such an interesting and understudied frontier of the Earth, where unique compounds could be isolated and used as important sources of industrial processes.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01342

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Coral-Associated Actinobacteria: Diversity, Abundance, and Biotechnological Potentials

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Marine Actinobacteria, particularly coral-associated Actinobacteria, have attracted attention recently. In this study, the abundance and diversity of Actinobacteria associated with three types of coral thriving in a thermally stressed coral reef system north of the Arabian Gulf were investigated. *Coscinaraea columna*, *Platygyra daedalea* and *Porites harrisoni* have been found to harbor equivalent numbers of culturable Actinobacteria in their tissues but not in their mucus. However, different culturable actinobacterial communities have been found to be associated with different coral hosts. Differences in the abundance and diversity of Actinobacteria were detected between the mucus and tissue of the same coral host. In addition, temporal and spatial variations in the abundance and diversity of the cultivable actinobacterial communities were detected. In total, 19 different actinobacterial genera, namely *Micrococcus*, *Brachybacterium*, *Brevibacterium*, *Streptomyces*, *Micromonospora*, *Renibacterium*, *Nocardia*, *Microbacterium*, *Dietzia*, *Cellulomonas*, *Ornithinimicrobium*, *Rhodococcus*, *Agrococcus*, *Kineococcus*, *Dermacoccus*, *Devriesea*, *Kocuria*, *Marmoricola,* and *Arthrobacter*, were isolated from the coral tissue and mucus samples. Furthermore, 82 isolates related to *Micromonospora*, *Brachybacterium*, *Nocardia*, *Micrococcus*, *Arthrobacter*, *Rhodococcus,* and *Streptomyces* showed antimicrobial activities against representative Gram-positive and/or Gram-negative bacteria. Even though *Brevibacterium* and *Kocuria* were the most dominant actinobacterial isolates, they failed to show any antimicrobial activity, whereas less dominant genera, such as *Streptomyces*, did show antimicrobial activity. Focusing on the diversity of coral-associated Actinobacteria may help to understand how corals thrive under harsh environmental conditions and may lead to the discovery of novel antimicrobial metabolites with potential biotechnological applications.

Keywords: culturable coral-associated Actinobacteria, Arabian Gulf, antimicrobial ability, temporal and spatial variation, *Platygyra daedalea*

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INTRODUCTION

The marine environment is currently recognized as the largest potential source of new actinobacterial species because more than 70% of the planet is covered by oceans (Lam, 2006). At present, the discovery of rare or novel marine Actinobacteria has become a major focus in the search for the next generation of pharmaceutical agents (Bull et al., 2000). Marine Actinobacteria are expected to differ in their characteristics from their terrestrial counterparts and may produce

new bioactive compounds (Manivasagan et al., 2013, 2014). In the literature, it is becoming evident that marine habitats are an abundant and novel source of Actinobacteria for new natural products because 716 new marine compounds were described in the Antibiotics Literature Database in 2004 (Blunt et al., 2006) and an additional 812 compounds were added in 2005 (Blunt et al., 2007). Culture-dependent and culture-independent molecular approaches have shown that marine Actinobacteria inhabit different marine habitats, including coastal and intertidal regions, marine sediments, seaweeds, fish, shrimps, mollusks and mangroves. Each of these environments has been found to harbor different members of Actinobacteria, some of which have antimicrobial activities (Mincer et al., 2002; Piza et al., 2004; Webster et al., 2004; Sivakumar et al., 2007).

Among marine systems, very little is known about actinobacterial diversity in coral reef systems. Corals, the most important members of the coral reefs, harbor abundant prokaryotic communities, including both Bacteria and Archaea (Rohwer et al., 2002) that inhabit coral mucus (Ducklow and Mitchell, 1979; Paul et al., 1986; Ritchie and Smith, 1997, 2004; Lampert et al., 2006), the tissue surface (Frias-Lopez et al., 2002; Bourne and Munn, 2005), the coral calcium carbonate skeleton and coral tissue (Williams et al., 1987; Shashar et al., 1994; Kushmaro et al., 1996; Banin et al., 2001). Lampert et al. (2006) have investigated the cultured bacteria associated with the mucus of the Red Sea coral *Fungia scutaria* and have found it to harbor different bacterial members, 23% of which were Actinobacteria. In addition, the mucus of *Fungia granulose* from the Red Sea (Kooperman et al., 2007), *Porites astreoides* from Bocas del Toro, Panama (Wegley et al., 2007), *Montipora capitata*, *Porites compressa* and *Porites lobata* (Ritchie and Lewis, 2005) has been found to harbor actinobacterial members. Furthermore, the culture-independent studies conducted by Yakimov et al. (2006) and Penn et al. (2006) have proven the presence of Actinobacteria in the deep-water corals of the Mediterranean Sea and the Gulf of Alaska Seamounts, respectively. Studies showed that healthy corals harbor larger numbers of Actinobacteria than their diseased counterparts (Frias-Lopez et al., 2002; de Castro et al., 2010). The capability of Actinobacteria to secrete a wide range of secondary metabolites against other microbes (Caundliffe, 2006; Piskorska et al., 2007) and their ability to fix nitrogen are expected to explain their dominance in healthy corals (Rohwer et al., 2002). Nithyanand et al. (2010, 2011) have found Actinobacteria associated with the branched coral *Acropora digitifera* from the Gulf of Mannar, India, with antibiotic activity against Gram-positive and Gram-negative bacteria. All of these studies investigated Actinobacteria associated with corals from tropical water bodies, but no information is available for thermally stressed corals, which are a potential reservoir for novel Actinobacteria species.

The Arabian Gulf is known as one of the hottest water bodies in the world (Kinsman, 1964; Sheppard et al., 1992), and corals of the Arabian Gulf are considered to be unique because they are able to survive extreme fluctuations in temperature (Riegl and Purkis, 2012). Corals usually perish when the water temperature exceeds 32°C or drops below 19°C; however, Gulf corals can survive water temperatures exceeding 35–39°C in the summer and falling below 11–9°C in the winter (Coles and Fadlallah, 1991; Spalding et al., 2001; Coles and Riegl, 2012; Riegl and Purkis, 2012). In addition, Gulf corals can survive at high salinity levels, which usually exceed 39 psu in most of the regions of the Arabian Gulf (Coles and Riegl, 2012; Riegl and Purkis, 2012). Very little information is available regarding Gulf coral holobionts, particularly the bacterial communities of these thermally stressed corals (Ashkanani, 2008; Al-Dahash and Mahmoud, 2013).

In our study, we investigated the variations in Actinobacteria associated with the tissue and mucus of various coral hosts thriving under the extreme thermal stress conditions found in the north portion of the Arabian Gulf. The ability of the coral-associated Actinobacteria to produce antimicrobial agents against certain Gram-positive and Gram-negative bacteria was assessed. Furthermore, the temporal and spatial variations in the abundance and diversity of Gulf coral-associated Actinobacteria were investigated.

MATERIALS AND METHODS

Sampling and Sample Processing

The cultured Actinobacteria associated with three different massive coral genera i.e., *Coscinaraea columna, Platygyra daedalea,* and *Porites harrisoni*, were investigated. *C. columna* and *P. daedalea* are listed in the IUCN red list as being of least concern, whereas *P. harrisoni* is listed as being near threatened. All of the species were sampled from the Qit'at Benaya inshore coral reef system north of the Arabian Gulf (N28 37021 E48 25702) in spring (March 2008). The spatial variation in the cultured Actinobacteria associated with the massive brain coral *P. daedalea* was investigated by sampling the tested coral from the Qit'at Benaya inshore reef and the Umm Al-Maradim offshore reef system (N28 40.792 E48 39.105) in autumn (October 2008). In addition, the temporal variation in the cultured Actinobacteria associated with *P. daedalea* was investigated by sampling the tested coral from the inshore reef in March 2008, October 2008, and March 2009. Five colonies of each type of coral were sampled, and three subsamples were collected from each colony. The seawater salinity, pH, temperature, dissolved oxygen, and conductivity were recorded for each site at each sampling day using a Horiba Water Quality Checker (Horiba, USA) (Supplementary Table S1).

Samples were collected during spring and autumn during which the corals were not subjected to much stress. It is more likely that the corals sampled at these two seasons would be healthy or at least recovering from the stress during the previous seasons.

Samples of coral tissue and mucus were collected by SCUBA diving. Mucus samples of the corals were collected by sterile syringes, whereas coral nubbins were removed from healthy coral colonies (1 cm^2 in size patches) and were collected in sterile bags. The coral mucus samples were transferred from the syringes to 15-ml sterile centrifuge tubes, and the volume of the collected mucus was determined. The volume was brought up to 10 ml with phosphate-buffered saline (PBS; Sambrook

et al., 1989). In contrast, the coral samples were washed by vigorously shaking the coral tissue with 10 ml of sterile saline water containing 3% NaCl for 2 min to remove the secreted mucus and any attached epiphytes. After washing the samples, the coral nubbin weight was determined, and the coral nubbins (coral tissue + skeleton + mucus) were macerated with a mortar and pestle in 20 ml of sterile PBS, the macerate were referred to through out the study by coral tissue.

Enumeration of Microbes in the Collected Samples Using the Direct Count Technique

The total numbers of microbes in coral tissue and mucus were determined using the 4'-6-diamidino-2-phenylindole (DAPI) (Sigma, USA) direct count method (Yu et al., 1995; Christensen et al., 1999). An aliquot of 0.25 ml of formaldehyde was added to 5 ml of the seawater samples and to 1 g of the sediment samples, which were suspended in 10 ml of sterile saline water. Additionally, 0.25 ml of formaldehyde was added to 5 ml of the coral tissue suspension and coral mucus samples. The samples were then stained with 0.1 ml of DAPI and incubated in the dark at room temperature for 40 min. Aliquots (50–100 μl) of the stained samples were filtered onto black polycarbonate 0.22-μm membrane filters (Millipore, Ireland) and enumerated by using an epifluorescent microscope (Zeiss, Germany).

Enumeration of Cultured Actinobacteria in Coral Tissue and Mucus

Serial dilutions of the coral mucus and tissue suspensions were prepared, and the 10^{-3} and 10^{-5} diluents were used. An aliquot of 0.1 ml of each diluent was inoculated on specialized media to promote and maximize the isolation of selected mucus- and coral-associated Actinobacteria. R2A medium (Oxoid, England), M2 medium (Mincer et al., 2002), M4 medium (Zhang et al., 2006), and Starch Casein Agar (SCA) medium (Atlas, 2004) were used, and the R2A and SCA media were modified to contain 3% (w/v) NaCl. The pH of each medium was set to 7.6, and all of the media were supplemented to obtain final concentrations of 50 μg ml^{-1} potassium dichromate ($K_2Cr_2O_7$), 15 μg ml^{-1} of nalidixic acid, 75 μg ml^{-1} cycloheximide and 75 μg ml^{-1} nystatin. Cycloheximide, potassium dichromate, and nystatin (Sigma, USA) were added to the media to inhibit fungal growth, whereas nalidixic acid was used to inhibit fast-growing Gram-negative bacteria, which would otherwise have overgrown the plates and prevented the isolation of slow-growing Actinobacteria. All of the plates were incubated at 28–30°C for 3–6 weeks. The developed colonies were categorized using morphological and cultural characteristics, counted, and purified.

Molecular Analysis of the Isolates

The total genomic DNA from the pure bacterial cultures was extracted using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, USA) following the manufacturer's protocol. The DNA extracted from each purified bacterial culture was amplified using PCR techniques. The 16S rRNA gene fragments were amplified using actinobacteria-specific primers. The 16S rRNA genes were amplified using Ready-To-Go PCR Beads (Amersham Biosciences, UK). Each tube contained 25 μl of a reaction mixture composed of 25 ng of the extracted DNA, 25 pmole of each of the forward S-C-Act-235-a-S-20 (CGCGGCCTATCAGCTTGTTG; Stach et al., 2003) and the reverse primers S-C-Act-878-a-A-19 (CCGTACTCCCCAGGCGGGG; Stach et al., 2003) and 23.5 μl of molecular-grade water. PCR amplification was performed in a thermocycler (Applied Biosystems, USA) using PCR programs comprised of an initial denaturation at 95°C for 4 min followed by 30 cycles of 95°C for 30 s, 70°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 7 min (Stach et al., 2003). The amplified PCR product with a size of 643 bp was purified using the QIA Quick Purification Kit (Qiagen, USA) following the manufacturer protocol, and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) was used for labeling and amplifying the purified product. Two microliters of the sequencing terminator and 2 μl of the 5X Big Dye Sequencing Buffer were mixed with 1 μl of each primer separately and 2 μl of the purified PCR product. The total mixture volume was supplemented with sterile molecular water to reach 10 μl. Using the Big Dye method, the labeling was completed in the GeneAmp PCR system 9700 thermocycler (Applied Biosystems, USA). The PCR program applied included 1 cycle of denaturation at 95°C for 1 min followed by 25 cycles of denaturation at 96°C for 1 min, annealing at 50°C for 5 s and extension at 60°C for 4 min. The labeled products were purified using 3 M sodium acetate (pH 5.2) and absolute ethanol and analyzed using a 3130x*l* genetic analyzer (Applied Biosystems, USA) and the Sequencing Analyzer v5.2 Software (Applied Biosystems, USA). The sequences obtained were compared with other sequences in the GenBank database using BLASTn (Altschul et al., 1997). The sequences were submitted to the GenBank under the accession numbers (KU579016-KU579199).

Antimicrobial Assays

The agar diffusion test (Isaacson and Kirschbaum, 1986) was used to examine the ability of actinobacterial isolates to produce antimicrobial products. The tests were conducted against indicator strains including Gram-positive (i.e., *Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative bacteria (i.e., *Escherichia coli*), which were cultured on marine agar. Two different modifications of the agar diffusion test were applied. The first method included placing disks (i.e., 2 mm in size) of the actinobacterial cultures, with the culture side facing the marine agar, on agar media containing the indicator strains. The second method was the agar-well diffusion test, which depended on making holes in the marine agar that contained the indicator organism and filling the holes with 0.1 ml of 0.45μm filtered marine broth containing the actinobacterial inoculum in the log phase of growth. Positive control (i.e., 100 mg ampicillin, Sigma) and negative control (sterile broth) was also included in the agar-well diffusion test. The plates were incubated at 26°C for 24–48 h, and the actinobacterial activity was evaluated by measuring the inhibition zones on the plates around the disks or the holes.

Statistical Analysis

Between-sites variations in the actinobacterial abundance was examined using *t*-test and by using SPSS (version 17) software. In addition, within-sites variations and between-hosts variations were examined using *t*-test and one-way ANOVA. Pearson correlation coefficient was used to examine the relationship between the microbial variables in the coral tissue and mucus.

RESULTS

Abundance and Diversity of Cultured Actinobacteria Associated with Various Coral Types

The total numbers of cultured Actinobacteria in *Platygyra daedalea, Porites harrisoni,* and *Coscinaraea columna* in tissue and mucus samples from the inshore reef system coral are shown in **Figure 1**. Different coral hosts were found to harbor equivalent numbers of cultured Actinobacteria in their tissues; in particular, the average numbers detected in tissues of *P. daedalea, P. harrisoni* and *C. columna* were 8.7×10^7 CFU g^{-1}, 8.3×10^7 CFU g^{-1}, and 7.7×10^7 CFU g^{-1}, respectively, and no significant difference was found among the tested corals. Significant differences ($P < 0.001$) in the numbers of cultured Actinobacteria were found in the comparison of mucus samples from various coral hosts; the highest numbers were found in *P. daedalea* mucus samples (9.6×10^7 CFU ml^{-1}), and lowest numbers were detected in *C. columna* (5.1×10^7 CFU ml^{-1}). In contrast, the comparison of the numbers of cultured Actinobacteria in the coral mucus and tissue samples showed that each coral host harbored significantly different numbers ($P < 0.01$) of Actinobacteria in their tissue and mucus; in particular, higher numbers were found in the coral mucus of both *P. harrisoni* and *P. daedalea* compared with its tissue, whereas *C. columna* harbored significantly less culturable bacteria in its mucus compared with its tissue.

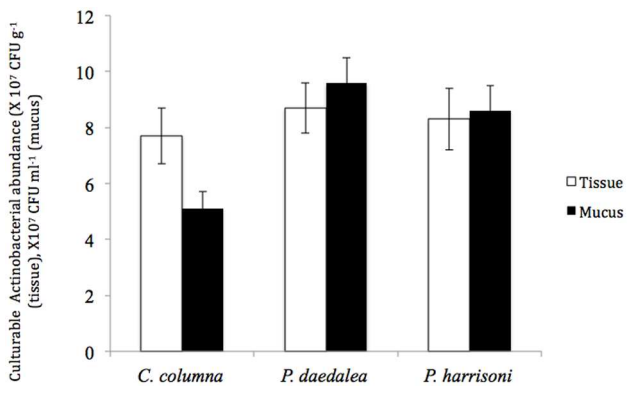

FIGURE 1 | The abundance of culturable Actinobacteria in coral tissue (□) and mucus (■) samples collected from the inshore reef system of Qit'at Benaya on March 2008.

In general, the M4 medium produced the highest numbers and diversity of isolates from the tissue and mucus samples of all of the corals sampled in the current study, whereas the R2A medium yielded the second-highest numbers and diversity, and the SCA medium gave the lowest numbers (Supplementary Figure S1).

The phylogenetic investigation of 169 isolates obtained from the three investigated hosts showed the dominance of 14 different actinobacterial genera. The similarity between the isolates and their nearest match in GenBank ranged from 97 to 100%. The 14 different genera to which the isolates belong are *Kocuria* sp., *Brevibacterium* sp., *Rhodococcus* sp., *Streptomyces* sp., *Marmoricola* sp., *Nocardia* sp., *Microbacterium* sp., *Arthrobacter* sp., *Micrococcus* sp., *Brachybacterium* sp., *Kineococcus* sp., *Dermacoccus* sp., *Devriesea* sp., and *Cellulomonas* sp. The abundance of different actinobacterial members varied across the samples such that some of these members were significantly more common in particular corals (**Figure 2**).

Kocuria sp. and *Brevibacterium* sp. were the most abundant cultured Actinobacteria in the three tested coral hosts. *Dermacoccus* sp. and *Devriesea* sp. were recovered only from the tissue of *C. columna*, whereas *Cellulomonas* sp. was found associated with *C. columna* mucus. *Brachybacterium* sp. and *Kineococcus* sp. were identified in *P. daedalea* mucus and tissue, respectively, whereas *Marmoricola* sp. was detected only in the tissues of both *P. daedalea* and *P. harrisoni*. The results showed that the *P. daedalea* samples harbored less diversity of cultured Actinobacteria than the *C. columna* and *P. harrisoni* samples (**Figure 2**).

Spatial and Temporal Variation in the Abundance and Diversity of *Platygyra daedalea*-Associated Cultured Actinobacteria

Among the three investigated coral genera, *Platygyra daedalea* showed the highest number but the lowest diversity of culturable Actinobacteria in both the tissue and mucus and was thus selected for further analysis to investigate the spatial and temporal changes in culturable Actinobacteria associated with this type of coral, which is very common in various coral reefs located in the northern section of the Arabian Gulf.

No significant differences were found in the total numbers of actinobacterial isolates obtained from *P. daedalea* tissue and mucus samples obtained from the inshore and offshore reef systems, despite the differences between the two environments. The tissue of *P. daedalea* was found to harbor 7.8×10^7 CFU g^{-1} and 8.5×10^7 CFU g^{-1} in the inshore and offshore reef samples, respectively, whereas the mucus samples obtained from the inshore and offshore reefs harbored 9.4×10^7 CFU ml^{-1} and 8.7×10^7 CFU ml^{-1}, respectively (**Figure 3**).

The investigation of the phylogenetic diversity of the cultured Actinobacteria associated with the tissue and mucus of *P. daedalea* samples obtained from the inshore (57 isolates) and offshore reef systems (58 isolates) in October 2008 showed a lower diversity in the mucus sample obtained from the inshore reef system (four different genera) compared with that observed in the

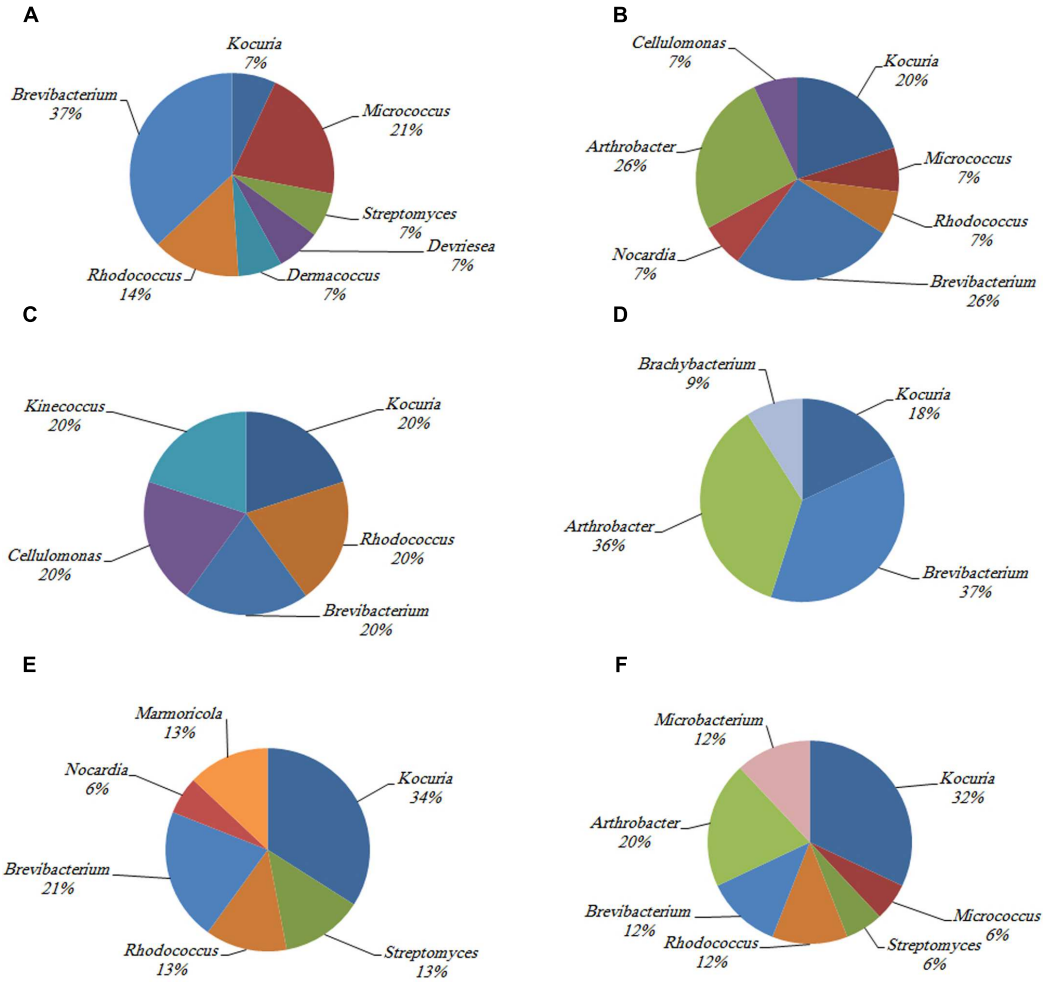

FIGURE 2 | The identity and percentage of actinobacterial isolates obtained from (A) *C. culumna* tissue, (B) *C. culumna* mucus, (C) *P. deadalea* tissue, (D) *P. deadalea* mucus, (E) *P. harrisoni* tissue, and (F) *P. harrisoni* mucus collected from inshore reef system on March 2008.

offshore reef samples (nine different genera; **Figure 4**). However, the tissue samples were found to harbor an equivalent level of diversity (six genera each). The dominance of *Brevibacterium* sp. in the inshore reef and offshore mucus and tissue samples point to the importance of this genus to the coral.

In contrast, the investigation of the temporal variation of Actinobacteria in the *P. daedalea* tissue and mucus samples obtained in March 2008, October 2008, and March 2009 showed significant differences in the total numbers of Actinobacteria in the coral tissue and mucus ($P < 0.01$). The highest numbers were recorded in the tissue (9.5×10^7 CFU g^{-1}) and mucus (10.8×10^7 CFU ml^{-1}) samples obtained in March 2009, whereas the lowest numbers were observed in both the tissue (7.8×10^7 CFU g^{-1}) and mucus (9.4×10^7 CFU ml^{-1}) samples obtained in October 2008 (**Figure 5**). The variation in the diversity of cultured Actinobacteria among the mucus and tissue samples of *P. daedalea* collected from the inshore reef system at different dates was apparent, as shown in **Figures 2, 4,** and **6**). The tissue samples collected in March 2009 were found to harbor seven different genera, whereas five and six different genera were recorded in the samples collected in March and October 2008, respectively. However, the mucus samples obtained in March 2009 presented the highest diversity with eight different genera, whereas the samples from March and October 2008 showed the presence of only four different genera. Some genera were isolated only once from the tissue samples obtained at the different sampling dates. For example, *Kineococcus* sp. and *Marmoricola* sp. were isolated in March 2008, *Renibacterium* sp. was isolated from the samples collected in March 2009, and *Micromonospora* sp. was isolated from the samples collected in October 2008. Distinctive genera, such as *Brachybacterium* sp. and *Ornithinimicrobium* sp., were found to be associated only with the mucus samples.

Total Microbial Abundance in Coral Tissue and Mucus

It was important to also quantify the total numbers of microbes in the investigated coral tissue and mucus to

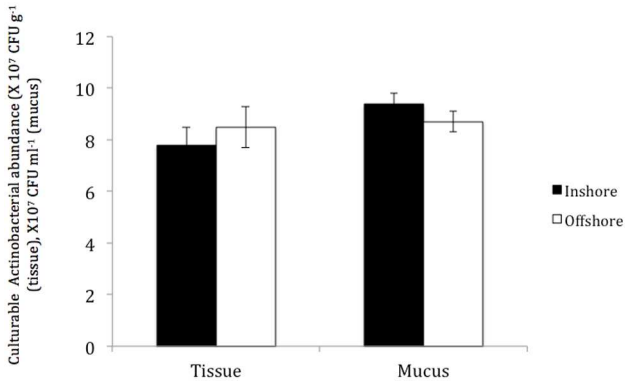

FIGURE 3 | The abundance of culturable Actinobacteria in coral tissue and mucus samples collected from the inshore reef system of Qit'at Benaya (■) and the offshore reef system of Umm Al-Maradim (□) on October 2008.

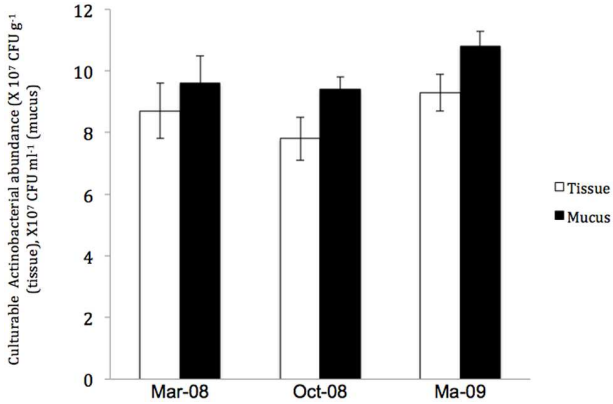

FIGURE 5 | The abundance of culturable Actinobacteria in *Platygyra daedalea* tissue (□) and mucus (■) samples collected from the inshore reef system of Qit'at Benaya on March 2009.

estimate the proportion of Actinobacteria in the total microbial community. No significant correlation ($P > 0.05$) was detected between the cultivable Actinobacteria and the total number of microbes in any of the investigated environmental samples. Furthermore, the comparison of the total microbial abundance in the three investigated corals sampled in March 2008 (**Table 1**) showed no significant differences between the total numbers of microbes detected in the tissue and mucus of the three investigated corals. The total numbers of microbes associated with the coral tissue and mucus samples of *P. daedalea* obtained in October 2008 from the inshore and offshore reef systems showed that the microbial numbers in the tested samples obtained from different sites on the same sampling date were significantly different ($P < 0.001$). The highest numbers were recorded in the inshore reef system samples. Significantly different numbers were found in the tissue samples of *P. daedalea* ($P < 0.001$) inhabiting the two sites. Significant differences in the total numbers of microbes were recorded in the tested samples, with the highest and lower numbers being recorded in March 2009 and March 2008, respectively.

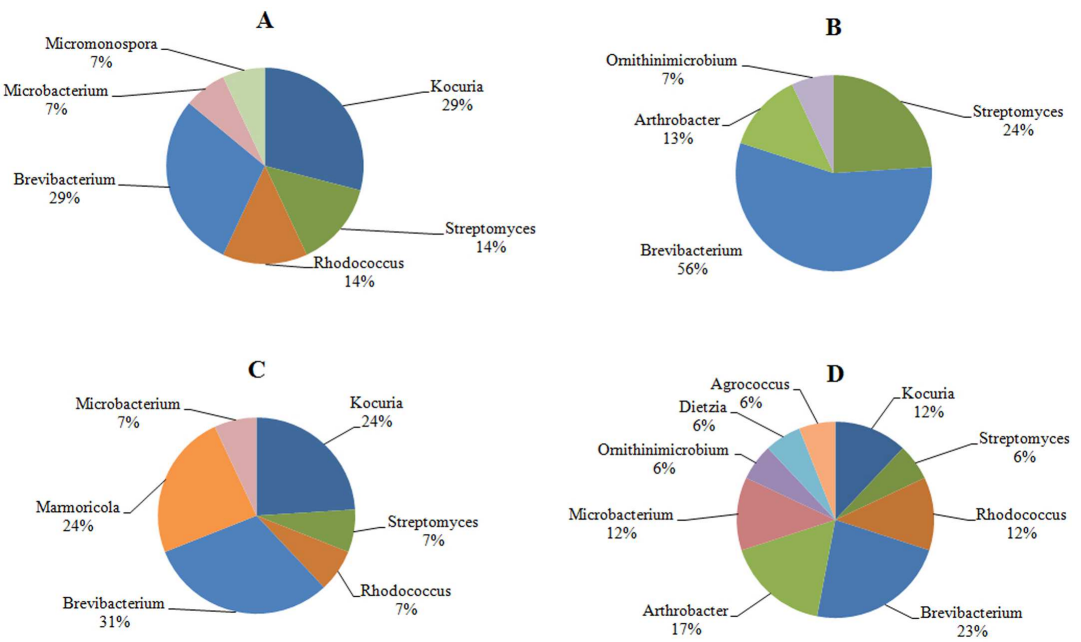

FIGURE 4 | The identity and percentage of actinobacterial isolates obtained from (A) *P. deadalea* tissue (B) *P. deadalea* mucus from inshore reef system of Qit'at Benaya (C) *P. deadalea* tissue (D) *P. deadalea* mucus collected from offshore reef system of Umm Al-Maradim (October 2008).

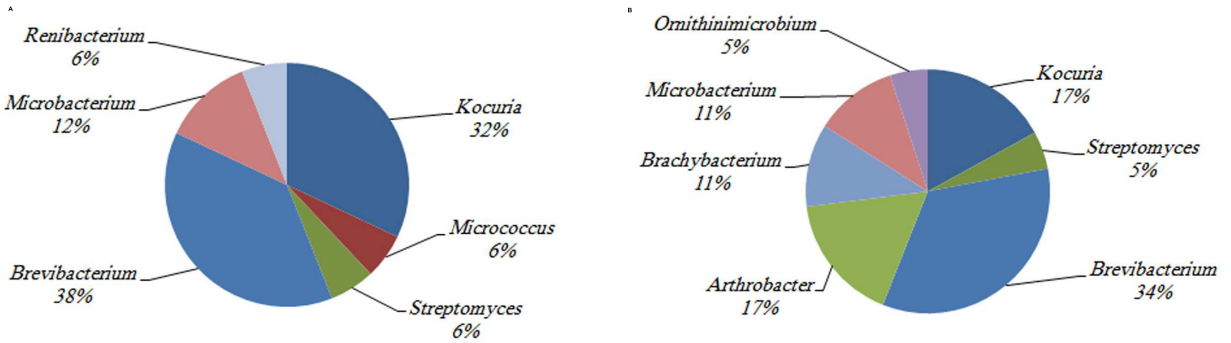

FIGURE 6 | The identity and percentage of actinobacterial isolates obtained from (A) *P. deadalea* tissue (B) *P. deadalea* mucus from Qit'at Benaya inshore reef system (March 2009).

Antimicrobial Activity Potential of Coral-Associated Actinobacteria

Among the 342 actinobacterial isolates obtained in the study, 82 exhibited antimicrobial activity against at least one tested bacterial culture, i.e., *Staphylococcus aureus, Bacillus subtilis,* or *Escherichia coli* as shown in **Figure 7**. The isolates that were able to produce antimicrobial activities included seven different genera, i.e., Streptomyces (38%), *Rhodococcus* sp. (16%), *Micrococcus* sp. (11%), *Arthrobacter* sp. (11%), *Micromonospora* sp. (10%), *Nocardia* sp. (8%), and *Brachybacterium* sp. (6%) (**Figure 8**). The majority of *Streptomyces, Micrococcus, Micromonospora,* and *Brachybacterium* were able to inhibit the growth of the three tested bacteria, whereas *Arthrobacter* and *Nocardia* were able to inhibit the growth of only the two tested Gram-positive bacteria. In addition, *Rhodococcus* isolates were able to inhibit the growth of *Bacillus subtilis* only. The majority of isolates showed strong antimicrobial activities against the tested organisms where the inhibition zone formed exceeded 15 mm (**Figure 7**). The isolates of each genus showed variations in the level of inhibition against the tested bacteria. For instance, among the 31 tested *Streptomyces* isolates some showed very strong inhibition against *S. aureus*, whereas others could not inhibit the growth of this bacterium (Supplementary Figure S2).

DISCUSSION

The analysis of the abundance and diversity of culturable Actinobacteria associated with *Platygyra daedalea* samples collected between March 2008 and March 2009 from inshore and offshore reef systems located in the north section of the Arabian Gulf revealed higher abundance and diversity of Actinobacteria in the tissue and mucus of this coral more than previously recorded for corals from tropical waters. The results obtained from two other massive Gulf corals, namely *Porites harrisoni* and *Coscinaraea columna*, sampled in March 2008 from the inshore reef system supported this finding. Gulf corals harbor threefold higher numbers of Actinobacteria in their mucus than the amounts that were previously reported by Nithyanand et al. (2011) for corals from the Gulf of Mannar in India. In addition, 82 different isolates belonging to seven different Actinobacterial genera showed antimicrobial activity against at least one Gram-positive or Gram-negative bacterium, and these included some isolates of marine origin that were rarely reported to exhibit antimicrobial activities. These include members of *Rhodococcus*.

Significant differences in the numbers of culturable Actinobacteria were obtained between the mucus and tissue samples of the same coral. Higher numbers were found in the mucus of both *P. daedalea* and *P. harrisoni* compared with the respective tissue samples. This finding opposes that reported by Bourne and Munn (2005), who found similar numbers of culturable bacteria in the coral tissue and mucus. However, the observation from *C. columna* samples, in which higher numbers were detected in the tissue, supports the findings reported by Koren and Rosenberg (2006), who found higher numbers of bacteria in *Oculina patagonica* tissues than in the mucus. Apparently, different coral hosts have their own mechanisms for controlling their symbiont numbers and diversity such that they achieve the maximum benefit from the symbiotic relationship.

Platygyra daedalea, *C. columna,* and *P. harrisoni* were found to harbor different numbers of cultivable actinobacteria in their mucus. The highest numbers were recorded in *P. daedalea*, whereas the lowest numbers were found in *C. columna* samples. This difference may be attributed to the amount and rate of mucus secretion by the corals. The rate of mucus production by massive spherical coral species, such as *Platygyra*, is higher than that by hemispherical corals, such as *Porites* (Richman et al., 1975). *Platygyra* contains thicker mucus layers (700-μm thick) than other members of the Faviidae family, which have thinner layers (~490 μm; Jatkar et al., 2010). The chemical composition of the mucus of the three different coral hosts may be different, thus favoring different microbial populations. This finding is supported by the study conducted by Rohwer et al. (2002), who have found that the mucus of different corals harbors different microbial populations depending on its chemical composition.

Despite harboring lower numbers of cultivable Actinobacteria, the *C. columna* tissue and mucus samples exhibited more Actinobacterial diversity than the *P. daedalea* samples obtained in March 2008. It is worth noticing that there are no contradictions

TABLE 1 | The total number of microbes in coral tissue and mucus, samples from Qit'at Benaya inshore reef and Umm Al-Maradim offshore reef system on various sampling dates.

Site	Date	Coral host	Total count for microbesMean (min–max) SD	
			Coral tissue ($\times 10^9$ cell g^{-1})	Coral mucus ($\times 10^9$ cell ml^{-1})
Inshore reef	March 2008	*Coscinaraea columna*	7.7 (7.0 – 8.6) 0.7	15.1 (14.3 – 17.1) 1.2
		Platygyra daedalea	5.1 (4.4 – 6.1) 0.6	12.0 (11.7 – 12.4) 0.2
		Porites harrisoni	4.4 (3.8 – 4.8) 0.3	15.6 (14.9 – 16.7) 0.8
	October 2008	*Platygyra daedalea*	7.3 (6.4 – 9.5) 1.2	14.8 (13.6 – 15.6) 0.7
	March 2009	*Platygyra daedalea*	8.3 (6.3 – 9.9) 1.3	17.2 (14.4 – 19.3) 2.4
Offshore reef	October 2008	*Platygyra daedalea*	6.9 (6.4 – 7.5) 0.4	7.3 (7.1 – 7.5) 0.1

Min, minimum; max, maximum; SD, standard deviation.

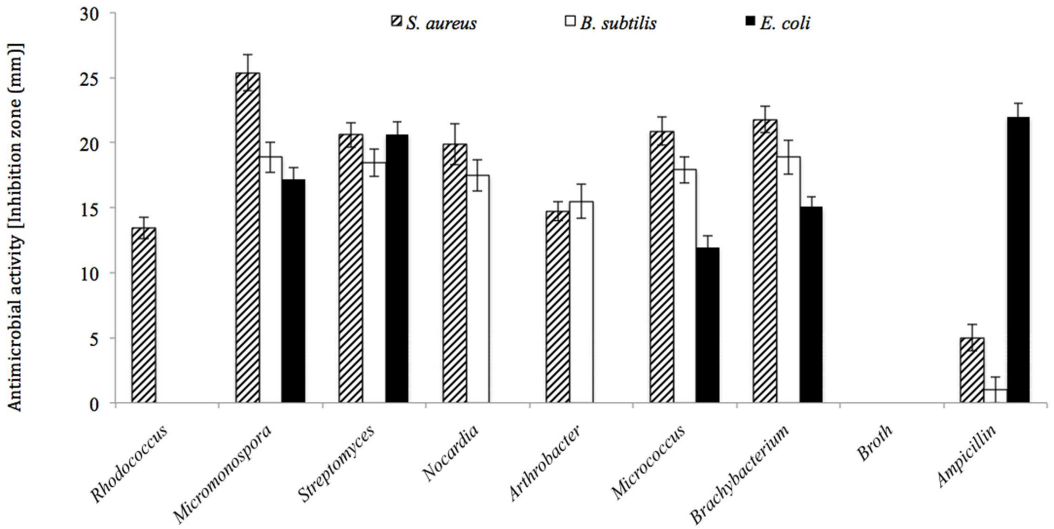

FIGURE 7 | Antimicrobial activities for the actinobacterial isolates against three tested bacteria (i.e., *Bacillus subtilis*, *Staphylococcus aureus,* and *Escherichia coli*). All actinobacterial isolates showed strong antimicrobial activities with inhibition zones higher than 15 mm at least to one of the tested bacteria.

in terms of the high bacterial numbers with low diversity observed in the *P. daedalea* samples. Other researchers have reported similar observations in other aquatic environments and have ascribed this phenomenon to the lack of competition for space and resources, resulting in microbial numbers equivalent to or even higher than those recorded in corresponding environments with higher microbial diversity (Mahmoud et al., 2005). The low Actinobacterial diversity in *Platygyra* samples obtained in March 2008 may suggest that this type of coral is more selective toward its symbionts than *C. columna* and *P. harrisoni*. It may also reflect the variation in the coral immunity levels between the tested corals. *Platygyra* may exhibit a stronger immunity level than the other two corals. Unfortunately, there are no published data to support or refute such an assumption. It is well known that corals are limited to innate immunity, through which they employ physiochemical barriers, such as mucus layers, which act as coral cellular defenses with the ability to distinguish between coral cells and other organism cells in the holobiont and produce both natural and inducible humoral defenses (Sutherland et al., 2004) to protect themselves. Kelman et al. (2006) suggested that scleractinian corals from the Red Sea may rely on non-chemical defenses against microorganisms that may include mucus production and sloughing. Because *Platygyra*, as mentioned previously, produce more and thicker mucus layers than the other corals examined in the current study, this coral may rely widely on this technique to defend itself against pathogens, whereas others that lack this feature depend largely on their symbionts to enhance their immunity.

As mentioned above, different coral hosts harbor similar numbers but present different diversities of cultivable Actinobacteria in their tissues. Ritchie and Lewis (2005) and Guppy and Bythell (2006) have shown that different coral hosts from the same sampling sites may harbor some or no similarities in their bacterial communities. This may also be attributed to coral innate immunity. Although there are no previous reports regarding the coral cellular defenses of the three tested corals, it is possible that the corals investigated in the current study allow selected symbionts to reach certain numbers in their tissue, where they keep these numbers under control and any excess can either be digested during feeding or repelled into the mucus. This is in agreement with the scenario suggested by Baghdasarian and Muscatine (2000), who have reported that

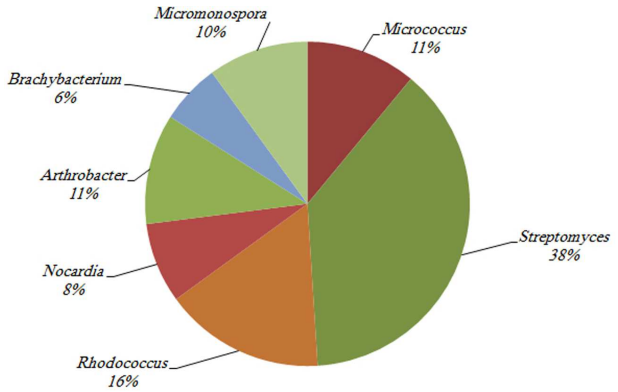

FIGURE 8 | The percentage of actinobacterial isolates that showed antimicrobial activities against at least one of the utilized tested organisms (i.e., *Bacillus subtilis*, *Staphylococcus aureus,* and *Escherichia coli*).

healthy cnidarians expel actively dividing zooxanthellae cells into the mucus to maintain a constant algal population density within the host tissue. However, the variation in the coral-associated actinobacterial diversity can be attributed to the individuality of each host.

Brevibacterium and *Kocuria* were the most dominant actinobacterial isolates in the investigated coral tissue and mucus samples. Phylogenetic trees constructed from Gulf-coral *Brevibacterium* and *Kocuria* and their counterparts from other environments revealed that the Gulf isolates are unique. The Brevibacterium phylogenetic tree (Supplementary data Figure S3) showed more than 70 Gulf coral-associated isolates clustering together and far from Brevibacterium from other environments. One exception when *B. mcbrellneri* (NZ-ADNU010000), an isolate from human urogenital tract, is considered. Kocuria on the other hand, showed variation among Gulf isolates but, all Gulf isolates clustered separately from their counterparts from other environments except for the airborne isolates *K. turfanensis* (DQ531634) and *K. flava* (EF602041) (Supplementary data Figure S4). Some studies have found an association between *Brevibacterium* and coral samples (Sabdono and Radjasa, 2008; Seemann et al., 2009). *Kocuria* has also been isolated from coral mucus (Ritchie, 2006) and tissue (Sabdono et al., 2005). The reason underlying why these two genera were found to dominate the cultivable actinobacterial groups is unknown. Mimura and Nagata (2001) have reported that *Brevibacterium* sp. JCM 6894 from seawater can more efficiently degrade the water-soluble fraction of jellyfish than other bacteria. These bacteria also degrade organophosphorus pesticides (Sabdono and Radjasa, 2008). In view of these abilities, *Brevibacterium* was suggested by Mimura and Nagata (2001) to be a strong candidate for use in bioremediation strategies. Could it be possible that the capability of *Brevibacterium* to degrade various chemical compounds facilitates their successful association with corals? Recent studies have shown that coral-associated *Brevibacterium* (Seemann et al., 2009) are able to produce palytoxin (PTX) such that it can accumulate in the tissue of the marine animals that feed on corals (Gleibs and Mebs, 1999; Seemann et al., 2009). Is it possible that corals accommodate these toxin producers to participate in reducing the grazing pressure exerted by other marine animals on corals? Or it is only a coincidence that the most dominant Actinobacteria are associated with corals that produce PTX? The literature has not revealed any special role of *Kocuria* in the marine system. *Kocuria* has been described as a marine organism (Kim et al., 2004), but only a few papers have reported its occurrence in the marine environment, and even fewer papers have reported its association with corals.

Although no significant differences were found in the number of culturable Actinobacteria between the inshore and offshore reef systems, a higher diversity was found in the offshore *P. daedalea* mucus samples collected in October 2008. Coral-associated microbial communities present differences with changing depth, water quality, and geographic location (Rohwer et al., 2001, 2002; Frias-Lopez et al., 2002; Reshef et al., 2006; Klaus et al., 2007). Therefore, variations would be expected in the actinobacterial diversity associated with the same coral host occupying different sites. In addition, changes in coral genotypes between the two sites may provide an explanation for the variation in their associated microbes, including Actinobacteria. This phenomenon of genotype variation is supported by the DGGE findings reported by Rohwer et al. (2001), who have shown that the microbial populations of 25 *Montastraea franksi* colonies from five different reef systems share only one common band due to variations in the coral genotypes. However, the species-specific microbiota principle suggested by Ritchie and Smith (1997) and Rohwer et al. (2001, 2002) should not be neglected. The results of the current study showed that the same coral samples of different individuals collected from two sites shared a number of identical actinobacterial genera, and this number was higher than that detected in both mucus and tissue samples of the same individual.

The total numbers of microbes in various environmental samples were higher in the inshore reef than the offshore reef system. This finding may be attributed to the high sewage input seeding the inshore water with high numbers of microbes, which may have an indirect effect on coral health in the area. It has been documented that the inshore reefs of Kuwait are less healthy than their offshore counterparts (Carpenter et al., 1997; Ashkanani, 2008; Al-Sarraf, 2009). Unfortunately, the correlation test did not reveal any significant correlation between the total numbers of microbes and the numbers of culturable Actinobacteria in the coral samples. Therefore, no direct relationship can be established between the two variables.

In contrast, the temporal investigation of *P. daedalea*-associated culturable Actinobacteria showed higher diversity and numbers of culturable Actinobacteria and total numbers of microbes in the mucus and tissue samples collected in March 2009, whereas the lowest numbers were recorded in the samples collected in November 2008. A natural variation in coral communities is expected to be observed over time, and many studies that monitored certain reef systems for a sufficiently long time have reported that disturbing these

systems due to various man-made or natural factors results in alterations in coral abundance and survival (Connell et al., 1997). However, few studies have attempted to explain how this alteration affects the microbial population of the corals themselves. The increment in water temperature to levels exceeding certain thresholds leads to significant changes in the mucus bacterial population (Ritchie and Smith, 1995; Guppy and Bythell, 2006) due either to decomposition of the coral mucus with extracellular proteases (Bourne and Munn, 2005) or to a reduction in the antibiotic content of the coral mucus (Ritchie, 2006). The "Coral Probiotic Hypothesis" suggested by Reshef et al. (2006) may provide an explanation for the changes in the actinobacterial abundance and diversity of *P. daedalea* sampled from the inshore reef system at different times. Under this hypothesis, corals experiencing changes in environmental conditions adapt rapidly by changing their microbial partners to accommodate more antimicrobial producers. By doing so, corals gain the ability to develop resistance to pathogens.

Nithyanand and Pandian (2009) reported that actinomycetes associated with corals and their produced metabolites had not yet been explored, and since then, few studies have focused on this topic, but all of these targeted corals from tropical regions. Our study targeted the actinobacterial community of the thermally stressed corals of the Arabian Gulf. The results showed that *Streptomyces*-related isolates dominated (∼38%) the group of isolates with antimicrobial activities, even though *Streptomyces* were not the cultured Actinobacteria that dominated the tissue and mucus of Gulf corals. This is expected because more than 500 species of *Streptomyces* account for 70–80% of secondary metabolites and it is well documented that marine *Streptomyces* are able to produce bioactive compounds with a range of activities, including anticancer, antimicrobial, and enzyme inhibition functions (Lam, 2006; Solanki et al., 2008). The second most dominant genus in this group was *Rhodococcus*, which made up 16% of the total isolates with antimicrobial activities. This is an interesting finding because papers reporting the ability of isolates of this genus from marine origin to produce antimicrobial products are few (Zhang et al., 2013). In addition to *Rhodococcus*, few have reported the antimicrobial activity potential of *Brachybacterium* (Radjasa, 2007). In the current study, 6% of the isolates with antimicrobial activities were related to *Brachybacterium*. Radjasa (2007) has investigated sponge-associated Actinobacteria that had 99% 16S rRNA-gene similarity to *Brachybacterium rhamnosum* and reported their ability to contain polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) responsible for structurally synthesizing bioactive secondary metabolites and to inhibit the growth of *E. coli*. It is likely that novel isolates and new findings will be obtained because the isolates in this study showed antimicrobial activities against *E. coli, S. aureus,* and *B. subtilis* and were related to *B. paraconglomeratum*, *B. phenoliresistens,* and *B. zhongshanense*. In other words, they are quite different from that reported by Radjasa (2007). *Arthrobacter*-related isolates, which made up 11% of the total isolates with antimicrobial activities, deserve attention. The ability of *Arthrobacter* to produce antibiotics has been reported previously by a few investigators working on isolates of marine origin. However, Shnit-Orland and Kushmaro (2008) reported that *Micrococcus* and *Arthrobacter* isolated from corals showed no antimicrobial activities. Hentschel et al. (2001) obtained an isolate from a Mediterranean sponge, whereas Radjasa et al. (2008) isolated an *Arthrobacter* species from corals of the North Java Sea that shows antimicrobial activities. Even though *Rhodococcus* and *Arthrobacter* are common soil Actinobacteria, their marine counterparts appear to have more antimicrobial potential than the terrestrial ones, which agrees with the conclusions reported by Lam (2006).

The other three actinobacterial genera that showed antimicrobial activities, namely *Micromonospora*, *Micrococcus*, and *Nocardia*, were previously isolated from various marine habitats and were reported to be a potential source of bioactive compounds (Bultel-Poncé et al., 1998; Hentschel et al., 2001; Lam, 2006, 2007; Radjasa et al., 2008; Solanki et al., 2008; Nithyanand and Pandian, 2009; Olano et al., 2009). It is likely that some of the isolates obtained in the current study contain novel compounds that have not previously been described. Even though *Brevibacterium* and *Kocuria* were the most dominant actinobacterial isolates, they failed to show any antimicrobial activity, whereas less dominant genera, such as *Streptomyces*, had antimicrobial activity.

CONCLUSION

The variations in the culturable actinobacterial populations associated with corals in inshore and offshore reef systems of the north section of the Arabian Gulf were observed. Different coral host types harbored different cultivable actinobacterial populations. Differences in the abundance and diversity of Actinobacteria were detected between the mucus and tissue of the same coral host. In addition, temporal and spatial variations in the abundance and diversity of the cultivable actinobacterial population were detected. Focusing on the diversity of coral-associated Actinobacteria may lead to the discovery of novel antimicrobial metabolites with potential biotechnological applications.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00204

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Environmental Controls Over Actinobacteria Communities in Ecological Sensitive Yanshan Mountains Zone

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The Yanshan Mountains are one of the oldest mountain ranges in the world. They are located in an ecologically sensitive zone in northern China near the Hu Huanyong Line. In this metagenomic study, we investigated the diversity of Actinobacteria in soils at 10 sites (YS1–YS10) on the Yanshan Mountains. First, we assessed the effect of different soil prtreatment on Actinobacteria recovery. With the soil pretreatment method: air drying of the soil sample, followed by exposure to 120°C for 1 h, we observed the higher Actinobacteria diversity in a relatively small number of clone libraries. No significant differences were observed in the Actinobacterial diversity of soils from sites YS2, YS3, YS4, YS6, YS8, YS9, or YS10 ($P > 0.1$). However, there were differences ($P < 0.05$) from the YS7 site and other sites, especially in response to environmental change. And we observed highly significant differences ($P < 0.001$) in Actinobacterial diversity of the soil from YS7 and that from YS4 and YS8 sites.. The climatic characteristics of mean active accumulated temperature, annual mean precipitation, and annual mean temperature, and biogeochemical data of total phosphorus contributed to the diversity of Actinobacterial communities in soils at YS1, YS3, YS4, and YS5 sites. Compared to the climatic factors, the biogeochemical factors mostly contributed in shaping the Actinobacterial community. This work provides evidence that the diversity of Actinobacterial communities in soils from the Yashan Mountains show regional biogeographic patterns and that community membership change along the north-south distribution of the Hu Huanyong Line.

Keywords: ecological sensitive zone, a Yanshan mountains, Actinobacteria, phylogenetic diversity, 16S rRNA Actinobacterial clone library

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INTRODUCTION

Microbial communities can diverge rapidly, and result in distinct biogeographic patterns (Green et al., 2008). However, based on different evolution, biogeographic patterns are posited to consist of dramatic range expansion as a result of effect at the genotype level (Ramette and Tiedje, 2007). For microbial biogeography, the traditional view has been that "Everything is everywhere,

but the environment selects" (Baas, 1934). There has been a debate over whether variation in microbial communities through space results from environmental, or from geographic barriers and other human activities that contribute to community structure (Eisenlord et al., 2012). If not all microbes are evenly dispersed over time, this would suggest that forces structuring the microbial communities are more complex than only adaptive evolution via natural selection (Bissett et al., 2010; Kuang et al., 2015; Yang et al., 2015). We addressed this issue by examining the community structure of a deeply diverse and divergent phylum, the Actinobacteria. Actinobacteria are important organisms that mediate plant litter decay and the subsequent formation of soil organic matter in terrestrial ecosystems. This phylum is phylogenetically divergent and the closest prokaryotic relative is yet to be identified (Ventura et al., 2007; Mendes et al., 2015). Actinobacteria express a variety of morphologies and life-history traits that could be advantageous for dispersal, including sporulation. Here, we evaluated by metagenomic technology whether environmental disturbance of an ecologically sensitive zone is associated with a highly structured community of soil Actinobacteria in the Yanshan Mountains of northern China. Microorganisms are the most diverse and abundant group of organisms on Earth; however, in soil microbial communities, work to understand this diversity has been primarily directed toward general rather than group-specific diversity. Actinobacteria, ubiquitously found in terrestrial (Han et al., 2015), freshwater (Mullowney et al., 2015), and marine (Sun et al., 2015) ecosystems, are the dominant soil bacterial phylum and they are believed to play multiple roles in the environment (Barka et al., 2016).

The construction of metagenomic libraries and other DNA-based metagenomic projects are initiated by the isolation of high-quality DNA that is suitable for cloning and that covers the microbial diversity present in the original sample. Since Pace et al. (1985) first proposed the direct cloning of environmental DNA, soil DNA extraction techniques, including both direct and indirect methods (Robe et al., 2003; Delmont et al., 2010), have been developed. These efforts have led to the development of various homemade DNA extraction protocols, as well as commercial kits, which have been used in more than 1000 studies reported yearly. Therefore, high quality DNA has been isolated from a variety of environments. In addition, cultivation-independent methodologies, particularly sequence analyses of cloned 16S ribosomal RNA genes (16S rDNA) are powerful tools to investigate microbial diversity. Most approaches target the 16S rRNA gene for PCR amplification and subsequent Sanger sequencing of the clone libraries (Sogin et al., 2006), ribosomal sequence tags (SARST; Poitelon et al., 2009), denaturing gradient gel electrophoresis (DGGE; Yim et al., 2015), terminal restriction fragment length polymorphism (T-RFLP; Lazzaro et al., 2015), Pyrosequencing (Schäfer et al., 2010), or 454 Life Sciences and Illumina analyses (Vasileiadis et al., 2012; Logares et al., 2014). However, there is no specific primers for Actinobacteria to construct a full or near full-length 16S rDNA clone libraries. And the Actinobacterial-specific primers used for high-throughput technique can obtain some information of Actinobateria, but sometimes the recovered sequence is too small to gain complete genetic information and detailed phylogenetic characterization of Actinobacteria, especially for a greater number of unclassified Actinobacteria.

Therefore, in this study, it was purpose to obtain a full or near full-length 16S rDNA sequence of Actinobacteria. To increase the proportion of Actinobacteria in the 16S rDNA library, we developed a method of soil pretreatment to concentrate the Actinobacterial community, and used a PCR primer system to capture Actinobacteria from prokaryotes in the 16Sr DNA full-length clone library. The purpose of the present study was to compare the community structure and phylogenetic diversity of Actinobacteria among various sites in the Yanshan Mountains.

METHODS

Sample Collection

Soil samples were collected from various locations in the Yanshan Mountains (**Figure 1**) on October 2–10, 2011. Descriptions of soil collection sites are presented in **Table 1**. In each of the 10 sites, there were 3 randomly selected 30 m × 30 m replicate plots 100–150 m apart. In each plot, we collected 10 soil samples using a 2.5 cm diameter soil core, which extended to a depth of 10 cm. The 10 soil samples in each plot were composited and passed through a 2-mm sieve in the field. By pooling the 10 soil cores, we aggregated spatial heterogeneity at the scale of individual plots. The 3 soil plot samples were combined into a representative sample for each site. From the sieved composite sample, a 5.0-g sample was removed for DNA extraction. This was done to allow a characterization of the Actinobacteria community at the scale of the entire Yanshan Mountains, and to explore regional trends in community similarity that may have been structured by environmental factors.

DNA Extraction Methods

We design three kinds of soil pretreatment method to improve the proportion of Actinobacteria DNA. (i) For protocol A, air dried soil sample were treated by 120°C 1 h (A1), 2 h (A2), 3 h (A3) respectively; (ii) For protocol B, soil sample were treated by air drying processing 15 days (B1), 30 days (B2), 45 days (B3) respectively; (iii) For protocol C, soil sample were treated by 0.1% Polymyxin B Sulfate immersion 1 h (C1), 2 h (C2), 3 h (C3) respectively; After pretreatment of soil samples, and centrifugal washing three times with sterile water for removing DNA of release, DNA extraction from 1.0 g soil samples was carried out using the PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories), according to the manufacturer's instructions. The yield and integrity of the environmental DNA obtained were confirmed through electrophoresis in 1% agarose gel.

Construction of 16S rRNA Gene Libraries

The purified DNA was used as a template to specifically amplify 16S rRNA gene fragments, a ∼1500 bp region using the bacteria-specific primers (Lane, 1991): 27F (5-AGAGTTTGATCC/ATGGCTCAG-3) and 1525R (5-AAGGAGGTGA/TTCCAA/GCC-3). To recondition the PCR product for elimination of heteroduplexes in mixed-template PCR (Janelle et al., 2002), the amplified reaction was diluted

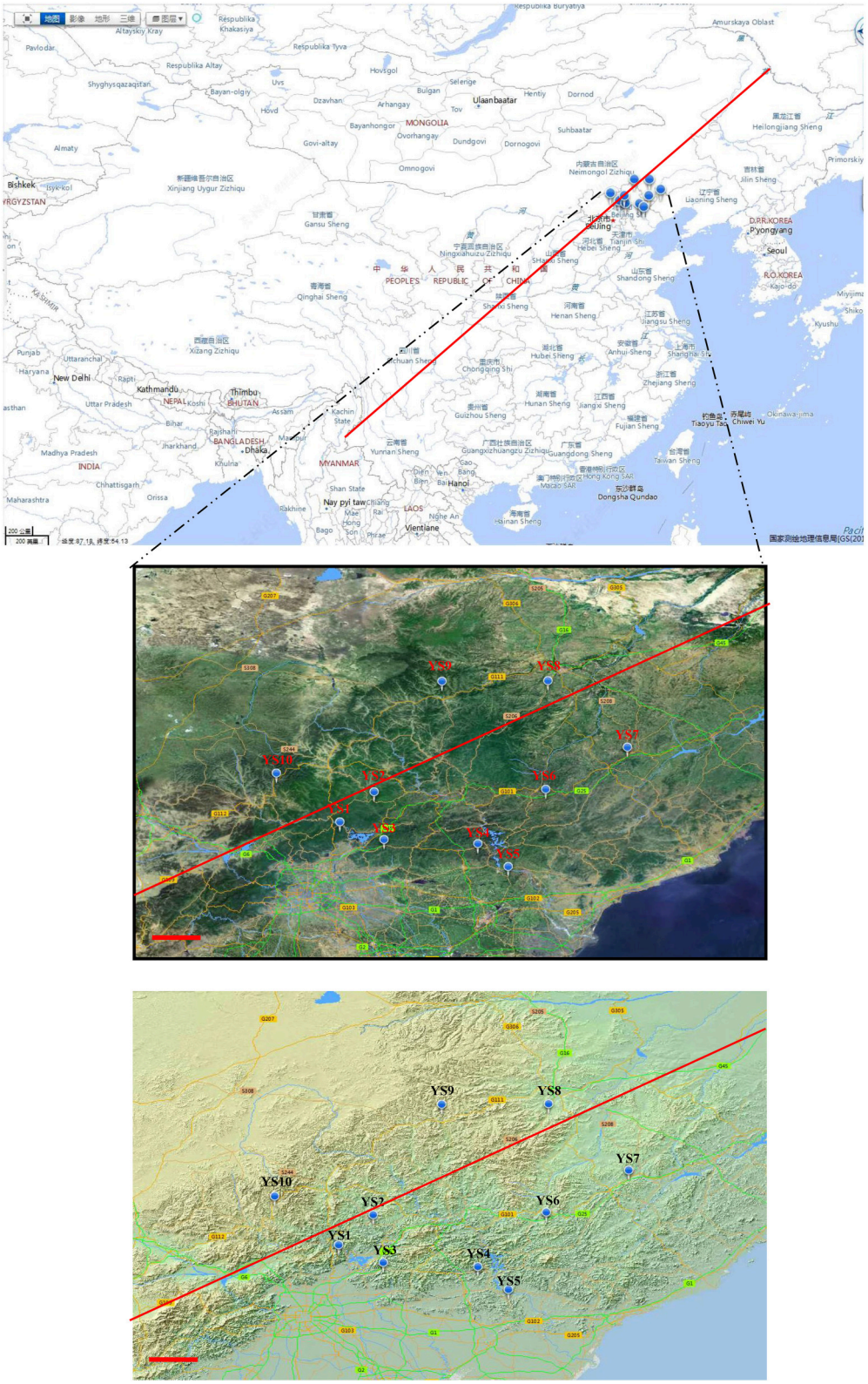

FIGURE 1 | Various sampling points along ecological sensitive Yanshan mountains zone. The circular mark denotes the stations. The photograph and topographic map were provided by the Mapword (http://map.tianditu.com/map/index.html. Red line is Hu Huanyong Line).

TABLE 1 | Actinobacteria sequencing statistics and α diversity measures of different pretreatment of soil samples.

Source[a]	Total no. of clones	Detection rate (%)	OTU[b]	Shannon diversity index (H/ (loge))	Equitability_J	Buzas and Gibson's evenness (E) index
A1	102	20.4	74	4.161	0.9668	0.866
A2	30	6.0	19	2.731	0.9674	0.807
A3	73	14.6	64	4.101	0.9762	0.943
B1	44	8.8	38	3.595	0.9783	0.958
B2	28	5.6	26	3.233	0.9724	0.975
B3	84	15.8	61	4.020	0.9779	0.913
C1	55	11.0	37	3.464	0.9693	0.863
C2	29	5.8	23	3.062	0.9767	0.929
C3	24	4.8	20	2.925	0.9765	0.931
CK	18	3.6	15	2.659	0.9720	0.952

[a]*Sources of data are from the following libraries: uncultured Actinobacteria are from treated samples A1, A2, A3, B1, B2, B3, C1, C2, C3, and CK.*
[b]*OUT were defined as clone sequences with <97% 16S rRNA gene sequence similarity to other clones.*

10-fold into a fresh reaction mixture of the same composition and cycled three times. The size and quality of the resulting PCR products was confirmed by agarose gel electrophoresis (1.4% agarose). They were then cloned into the pUCm-T linear plasmid vector (Takara Bio Group, Code D101A) and then into *E. coli* DH5a competent cells (Takara Bio Group). After the transformants were grown overnight, single-clone colonies were picked up with sterile toothpicks and transferred into 1.5 mL microcentrifuge tubes containing 50 mL of TE buffer. The tubes were heated for 15 min at 95 C to lyse the cells, and then chilled on ice. Insert 16S rDNA sequences were identified by M13/pUC sequencing primer and M13/pUC reverse primer (approximately 1.5 kb).

Amplification and Sequencing of Actinobacteria *16S rRNA* Genes

Two different Actinobacteria-specific primer sets specifically targeting 16S rRNA gene were used to confirm the presence of selected Actinobacteria genotypes in soil DNA. The first primer set, Com2xf /Ac1186 (Schäfer et al., 2010), was used to detect most Actinobacteria species. The 25-μL PCR reaction mixture contained 2.5 μL PCR buffer, 2 μL $MgCl_2$ (25 mM), 2 μL dNTPs (2.5 mM), 0.5 μL each primer (10 μM, Shenggong Biotech, Shanghai, China), 17.7 μL H_2O, 0.2 μL BSA (20 mg/mL^{-1}), and 0.1 μL Taq polymerase (5 U/μL^{-1}) (Takara, Japan). This mixture was added directly to cloned cells. PCR was carried out in a thermocycler (Bio-Rad, München, Germany) with an initial denaturation step at 95°C for 10 min, followed by 25 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, followed by a final extension at 72°C for 5 min. A second PCR using the primer set SC-Act235-aS-20/SC-Act878-aA-19 (Stach et al., 2003) was carried out to increase the amount of detectable Actinobacteria DNA. The 25-μL reaction mixture contained 2.5 μL PCR buffer, 2.5 μL $MgCl_2$ (25 mM), 2 μL dNTPs (2.5 mM), 0.5 μL each primer (10 μM, Shenggong Biotech, Shanghai, China), 17.7 μL H_2O, 0.2 μL BSA (20 mg/mL^{-1}), and 0.1 μL Taq polymerase (5 U/μL^{-1}) (Takara, Japan). The reaction mixture was also added directly to cloned cells. PCR was performed with an initial denaturation step at 95°C for 10 min, followed by 25 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, followed by a final extension at 72°C for 5 min. The success of PCR reactions were determined by subjecting the amplified products to 1% agarose gel electrophoresis and ethidium bromide staining. All positive clones and the A3 clone library were recultured in LB broth, and sequenced using Shenggong Biotech, Shanghai, China.

Phylogenetic Analyses

The 16S rRNA gene sequences were taxonomically assigned using the Naïve Bayesian rRNA classifier of the Ribosomal Database Project II (RDP; Wang et al., 2007). Sequences from this study were subsequently aligned using the ClustalW multiple alignment tool from BioEdit v7.0.5.3. The program DNADIST v3.5c in BioEdit was used to compute a distance matrix from the aligned nucleotide sequences. The distance matrix was input into the DOTUR program (v1.53) to assign the sequences to operational taxonomic units (OTUs) using the furthest-neighbor clustering algorithm (Schloss and Handelsman, 2005) at 97, 95, and 90% identities. Sequences from each clone library were aligned separately, and OTUs were identified at 97% identity. One representative sequence was selected for each OTU. Representative sequences from each OTU (97%) in 10 libraries determined in this study were deposited in the NCBI database under accessions no. KC554071–KC554721. Coverage (C) was used as a measure of captured diversity, where C is expressed by 1_n1/N, in which n1/N is the ratio of the number clones that appeared only once (n1) to the total number of clones (N). Rarefaction curves were produced by standard calculations by comparing the total number of clones obtained to the number of clones representing unique OTUs. Sampling sufficiency of each library was determined as described by Kemp and Kemp and Aller (2004) using the "Large Enough" estimator available online at http://www.aslo.org/lomethods/free/2004/0114a.html. The Shannon index, Simpson's diversity index, and nonparametric richness estimators ACE and Chao1 were calculated using the DOTUR program (Schloss and Handelsman, 2005). A neighbor-joining tree was created using MEGA version 4 software. The bootstrap values represent 1000 samplings. Multiple environments were simultaneously analyzed using phylogenetically comparing the microbial communities using weighted and unweighted UniFrac to conduct a principal

coordinates analysis (Lozupone et al., 2006). The neighbor-joining tree generated for input to UniFrac was limited to 999 sequences. The environmental input file for UniFrac contained a count of how many times the selected sequence appeared in the clone library. The UniFrac significance test with abundance weights was used to determine significant differences in the Actinobacteria community structure. *P*−values were corrected for multiple comparisons by multiplying the calculated *P*−value with the number of comparisons made (Bonferroni correction; Lozupone et al., 2006).

Environmental Variables and Multivariate Statistical Analysis

Environmental characteristics were assembled into two data sets: (1) a biogeochemical data set composed of factors, and (2) climatic characteristics. The biogeochemical data matrix included soil pH and total nitrogen (TN); total phosphorus (TP); available phosphorus (AP); available potassium (AK); organic matters (OM) (Supplementary Table 1). The second matrix characterized climatic variation by including annual mean temperature (MT); annual mean precipitation (MP); mean sea level elevation (ME); annual mean sunshine duration (SD); mean active accumulated temperature (>10°C) (AAC) (Supplementary Table 2). The climatic data used in this study were averages from the years 1981 to 2012. Environmental vectors, of biogeochemical and climatic data sets, were fit to nMDS ordinations of biological data, which identified the individual variables correlated with community patterns. Redundancy analysis (RDA) was used to examine the correlations between species patterns and environmental variables to evaluate which variables explained significant proportions of variation in Actinobacteria community composition. Additional statistics were conducted in the R package vegan (Oksanen et al., 2011).

RESULT

Testing of an Actinobacteria Primer System

The Actinobacteria specific primer systems detected 75 positive clones from the 16S rDNA clone library of the A3 sample. To determine the validity and specificity of the primer system, all clones in the library were sequenced and classified. Two out of 75 positive clones and another 425 clones belonged to the Acidobacteria, Proteobacteria and Firmicutes, and 73 positive clones were Actinobacteria belonging to 20 known and 34 unknown genera.

Effect of Different Soil Pretreatments on Actinobacteria Recovery

After soils were pretreated, we detected a larger number and phylotype s of Actinobacteria in the same numbers of prokaryotic microorganisms in the 16S rDNA cloned library (**Table 1**). It were corroborated by the diversity indices, which were significantly higher than direct extraction of Kit (CK). In addition, the number Actinobacteria clones detected was significantly different among samples treated by the 3 pretreatment methods. The total detection rate using each of these methods were: A (13.7%) > B (10.4%) > C (7.2%). Protocol A1 yielded 102 clones with a 20.4% detection rate (from 500 clones); protocol B3 yielded 84 with 15.8% detection; protocol A3 yielded 73 with 14.6% detection; CK yield 18 with 3.6% detection. Sequences with 97% similarity in the 16S rRNA gene used for phylogenetic analyses were combined into OTUs. A total of 252 OTUs were present in the 10 clone libraries. Most of them were A1 (74 out of 102 clones), next were A3 (64 out of 73 clones), third were B3 (62 out of 84 clones), while CK had only 15 (out of 18 clones). In addition, only A1 contained all OTUs recovered by CK. Even if the rarefaction curves did not approach an asymptote (**Figure 2**), meaning that we did not capture the full diversity of the Actinobacterial community, 10 clones representing 37 known genera out of a total of 186 genera were detected, with A1 yielding 21 (out of 56) known genera; A3 yielding 20 (out of 54) genera; B3 yielding 17 (out of 54) genera. They were all far more than CK, 8 out of 12 genus. *Nocardioids* and *Conexibacter* and some unclassified groups were detected in the 10-clone library. Unique known genera detected using the A1 method were *Dactylosporangium, Lechevalieria,* and *Amycolatopsis*; A3 resulted in the detection of *Kineosporia* and *Angustibacter*; B2, *Microlunatus* and *Actinoplanes*; B3, *Geodermatophilus* and *Kribbella*; C1, *Acidothermus* and *Phycicoccus*; and C2, *Nesterenkonia* and *Aeromicrobium.* The A2, B1, and C3 had not unique known genus but unclassified group (**Figure 3**). The Actinobacterial compositions at the order/suborder levels were significantly different between the pretreated or untreated soil samples (**Figure 4**). The pretreated soil allowed increased detection of specific orders/suborders, including *Solirubrobacterales, Propionibacterineae, Frankineae, Acidimicrobiales*, and *Micrococcineae*. However, *Corynebacterineae, Kineosporiineae*, and *Rubrobacterales* were only detected in the A3 library, which clearly indicated that pretreatment of soil could lead to an underestimation of some Actinobacteria groups. Furthermore, the Actinobacterial library was dominated by the *Solibubrobacterales* (A2, 6.8%; C1, 38.2%) of the Actinobacteria clones and A2, 0.4%; B3,−6.4% of all 16S rDNA clones, whereas *Propionibacterineae* dominated the A1 and A2 libraries (24.0% of the Actinobacterial clones and 24.1% of all 16S rDNA

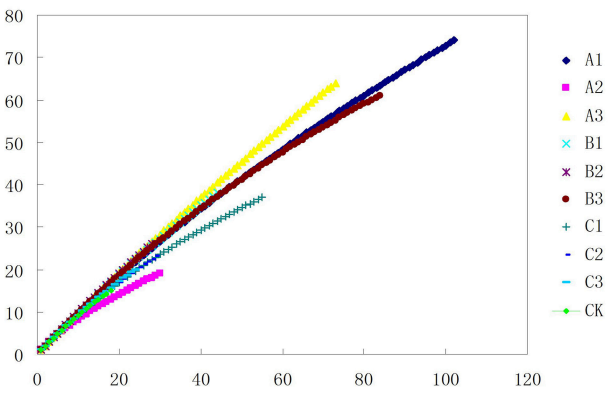

FIGURE 2 | Phylotype richness curves for clone and culture libraries. Sampling curves were calculated by rarefaction[56,57].

	A1	A2	A3	B1	B2	B3	C1	C2	C3	CK
Actinobacteria										
» *Actinobacteridae*										
» » *Actinomycetales*										
» » » *Corynebacterineae*										
» » » » *Mycobacteriaceae*										
» » » » » *Mycobacterium*	1	0	1	0	0	0	0	0	0	0
» » » *Frankineae*										
» » » » *Geodermatophilaceae*										
» » » » » *Blastococcus*	1	1	1	1	1	1	1	1	1	1
» » » » » *Geodermatophilus*	0	0	0	0	0	1	0	0	0	0
» » » » » unclassified *Geodermatophilaceae*	1	1	0	0	0	1	1	0	0	0
» » » » *Sporichthyaceae*										
» » » » » *Sporichthya*	0	0	0	0	1	1	0	0	0	0
» » » » *Acidothermaceae*										
» » » » » *Acidothermus*	0	0	0	0	0	0	1	0	0	0
» » » unclassified *Frankineae*	1	0	0	0	0	0	1	0	0	0
» » *Micrococcineae*										
» » » *Intrasporangiaceae*										
» » » » *Knoellia*	1	0	1	1	0	0	1	0	0	0
» » » » *Terrabacter*	1	0	1	0	0	1	0	0	0	0
» » » » *Intrasporangium*	0	0	1	0	0	1	0	0	0	0
» » » » *Phycicoccus*	0	0	0	0	0	0	1	0	0	0
» » » » unclassified *Intrasporangiaceae*	1	0	1	0	1	1	1	1	1	1
» » » *Microbacteriaceae*										
» » » » *Agromyces*	1	0	1	1	0	1	0	0	0	0
» » » » unclassified *Microbacteriaceae*	0	0	0	1	0	1	0	0	0	0
» » » *Micrococcaceae*										
» » » » *Arthrobacter*	1	1	1	1	0	1	0	1	1	1
» » » » *Nesterenkonia*	0	0	0	0	0	0	0	1	0	0
» » » » unclassified *Micrococcaceae*	0	0	0	1	0	1	0	1	0	0
» » » *Promicromonosporaceae*										
» » » » unclassified *Promicromonosporaceae*	0	1	0	0	0	1	0	0	0	0
» » *Micromonosporineae*										
» » » *Micromonosporaceae*										
» » » » *Asanoa*	1	0	1	0	0	0	0	0	0	0
» » » » *Dactylosporangium*	1	0	0	0	0	0	0	0	0	0
» » » » *Micromonospora*	0	1	0	0	0	0	1	0	0	0
» » » » *Actinoplanes*	0	0	0	0	1	0	0	0	0	0
» » » » *Catenuloplanes*	0	0	0	0	0	0	0	0	1	1
» » » » unclassified *Micromonosporaceae*	1	0	1	1	1	1	1	1	0	0
» » *Propionibacterineae*										
» » » *Nocardioidaceae*										
» » » » *Jiangella*	1	0	0	0	0	0	1	0	0	0
» » » » *Marmoricola*	1	0	1	0	0	1	1	1	1	1
» » » » *Nocardioides*	1	1	1	1	1	1	1	1	1	1
» » » » *Pimelobacter*	0	0	1	0	0	1	0	0	0	0
» » » » *Kribbella*	0	0	0	0	0	1	0	0	0	0
» » » » *Aeromicrobium*	0	0	0	0	0	0	0	1	0	0
» » » » unclassified *Nocardioidaceae*	1	1	1	0	1	1	0	1	0	0
» » » *Propionibacteriaceae*										
» » » » *Microlunatus*	0	0	0	0	1	0	0	0	0	0
» » *Pseudonocardineae*										
» » » *Actinosynnemataceae*										
» » » » *Lechevalieria*	1	0	0	0	0	0	0	0	0	0
» » » *Pseudonocardiaceae*										
» » » » *Amycolatopsis*	1	0	0	0	0	0	0	0	0	0
» » » » *Kibdelosporangium*	1	0	1	0	0	0	0	0	0	0
» » » » *Pseudonocardia*	1	0	1	0	0	1	1	0	0	0
» » *Streptomycineae*										
» » » *Streptomycetaceae*										
» » » » *Streptomyces*	1	1	1	0	1	1	0	1	0	0
» » *Streptosporangineae*										
» » » *Thermomonosporaceae*										
» » » » *Actinomadura*	1	0	0	0	1	0	0	0	0	0
» » » *Streptosporangiaceae*										
» » » » unclasseified *Streptosporangiaceae*	0	0	1	0	0	0	0	0	0	0
» » » unclassified *Streptosporangineae*	0	0	1	0	0	0	0	0	0	0
» » *Kineosporiineae*										
» » » *Kineosporiaceae*										
» » » » *Kineosporia*	0	0	1	0	0	0	0	0	0	0
» » » » *Angustibacter*	0	0	1	0	0	0	0	0	0	0
» » unclassified *Actinomycetales*	1	1	0	1	1	1	1	0	0	0
» *Acidimicrobidae*										
» » *Acidimicrobiales*										
» » » *Acidimicrobineae*										
» » » » *Iamiaceae*										
» » » » » *Iamia*	1	0	0	0	1	1	0	0	0	0
» » » *Acidimicrobiaceae*										
» » » » *Aciditerrimonas*	1	1	1	1	1	0	0	1	1	1
» » » » *Ilumatobacter*	0	0	0	0	0	1	1	0	0	0
» » » unclassified *Acidimicrobineae*	1	1	1	1	1	1	0	0	0	0
» *Rubrobacteridae*										
» » *Solirubrobacterales*										
» » » *Solirubrobacteraceae*										
» » » » *Solirubrobacter*	1	1	1	1	1	1	1	0	1	1
» » *Conexibacteraceae*										
» » » *Conexibacter*	1	1	1	1	1	1	1	1	1	1
» » unclassified *Solirubrobacterales*	1	0	1	1	0	1	1	1	1	1
» *Rubrobacterales*										
» » *Rubrobacterineae*										
» » » *Rubrobacteraceae*										
» » » » *Rubrobacter*	0	0	1	0	0	0	0	0	0	0
» unclassified *Rubrobacteridae*	1	0	1	1	1	0	1	1	1	1
unclassified *Actinobacteria*	1	1	1	1	1	1	1	1	1	1

FIGURE 3 | Distribution of Actinobacteria clones from different pretretment in taxonomy.

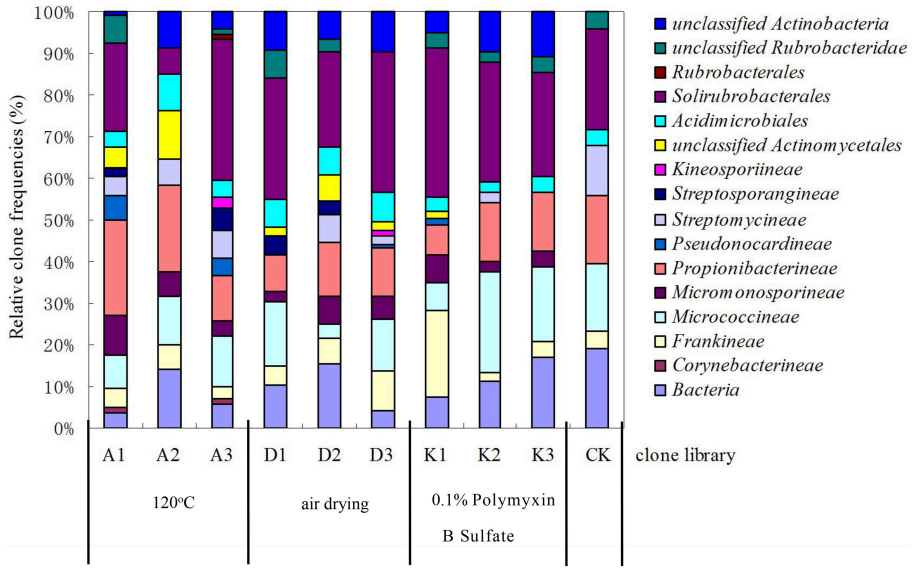

FIGURE 4 | Relative clone frequencies in major phylogenetic groups of the clone libraries from different pretreatment of soil sample.

clones; **Figure 4**). The B3 library allowed the detection of a greater number of unclassified Actinobacteria, unclassified *Rubrobacteridae*, and unclassified *Actinomycetales* than the A1 library.

Actinobacteria Community Composition at Stations in the Yanshan Mountains

Soil samples from 10 stations were treated at 120°C for 1 h, then the bacterial 16S rDNA clone library was constructed. We randomly selected 1000 clones (for sufficient Actinobacterial coverage) from each station to detect Actinobacteria using 2 Actinobacteria-specific primer sets. From the 10,000 clones generated, approximately 13% ($n = 1327$) resulted in PCR products from the Actinobacteria-specific primers.

Depending on the station surveyed, the proportion of Actinobacteria among total clones varied between 10.8 and 20.4% (**Table 2**), and resulted in 575 OTUs grouped at the 97% similarity level. The "Large Enough" calculator was used to determine whether individual clone libraries were sampled sufficiently. If the estimated phylotype richness reached an asymptote, we inferred that the library was large enough to yield a stable estimate of phylotype richness. According to the figure, all sites appeared to have been sufficiently sampled (Supplementary Figure 1). We identified OTUs in 28 of 39 Actinobacterial families, classified by the RDP (**Figure 5**). For the most abundant OTUs, the closest similarity to known organisms was 100% to members of the *Blastococcus* genus, *Frankineae* family. UniFrac metrics were used to assess community similarity between 2 or more samples according to their structure (weighted/quantitative) and membership (unweighted/quantitative). In the 2-dimensional plot visualized by the UniFrac weighted distance matrix principle coordinates analysis (3% dissimilarity), the samples of each system distinctively responded to the majority of the variation detected in the samples across 2 axes (**Figure 7A**). Axis 1 accounted for 21.82% of the variation, and Axis 2 accounted for 19.29% of the variation. In **Figure 7B**, the same 2-dimensional plot was shown for the unweighted method, which showed that samples from the same type, were in consideration of community membership, although less distinctive (Axis 1 = 16.72%, Axis 2 = 13.14%). The results from the UniFrac weighted and unweighted PCA plots demonstrate distinctions in structure and composition of the Actinobacterial communities from different stations. Furthermore, the UniFrac significance test results revealed significant differences in community membership between sites YS1 and YS6 ($P < 0.001$), sites YS4 and YS7 ($P < 0.001$), and sites YS7 and YS8 ($P < 0.001$) (Supplementary Figure 2). Diversity estimates, Ace and Chao1, indicated that YS6, YS7, and YS9 were more diverse than the other sites.

Conexibacteraceae, Geodermatophilaceae, Micrococcaceae, Micromonosporaceae, Nocardioidaceae, Propionibacteriaceae, Pseudonocardiaceae, and *Solirubrobacteraceae* represented 46.4–66.9% of the bacterial community in each station. These taxa together accounted for an average of 55% of the Actinobacterial clones obtained from soil of the 10 stations in the Yanshan Mountains. *Geodermatophilaceae, Micromonosporaceae, Nocardioidaceae, Propionibacteriaceae, Pseudonocardiaceae, Streptomycetaceae,* and *Solirubrobacteraceae* were common to the 10 libraries, and they were identified as contributing substantially to the relative abundance of Actinobacteria (**Figure 5**). To demonstrate the differences in Actinobacterial community composition, relative abundances of Actinobacteria were also assessed. **Table 3** displays the relative abundances and Shannon diversity indices of the salient families of Actinobacteria identified in the soils from the 10 stations. Groups of family, YS10 were fewest, YS4 were most. The UniFrac

metric identified the unique phylogenetic branch belonging to Actinobacterial communities within each site compared to the entire community ($P = 0.001$). The unique family of site YS3 was *Cryptosporangiaceae*, YS4 was *Rarobacteraceae*, and YS6 was *Jiangellaceae*, and the unique families of YS7 were *Acidothermaceae* and *Cellulomonadaceae*.

Non-metric multi-dimensional Scaling of a Bray-Curtis distance matrix demonstrated that some soil properties and/or spatial factors resulted in greater divergence within the Actinobacteria population (**Figure 8**). Axes 1 and 2 explained 71.8% of the Actinobacteria community variation. Concentrations of MP, MT, TN, AP, and ME were strongly associated with Axis 1 (loadings of −0.66, −0.63, 0.63, 0.58, and 0.56, respectively). MT, MP, ME, and TP were also strongly associated with Axis 2 (−0.59, −0.53, 0.47, and −0.40 respectively), and the pH (0.29, −0.15), OM (0.25, 0.25), and AK (0.26, −0.22) content had lower loadings than the other factors on both axes. AAC, MP, MT, and TP were correlated with YS1, YS3, YS4, and YS5 samples. OM, AP, TN, SD, and ME were correlated with YS2, YS7, YS8, YS9, and YS10 samples. An RDA analysis was employed to determine the influence of environmental factors on the Actinobacteria community (**Figure 9**). The first and second dimensions explained 42.2% of the total variance. The RDA analysis revealed that the Actinobacteria community compositions were related to multiple environmental factors, and other factors that were not studied in this paper.

TABLE 2 | Actinobacteria sequencing statistics and α diversity measures of soil samples of Yanshan mountains zone.

Source[a]	Total no. of clones	OTU[b]	Shannon diversity index (H′)	Pielou's evenness (J') index	Chao 1	ACE
YS1	114	76	4.127	0.9529	198.04	255.21
YS2	125	54	3.563	0.8932	161.51	154.02
YS3	108	77	4.151	0.9557	171.64	321.31
YS4	115	65	3.869	0.9269	159.91	209.37
YS5	121	59	3.741	0.9275	106.86	142.94
YS6	149	66	3.771	0.9001	466.81	200.04
YS7	149	107	4.459	0.9543	373.64	579.72
YS8	129	62	3.834	0.9290	126.77	131.93
YS9	204	138	4.713	0.9565	498.87	548.14
YS10	113	51	3.495	0.8889	80.75	126.56

DISCUSSION

Actinobacteria is one of the major phyla within the domain Bacteria. Because of the high diversity of members in this phylum, it is very difficult to develop a primer system that amplifies full-length, 16S rRNA gene sequences from all Actinobacteria. In spite of this, in the present study, it was possible to adopt indirect methods so that a larger number of full sequences could be screened from the bacterial 16S rDNA clone libraries. To simplify the screening process, we used 4 primers

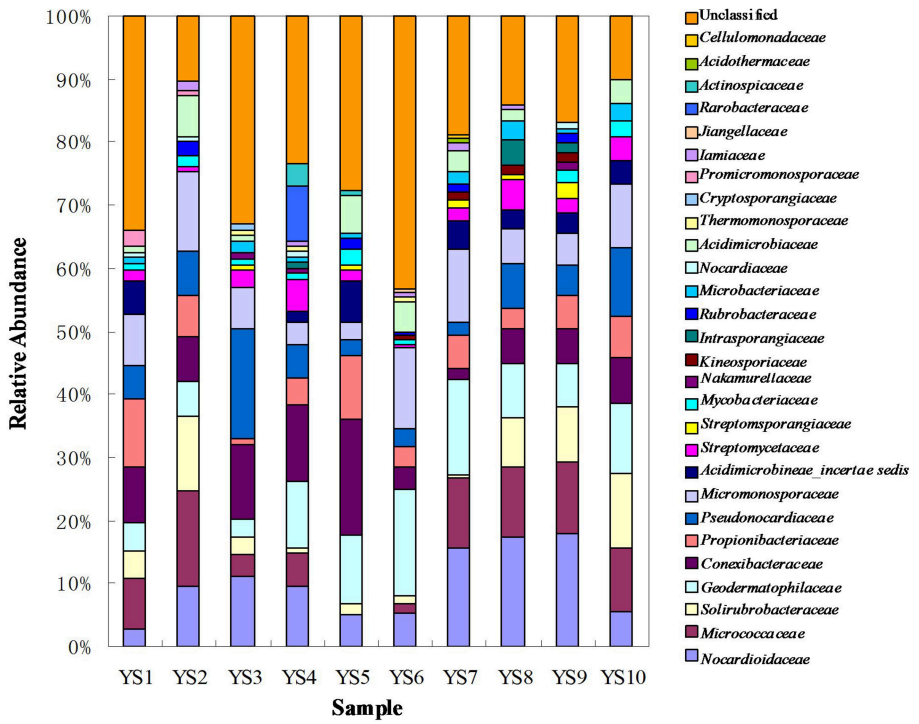

FIGURE 5 | Composition of different family based on classification of 16S rRNA sequences of Actinobacteria from soil of ten sites.

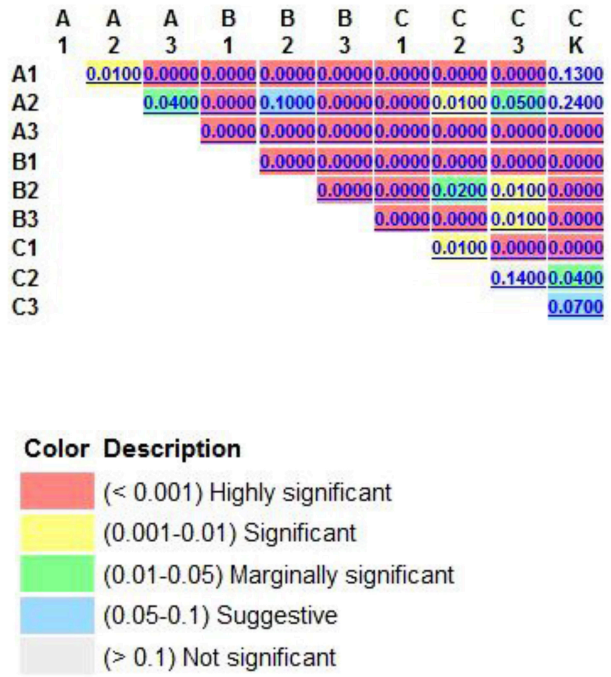

FIGURE 6 | The UniFrac significance were calculated by way of each pair of pretreatmental procotol, which tests whether each pair of environments differs from one another. Air dried soil sample were treated by 120°C 1 h (A1); 2 h (A2); 3 h (A3); soil sample were treated by air drying processing 15 days (B1); 30 days (B2); 45 days (B3), soil sample were treated by 0.1% Polymyxin B Sulfate immersion 1 h (C1); 2 h (C2); 3 h (C3).

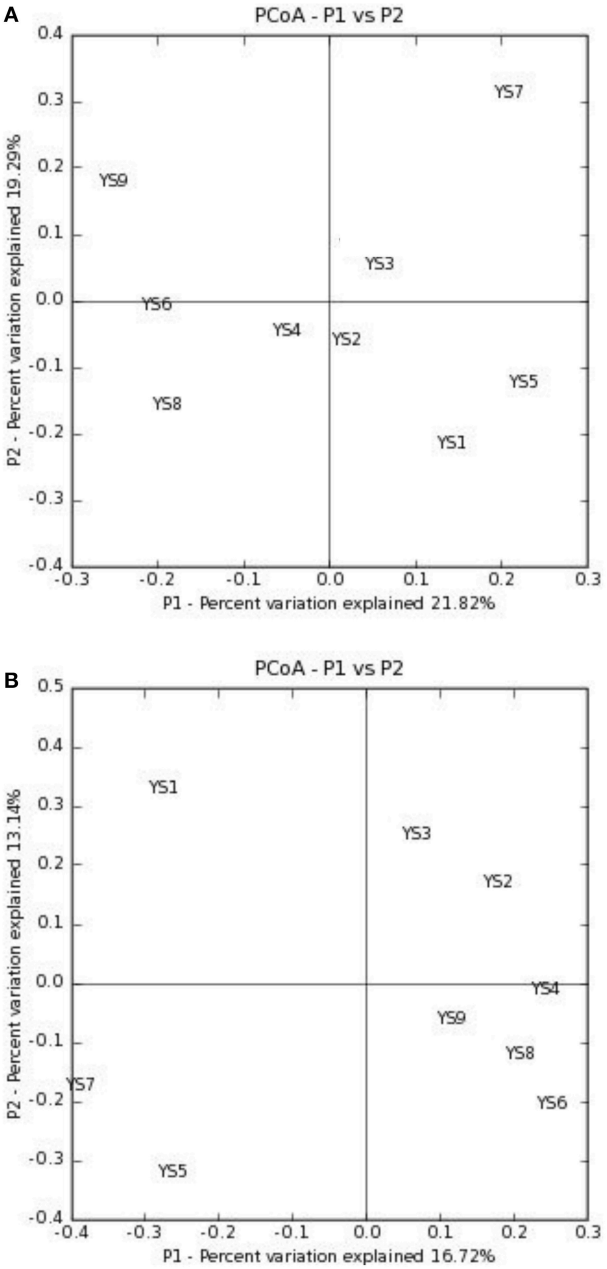

FIGURE 7 | PCoA plots are presented of the first two axes based on (A) weighted and (B) unweighted Unifrac distance matrices showing the quantitative and qualitative clustering of samples.

at the same time, and selected clones showed amplification bands (∼270 and/or ∼640 bp) for sequencing. Sequencing of the clone libraries clearly indicated that Actinobacteria DNA was primarily detected, with a false positive rate of 2.5%. The primer systems, Com2xf/Ac1186r/SC-Act235-aS-20/SC-Act878-aA-19, were suitable to screen for Actinobacteria in the 16S rDNA clone libraries.

One of the aims of this study was to improve methods for detection and identification of Actinobacteria represented in 16S rDNA clone libraries derived from environmental samples. In the soil, the majority of bacterial 16S rDNA products were from non-Actinobacterial strains; Actinobacteria from the 16S rDNA clone library were relatively rare. In this study, we studied the effect of air-drying, heating, or 0.1% Polymyxin B Sulfate on analysis of Actinobacteria diversity using culture-independent methods. These pre-treatment methods for the culture and isolation of Actinobacteria have been suggested by several researchers (Demain and Davies, 1999; Seong et al., 2001; Jiang et al., 2010; Jensen et al., 2015; Sun et al., 2015). Employing pretreatments of soil by drying and heating has been shown to increase the number of actinomycetes that were isolated. In this study, when the total DNA of untreated soils was extracted, the colonies recovered was mainly from other orders of bacteria (**Table 1**). However, no matter which pretreatment method was applied, pretreatment significantly increased the numbers of Actinobacterial colonies ($P < 0.01$), while drastically reducing the numbers of other bacterial colonies ($P < 0.01$). The rarefaction analysis of OTUs at the 97% level suggested that the number of clones screened (500) was insufficient to cover the diversity of Actinobacteria and the data were rarefied (**Figure 2**). Therefore, in our analysis of Actinobacteria diversity in the Yanshan Mountains, we increased the number of clones screened to 1000. Data confirmed that the pretreatment of soil led to an increase in the detection of Actinobacteria taxa and access to a more genetically diverse community of Actinobacteria.

At the same time, we found that each of the soil pretreatments could not only increase the detection rate of Actinobacteria, but showed a bias toward the detection of some groups of Actinobacteria. It provides a reference for the separation of the corresponding groups of Actinobacteria. Each of these treatment methods has both positive and negative aspects, in terms of their efficiency and ability to yield DNA extracts that truly represent the natural microbial community. Altogether, our results indicate that the Actinobacterial abundance and diversity that was detected might be affected by pretreatment procedures used to recover soil metagenomic DNA. Understanding these biases has become critical with the expansion of 16S rDNA technologies, which allow a more comprehensive investigation of specific microbial diversity. Our study confirms the pivotal importance of soil sample pretreatment in the DNA extraction procedure. It also emphasizes the need for thorough technical surveys to increase species richness per sequencing effort to be useful in microbial diversity studies. Consequently, we need to revisit our choice of pretreatment protocols to ensure that the DNA recovered from soil is not only of good quality, but also sufficiently representative in terms of richness and evenness of the Actinobacterial populations. In contrast to untreated soils, where Actinobacteria are believed to represent only about 3.6% of the total bacterial community, investigations of pretreated soils revealed that Actinobacterial 16S rRNA genes accounted for between 4.8 and 20.4% of the total community. The detected Actinobacteria were highly diverse (A1). Compared with other pretreatment methods, Actinobacteria diversity from methods A1 and A2 were not different with CK, as determined by the UniFrac significance test (0.13 and 0.24, respectively; **Figure 6**). Moreover, A1 yielded 102 clones with a detection rate of 20.4%, which was much higher than those found with the other pretreatment processes. Therefore, in order to gain accurate and representative phylogenetic information on Actinobacteria in the Yanshan Mountains, we chose the A1 soil pretreatment method: air drying of the soil sample, followed by

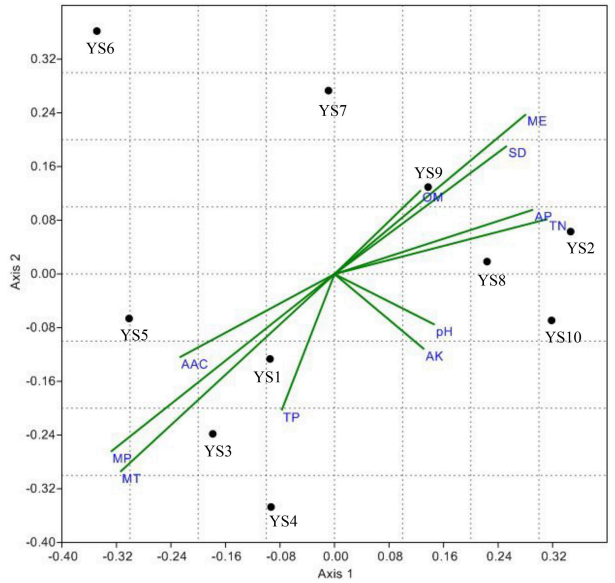

FIGURE 8 | Non-metric Multidimensional Scaling (NMDS) projection of a Bray–Curtis distance matrix showing the response of *Actinobacteria* communities to environmental vector. Axis 1 explains 45.6% of variance, while Axis 2 describes an additional 26.2% of variance among samples. Environmental variables abbreviations are TN, total nitrogen; TP, total phosphorus; AP, available phosphorus; AK, available potassium; OM, organic matters; MT, mean temperature; MP, mean precipitation; ME, mean sea level elevation; SD, mean sunshine duration; AAC, mean active accumulated temperature.

TABLE 3 | Abundance and diversity of main family from Actinobacteria.

	YS1		YS2		YS3		YS4		YS5		YS6		YS7		YS8		YS9		YS10	
	RA[a]	SI[b]	RA	SI	RA	SI	RA	SI	RA	SI	RA	SI	RA	SI	RA	SI	RA	SI	RA	SI
Nocardioidaceae	2.1	1.39	8.5	1.77	8.5	1.91	7.8	1.91	4.2	1.33	5.7	1.56	4.3	2.20	17.0	2.82	15.6	1.91	26.2	3.02
Micromonosporaceae	8.7	1.58	15.4	1.06	6.7	1.95	3.8	1.39	2.9	1.10	18.3	1.77	10.6	1.70	17.3	2.51	6.7	1.55	9.6	2.39
Conexibacteraceae	9.7	1.75	8.7	1.87	12.6	2.49	13.6	1.58	21.4	1.64	4.9	1.73	7.8	1.61	2.9	0.69	6.8	0.00	11.7	2.06
Pseudonocardiaceae	7.4	1.79	11.1	0.00	23.5	1.32	7.4	1.56	3.7	1.10	4.9	1.10	14.8	0.50	3.7	1.10	11.1	1.58	12.4	2.04
Propionibacteriaceae	16.4	1.10	11.0	0.74	1.4	0.00	6.8	1.33	16.4	0.72	6.8	0.60	9.6	0.50	11.0	1.39	5.5	0.00	15.1	2.03
Acidimicrobineae_incertae_sedis	15.8	1.79	0.0	0.00	0.0	0.00	5.3	0.69	21.1	0.41	0.0	0.69	10.5	0.00	18.4	1.33	10.5	0.00	18.4	1.75
Micrococcaceae	8.5	1.00	17.9	0.69	3.8	1.39	5.7	0.87	0.0	0.00	1.9	0.38	10.4	0.64	16.0	1.66	13.2	0.69	22.6	1.71
Geodermatophilaceae	4.0	0.50	5.6	0.41	2.4	1.10	9.6	0.84	10.4	0.91	20.0	0.43	9.6	1.44	18.4	1.62	8.8	1.30	11.2	1.43
Streptomycetaceae	6.1	0.69	3.0	0.00	9.1	0.64	18.2	0.87	6.1	0.69	3.0	0.64	12.1	0.69	9.1	0.00	18.2	0.69	15.2	1.39
Solirubrobacteraceae	7.1	1.61	21.4	0.41	4.3	0.00	1.4	0.00	2.9	0.69	2.9	0.69	18.6	0.69	1.4	0.00	14.3	1.23	25.7	1.21
Streptosporangiaceae	0.0	0.00	0.0	0.00	10.0	0.00	0.0	0.00	10.0	0.00	0.0	0.00	0.0	0.00	20.0	0.69	10.0	0.00	50.0	0.95
Mycobacteriaceae	6.2	0.00	12.5	0.64	6.3	0.00	6.3	0.00	18.7	0.64	6.2	0.64	18.8	0.69	0.0	0.64	0.0	0.00	25.0	0.56
Acidimicrobiaceae	2.9	0.00	22.8	0.74	2.9	0.00	0.0	0.00	20.0	0.41	20.0	1.04	11.4	0.41	14.3	1.61	5.7	0.69	0.0	0.00
unclassdified	12.5	3.05	4.3	2.51	11.8	3.14	8.9	2.65	10.9	2.91	21.0	1.85	3.6	2.54	9.6	3.15	5.9	2.29	11.5	3.26

[a] *Relative abundance (%) of taxonomic group with respect to total OTUs observed for community.*
[b] *Shannon diversity index.*

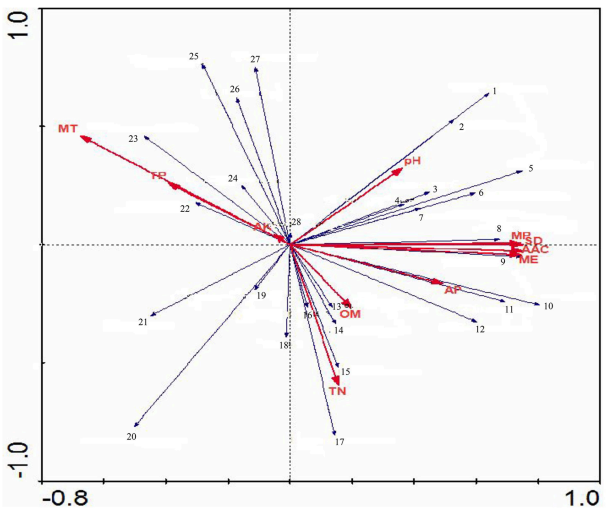

FIGURE 9 | The RDA ordination plot for the relationship between the family patterns of *Actinobacteria* community Clusters and environmental factors in the Yanshan mountains zone. Correlations between pattern or environmentasl factors and RDA axes are represented by the length and angle of arrows.

exposure to 120°C for 1 h. With this method, we observed high Actinobacterial diversity in a relatively small number of clone libraries.

Yanshan Mountains is a famous mountain range in north China, located at N 39°40′ ∼ 42°10′, E 115°45′ ∼ 119°50′ in the Inner Mongolia platform anteclise and subsidence zone. The eastern slope of the mountains has low mountains and hills, and lush vegetation, including shrubs, weeds, and a vast forest area. The western slope has low and medium mountains and sparse vegetation, including shrubs and grass. The Yanshan Mountains lie in an ecologically sensitive zone of north China near the Hu Huanyong Line (Hu, 1985). It is an ecosystem that has been adversely affected by forces of nature resulting in the destabilization of the balance of the living and non-living organisms in it and making it vulnerable to destruction. The ecosystem is facing changes due to climate change and destructive human activity, such as the mass cutting of trees. Living organisms interact with one another in an ecosystem in a cyclic manner; therefore, when one organism is destroyed, it affects the remaining organisms (Montoya et al., 2006).

Using a 16S rRNA gene clone library as a culture-independent method to survey the Actinobacterial community of Yanshan Mountains, we found that the overall diversity observed at the different stations was very high. The high number of novel Actinobacteria detected in the environmental samples is also significant. The Antibiotic Literature Database indicates that 57.8% of the known bioactive microbial products are produced by members of the class Actinobacteria. In this study, based on a comparison of signature nucleotides with higher taxa described by Zhi et al. (2009). we identified a total of 23 unclassified Actinobacteria, representing 2 novel orders, 10 novel suborders, and 39 novel families from Yanshan Mountains. It is reasonable to conclude that these new lineages may produce novel bioactive compounds, similar to other Actinobacteria. Clearly, the diversity of Actinobacteria greatly exceeds that predicted based on culture-based estimates, and this highlights the great biotechnological value in continuing efforts to isolate novel Actinobacteria genera. The genera *Conexibacter*, *Solirubrobacter*, *Microlunatus*, *Blastococcus*, and *Streptomyces* were common to all stations surveyed in this study. These groups are conserved in the Yanshan Mountains. Despite changing ecologies in the different stations, they were always present. Although members of the order Solirubrobacterales have not been extensively studied, recent studies have shown their ability to adapt and colonize different ecosystems, including fungal growing ant colonies (Ishak et al., 2011), spinach phyllosphere (Lopez-Velasco et al., 2011), desert, and Antarctic soil (Chong et al., 2012). Members of the genus *Blastococcus* were recovered from different latitudes and climates in dry and/or extreme conditions (Salazar et al., 2006), these microorganisms have the potential to colonize and alter stone and monument surfaces. *Microlunatus* spp. have been isolated from marine sediments (Yuan et al., 2014), a soil sample collected from Alu, an ancient cave (Cheng et al., 2013), and from conventional farming (Li et al., 2012). Some *Microlunatus* spp. have phosphorus-accumulating functions and phosphate uptake/release activities (Akar et al., 2005) in the enhanced biological phosphate removal (EBPR) process, and they are believed to play a pivotal role in phosphorus removal. The EBPR process is attracting interest for its potential use in phosphorus recycling (Hirota et al., 2010). In this study, some groups seemed to be more adaptive, based on their ability to survive in various environments. In contrast, there were unique genera identified in specific site: YS1, *Longispora*, *Propionibacterium*, and *Xylanimonas*; YS2, *Nocardia*; YS3, *Actinaurispora*, *Actinomadura*, *Actinomycetospora*, *Cryptosporangium*, *Humicoccus*, and *Phytohabitans*; YS4, *Actinocorallia*, *Actinospica*, *Hamadaea*, *Millisia*, and *Phycicoccus*; YS5, *Actinokineospora*; YS6, *Jiangella*; YS7, *Cellulomonas* and *Okibacterium*; YS8, *Rothia*, *Saccharothrix*, and *Terrabacter*; YS9, *Amycolatopsis*, *Cryobacterium*, *Knoellia, Nakamurella*, and *Rhodococcus*. Endemic taxa of these different stations reflect the Actinobacterial response to different environments.

The UniFrac analysis of the stations showed that the Actinobacterial compositions of YS2, YS3, YS4, YS6, YS8, YS9, and YS10 did not differ ($P > 0.1$). It is indeed "everything is everywhere, but the environment selects," with no evident dispersal limitations on Actinobacteria, This theory suggests that each ecologically equivalent study site will have similar Actinobacterial communities due to near identical environmental variables, which eliminate environmental filtering as well as constant additions by the regional species pool. Conversely, Bissett et al. (2010) described a hypothesis of "wherever you go, that's where you are" implying that beyond strong environmental selection, other factors (i.e., dispersal or colonization limitations and evolutionary events) play a significant role in shaping microbial communities. Between YS7 and most other sites, there were significant different in Actinobacterial community composition, with YS4 and YS8 showing highly significant differences ($P < 0.001$). It has been suggested that microbial biogeographical patterns are shaped by

environmental factors (Fierer, 2008). For instance, pH (Fierer and Jackson, 2006), temperature (Yergeau et al., 2007), and precipitation (Clark et al., 2009) have been found to be the best predictors of continent-scale patterns. It is also believed to be globally distributed by prevailing winds and community patterns are thought to result from barriers to dispersal, physiological requirements, resource availability, competition, or some combination thereof. However, Actinobacteria do not display a cosmopolitan distribution: their communities remain distinct not only over large geographical distances (Wawrik et al., 2007; Eisenlord et al., 2012) and seasonal differences (Cho et al., 2008), but also vary with local environmental factors[54] and within a single sampling location (Abdulla and El-Shatoury, 2007; Van der Gucht et al., 2007). This work provides evidence that soil Actinobacterial communities exhibit regional biogeographic patterns, wherein community membership changes across the north-south distribution of Hu Huanyong Line. Stations YS1, YS3, YS4, and YS5 are located at the edge of the ecologically sensitive zone, the southern Yanshan Mountains, in the rain belt, and these sites are affected by the continental climate significantly. The climatic characteristics of AAC, MP, and MT and biogeochemical data of TP likely contributed to Actinobacterial communities at these stations. The ecological environments of other stations were not stable and fragile. It was clear that biogeochemical factors contributed more to Actinobacterial community structure than chemical factors. The stability of Actinobacterial communities in different ecological environments was largely correlated with biogeochemical factors and less with climate factors, such as *Streptomycelaceae* and pH, *Solirubrobacteraceae* and AP, *Propionibacteriaceae* and OM, *Geodermatophilaceae* and TN.

AUTHOR CONTRIBUTIONS

HT, implementation of the experiment. XS, English check. XW, English check. HH, English check. XZ, sampling. LZ, designing experimental program.

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SUPPLEMENTARY MATERIAL

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Characterization and evaluation of antimicrobial and cytotoxic effects of *Streptomyces* sp. HUST012 isolated from medicinal plant *Dracaena cochinchinensis* Lour.

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A highly potent secondary metabolite producing endophytic strain, *Streptomyces* sp. HUST012 was isolated from the stems of the medicinal plant *Dracaena cochinchinensis* Lour. Strain HUST012 showed antimicrobial and antitumor activities which were significantly much higher than those of dragon's blood extracted from *D. cochinchinensis* Lour. On further analysis, the strain was found to produce two metabolites, SPE-B11.8 (elucidated to be a novel metabolite (*Z*)-tridec-7-ene-1,2,13-tricarboxylic acid) and SPE-B5.4 (elucidated as Actinomycin-D). The Minimum Inhibitory Concentration values of SPE-B11.8 against a set of test bacterial organisms (Methicillin-resistant *Staphylococcus epidermis* ATCC 35984, Methicillin-resistant *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 13883) ranged between 15.63 and 62.5 μg/ml while that for SPE-B5.4 ranged between 0.04 and 2.24 μg/ml. The compound SPE-B11.8 showed cytotoxic effect at 41.63 and 29.54 μg/ml IC_{50}-values against Hep G2 and MCF-7, respectively, while the compound SPE-B5.4 exhibited stronger activities against them at 0.23 and 0.18 μg/ml IC_{50}-values.

Keywords: endophytic, *Streptomyces* sp. HUST012, *Dracaena cochinchinensis* Lour., antimicrobial and cytotoxic activities, (*Z*)-tridec-7-ene-1,2,13-tricarboxylic acid, Actinomycin-D

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Introduction

Streptomyces spp. have been shown to possess the ability to synthesize antibacterial, antifungal, insecticidal, antitumor, anti-inflammatory, anti-parasitic, antiviral, anti-infective, antioxidant, and herbicidal compounds (Qin et al., 2011; Kawahara et al., 2012). Nearly 70% of the natural antibiotics used in clinical practices were produced by actinobacteria (Subramani and Aalbersberg, 2012) of

which 75–80% have been derived from *Streptomyces* alone (Inbar and Lapidot, 1988; Olano et al., 2004; Rehm et al., 2009; Crnovcic et al., 2013).

The plant *Dracaena cochinchinensis* Lour. has been used as a traditional medicine since ancient times in the form of Dragon's blood, a deep red resin. Dragon's blood has been shown to illustrate antimicrobial, antiviral, antitumor, cytotoxic, analgesic, antioxidative, anti-inflammatory, haemostatic, antidiuretic, anti-ulcer and wound healing activities (Gupta et al., 2008). It also finds application as coloring materials and wood varnish (Gupta et al., 2008). However, the slow growth in combination with its low dragon's blood yield results in the destruction of large number of century old plant for harvesting a few milligrams of dragon's blood (Fan et al., 2008). This current study was conducted to explore a sustainable way of utilizing the medicinal plant by studying the endophytic actinomycetes associated with the plant. This paper incorporated the results of the characterization and the evaluation of cytotoxic and antimicrobial effects of an endophytic *Streptomyces* sp. strain, isolated from the medicinal plant *D. cochinchinensis* Lour. in comparison with those of dragon's blood extracted from the host plant. The paper also reported the structure elucidation of the bioactive metabolites extracted from the endophytic actinobacteria.

Materials and Methods

Sample Collection and Isolation of Endophytic Actinomycete

Healthy stems of *D. cochinchinensis* Lour. plant were collected from Cuc Phuong National Park, Ninh Binh province, Vietnam (20° 19′ 8″N, 105° 37′ 20″E; 338 m). The samples were surface sterilized and plated on Sodium propionate medium (Qin et al., 2009). The medium was supplemented with nalidixic acid (25 mg/l), nystatin (50 mg/l), and $K_2Cr_2O_7$ (50 mg/l) to inhibit the growth of Gram-negative bacteria and fungi and polyvinyl pyrolidone (PVP) 2% and tannase 0.005% to improve the growth of colonies. Actinomycetes colonies grown on this culture media were selected and purified by repeated streaking onto International *Streptomyces* Project (ISP) 2 medium. The purified strain HUST012 was preserved as glycerol suspensions (20%, v/v) and as lyophilized spore suspensions in skim milk at −80°C (Zhang et al., 2010).

Characterization of the Endophytic Isolate HUST012

The endophytic isolate HUST012 was characterized on the basis of the physiological and biochemical properties and the analysis of 16S rRNA gene sequence. Morphological and growth patterns were observed on different media (Shirling and Gottlieb, 1966). Morphological characteristics were observed by light microscopy (Olympus BH2) and scanning electron microscopy (JSM-6610LV, JEOL Ltd.) (Anderson and Wellington, 2001). The ability of the isolate to grow at different pH (4.0–10.0, at intervals of 1.0 pH unit using the buffer system as described by Xu et al., 2005) and concentration of NaCl (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 10.0, 11.0, 12.0, 15.0%, w/v) was examined on ISP 2 medium. Growth was tested at 4, 10, 20, 25, 28, 37, 45, and 55°C using ISP2 medium. The hydrolysis of starch, casein and gelatin was carried out according to the methods described by Tindall et al. (2007). Nitrate reduction and H_2S production were determined using conventional procedures (Goodfellow, 1971; Athalye et al., 1985). Utilization of the carbon source was performed as previously described (Shirling and Gottlieb, 1966; Athalye et al., 1985; Mechri et al., 2014) using the basal medium recommended by Pridham and Gottlieb (1948).

The isolation of genomic DNA and PCR amplification for 16S rRNA gene was performed as previously described (Li et al., 2009). The identification of phylogenetic neighbors and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (http://www.eztaxon.org/) (Kim et al., 2012) and BLAST analysis (http://blast.ncbi.nlm.nihgov/Blast.cgi). Multiple sequence alignment was done using CLUSTALW (Thompson et al., 1997). The phylogenetic tree was constructed using the aligned sequences by the neighbor-joining method (Saitou and Nei, 1987) using Kimura-2-parameter distances (Kimura, 1983) in the MEGA 6 software (Tamura et al., 2013). To determine the support of each clade, bootstrap analysis was performed with 1000 replications (Felsenstein, 1985).

The GenBank accession number for the partial 16S rRNA gene sequences of strain HUST012 is KP330557.

Evaluation of Antimicrobial Activities

The antibacterial activities was evaluated against Methicillin-susceptible *Staphylococcus aureus* (MSSA) ATCC 29213, Methicillin-resistant *Staphylococcus epidermidis* (MRSE) ATCC 35984, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Aeromonas hydrophila* ATCC 7966, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 11105, and *Enterococcus faecalis* ATCC 29212 using the agar well diffusion method (Holder and Boyce, 1994). The Minimum Inhibitory Concentration (MIC) was determined as previously described (Andrews, 2001).

The animal fungal pathogens *Fusarium graminearum*, *Aspergillus carbonarius*, and *Aspergillus westerdijkiae* which were known to produce strong toxic deoxynivalenol (DON) and ochratoxin A (Khamna et al., 2009; Huffman et al., 2010) were kindly provided by UMR Qualisud, CIRAD, France. These strains were maintained on Potato Dextrose Agar (PDA) medium (Liu et al., 2002). For the determination of antifungal activity, culture broth of HUST012 (100 ml) was centrifuged at 7000 *g* for 10 min. The supernatant was collected and added to the PDA medium (pH 5.5) at a concentration of 15% (v/v). Sterilized water was used as control. The resulting PDA plates were inoculated with the different fungal strains and incubated for 5 days at 28°C. The fungal radial growth was measured. Each experiment was carried out in triplicates.

Determination of Cytotoxic Activity

The cytotoxicity against human hepatocellular carcinoma Hep G2 and human breast adenocarcinoma MCF-7 cell lines was tested by using sulforhodamine B (SRB) assay as previously described (Thao et al., 2014). Ellipcitine was used as the positive control. The test was done in triplicates to ensure accuracy.

Fermentation

A small-scale liquid fermentation was performed with YIM 61 medium (Qin et al., 2009) as the antibiotic producing medium (200 rpm, 28°C, 5 days). The scale up fermentation (20 L) was done using the New Brunswick BioFlo®/CelliGen® 115 Benchtop Fermentor & Bioreactor (28°C, 5 days). In both cases, seed culture for inoculation was prepared in ISP2 medium (200 rpm, 28°C, 4 days)

Extraction and Purification of the Active Compounds

The fermentation broth was centrifuged at 7000 rpm for 10 min. The supernatant fraction was then extracted thrice with ethyl acetate. The ethyl acetate layer was concentrated *in vacuo* to give ethyl acetate extract (SPA-E). The aqueous phase was filtered through a diaion HP20 column and eluted with water and methanol subsequently. The water eluent fraction was evaporated to give the extract designated as SPA-W1 while the methanol eluent was concentrated *in vacuo* to obtain a brown solid (SPA-W2).

Similarly, the mycelium cake obtained after centrifugation of the fermentation broth was processed to obtain a ethyl-acetate extract (SPB-E), a water eluent extract (EPB-W1) and a methanol eluent extract (SPB-W1). All these fractions were analyzed in Silica gel TLC sheet (Merck, Germany) using the dichloromethane-methanol (30:1, v/v) solvent system. Based on the similarity profiles in the TLC (Koup et al., 1978), SPA-E/SPB-E, SPA-W1/SPB-W1, and SPA-W2/SPB-W2 were pooled together and were designated as SP-E, SP-W1, and SP-W2, respectively. A schematic diagram representing the extraction protocol is shown in **Figure 1**. Each of these fractions was evaluated for antimicrobial and cytotoxic activities. The bioactive fractions were further purified using different solvent systems to obtain pure metabolite(s) as represented in **Figure 1B**.

Structure Elucidation of the Pure Active Compounds

The structure of the bioactive compound(s) was analyzed using mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (Booth et al., 1976; Hamza et al., 2013). The results were compared with the available reference compounds and published literatures.

Determination of Antibacterial and Cytotoxic Effects of the Dragon's Blood Extracted from Medicinal Plant *D. cochinchinensis*

Dragon's blood in the xylem of the host plant was extracted as described by Wang et al. (2011). The dry weight of the extract was dissolved in 95% (v/v) alcohol and filtered through sterile filter membrane (0.22 μm). The solution was then used for antibacterial and antitumor tests.

The MIC of Dragon's blood against MSSA ATCC 29213, MRSE ATCC 35984, *K. pneumoniae* ATCC 13883, and *E. coli* ATCC 25922 was determined by broth dilution method on 96-well plate as previously described (Andrews, 2001). The MIC against *F. graminearum* was determined according to Gopal et al. (2012). The SRB assay was used for determination the cytotoxic effect of the Dragon's blood on human breast adenocarcinoma (MCF-7) and human hepatocellular carcinoma (Hep G2) cells (Thao et al., 2014).

Results

Characterization of Strain HUST012

Cells of the strain HUST012 was Gram-positive and aerobic. The strain formed extensively branched, non-fragmented substrate and aerial mycelia. Strain HUST012 formed straight or rectiflexibile spore chains with smooth surface. However, these spore chains generally contained less than 50 spores (Figure S1). The strain grew well on ISP 2, ISP 3, ISP 5, TSA, Czapek, and Nutrient agar media, with a gray color aerial mycelium. It produced green-yellow and yellow pigments on ISP2 and Czapek agar media respectively. Strain HUST012 was found to grow over a wide range of temperature (4–45°C) and pH (4.0–9.0) with optimal growth at 28°C and pH 6.0–7.0, and in the presence of upto 10% NaCl (w/v) with optimum at 1–3% NaCl.

HUST012 could utilize DL-alanine, L-arginine, L-asparagine, Glycine, DL-leucine, L-lysine, DL-serine, L-glutamic acid, DL-methionine, L-cystine, L-histidine as nitrogen resources; D-fructose, D-galactose, D-glucose, D-mannose, D-trehalose, D-sorbose, D-xylose, glycerol, and sodium acetate as carbon sources. The strain was positive for amylase and catalase activities, but was negative for nitrate reduction, H_2S production and gelatin reduction tests. Strain HUST012 showed highest 16S rRNA gene sequence similarities with *Streptomyces parvulus* (99.26%). Phylogenetic tree (**Figure 2**) based on neighbor-joining method also indicated its closest similarity to *Streptomyces parvulus*. The phenotypic and genomic data indicated that the strain HUST012 represented a strain of the genus *Streptomyces* for which the strain was referred to as *Streptomyces* sp. strain HUST012.

Antimicrobial and Cytotoxic Effects of Strain HUST012

The culture filtrate of strain HUST012 exhibited antibacterial activity against all tested Gram positive and Gram negative bacterial strains. The maximum activity was found against MRSE ATCC 35984 (inhibition zone of 35 mm diameter), followed by *A. hydrophila* ATCC 7966 (26 mm) and MSSA ATCC 29213 (25.80 mm). The detailed antimicrobial profiles are shown in **Table 1**.

The antifungal activity of *Streptomyces* sp. strain HUST012 was examined against three mycotoxin producing fungal strains. The fungal growth inhibition was observed in the order: *F. graminearum* (9.7 mm), *A. carbonarius* (7.7 mm), and *A. westerdijkiae* (1.8 mm).

Fermentation, Antimicrobial and Cytotoxic Effects of Bioactive Metabolites of Strain HUST012

Among the crude metabolites extracts of strain HUST012, the fraction SP-E showed the highest antibacterial and cytotoxic activities. This fraction was further purified by column chromatography with different gradient solvent systems as

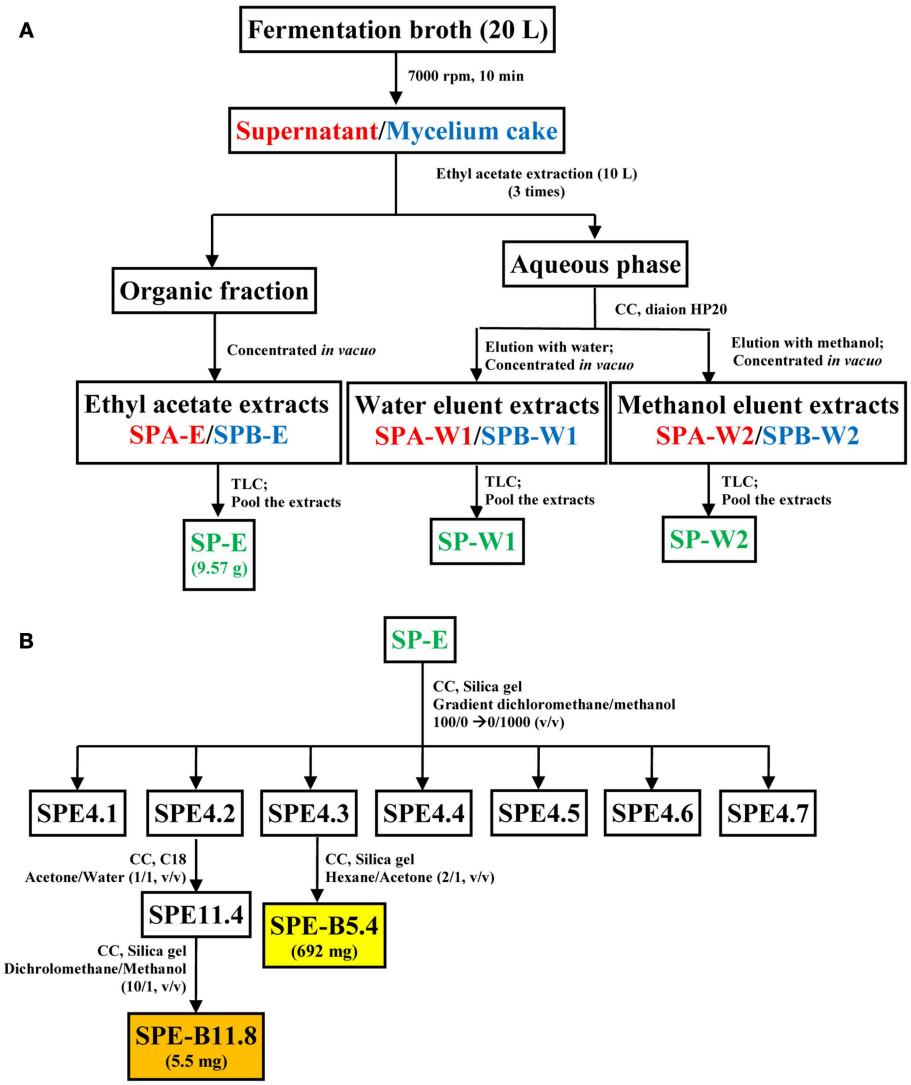

FIGURE 1 | Schematic representation of the process for metabolite extraction from strain HUST012. (A) Extraction of the fermentation medium into crude metabolite extracts; **(B)** fractionation protocol for pure compounds SPE-B11.8 and SPE-B5.4 from the crude ethyl acetate extract. Note: CC, Column chromatography; Font in red color indicates supernatant, blue mycelium and green pooled fractions; Color boxes indicate pure compound.

depicted in **Figure 1**. Two bioactive metabolites, designated SPE-B11.8 and SPE-B5.4, were purified.

The MIC values of the metabolite SPE-B11.8 against the test bacterial organisms ranges between 15.63 and 62.5 μg/ml while those for SPE-B5.4 ranges between 0.04 and 2.24 μg/ml (**Table 2**).

Human hepatocellular carcinoma Hep G2 and human breast adenocarcinoma cell MCF-7 lines were used as model systems to examine the cytotoxic effect of *Streptomyces* sp. HUST012. The culture filtrate, crude metabolite extracts (SP-E, SP-W1, SP-W2) and the pure metabolites (SPE-B11.8 and SPE-B5.4) were examined for their cytotoxic effect on the two human cancer cell lines Hep G2 and MCF-7. The cytotoxic assay results showed that the culture filtrate of the strain HUST012 had significant inhibition toward Hep G2 and MCF-7 cells with IC_{50}-values of 4 and 3 μg/ml, respectively. Among the crude metabolites extracts, SP-E showed the strongest cytotoxic effect with IC_{50}-values of 0.31 and 0.18 μg/ml. The pure metabolites SPE-B11.8 showed cytotoxic effect at 41.63 and 29.54 μg/ml IC_{50}-values against Hep G2 and MCF-7, respectively, while the metabolite SPE-B5.4 exhibited the same at 0.23 and 0.18 μg/ml IC_{50}-values (**Table 3**).

Structure Elucidation of Bioactive Compounds

The structure of the compounds SPE-B11.8 and SPE-B5.4 were analyzed through the techniques of MS and NMR spectroscopy.

The compound **SPE-B11.8** was obtained as a colorless solid. Its HRESIMS spectrum showed a peak at *m/z* 315.1814 $[M+H]^+$, corresponding to the molecular formula $C_{16}H_{27}O_6$. The 1D and 2D-NMR spectra of **SPE-B11.8** showed signals characteristic

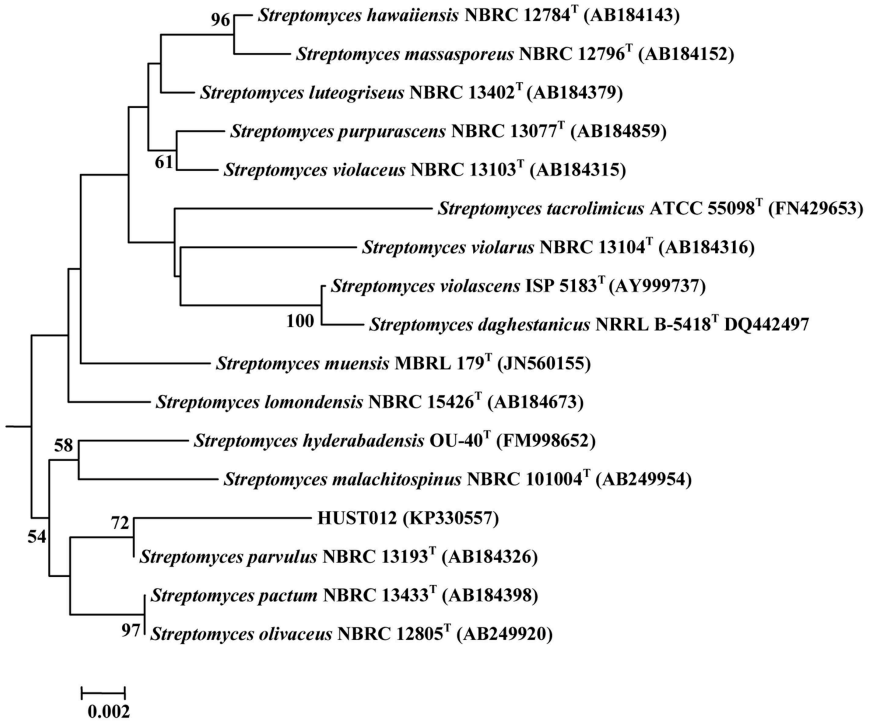

FIGURE 2 | Neighbor-joining tree showing the phylogenetic relationships based on 16S rRNA gene sequence of the strain HUST012 and closest species. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% was given at the node.

TABLE 1 | Antimicrobial activities of the strain *Streptomyces* sp. HUST012 against bacterial and fungal strains.

Test strains	Inhibition zone (mm diameter)
GRAM POSITIVE BACTERIA	
Methicillin-resistant *S. epidermidis* ATCC 35984	35.00 ± 0.80
Methicillin-resistant *S. aureus* ATCC 25923	18.90 ± 1.67
Methicillin-susceptible *S. aureus* ATCC 29213	25.80 ± 1.47
Enterococcus faecalis ATCC 29212	20.00 ± 0.20
GRAM NEGATIVE BACTERIA	
Escherichia coli ATCC 25922	18.90 ± 1.00
Escherichia coli ATCC 11105	12.40 ± 0.73
Klebsiella pneumoniae ATCC 13883	19.80 ± 2.20
Aeromonas hydrophila ATCC 7966	26.00 ± 0.47
FUNGAL STRAINS	
Fusarium graminearum	9.70 ± 0.73
Aspergillus westerdijkiae	1.80 ± 0.47
Aspergillus carbonarius	7.70 ± 0.80

TABLE 2 | Antibacterial and cytotoxic effects of the compounds HPE-B11.8 and SPE-B5.4 in comparison with Dragon's blood extracted from medicinal plant *D. cochinchinensis* Lour.

Test organisms/cancer cell line	SPE-B11.8	SPE-B5.4	Dragon's blood extract
	MIC/IC_{50} (μg/ml)		
MRSE ATCC 35984	15.63 ± 1.18	0.04 ± 0.00	4.88 ± 0.05
MRSA ATCC 25923	62.5 ± 2.26	0.04 ± 0.00	4.88 ± 0.05
E. coli ATCC 25922	Inactive	2.24 ± 0.01	9.77 ± 0.23
K. pneumoniae ATCC 13883	62.5 ± 2.26	0.04 ± 0.00	4.88 ± 0.05
F. graminearum	Inactive	9.77 ± 0.23	19.53 ± 0.80
Hep G2	41.63 ± 0.61	0.23 ± 0.05	77.91 ± 0.22
MCF-7	29.54 ± 2.89	0.18 ± 0.05	70.00 ± 7.08

for a monounsaturated fatty acid with the double bond at δ_H 5.33/δ_C129.1–129.9, three carboxylic groups at δ_C 175.1, 178.1, and 181.1, and a cluster of methylenic protons at δ_C in the range of δ_C 24.6–35.1. The COSY and HMBC spectra led to the identification of the fragments of SPE-B11.8 structure (see Figure S2 for the complete NMR spectra). The position of the double bond was also confirmed by the MS data with the fragment at *m/z* 128.08 and 187.08 corresponding to the breakdown at C-7 and C-8 liason. The configuration of the double bond was determined based the on the chemical shifts of vicinal carbon atoms. Both C-6 and C-9 appeared at δ_C 26.5 and 26.3 ppm indicating the *Z* configuration. Thus, compound SPE-B11.8 was newly elucidated to be (*Z*)-tridec-7-ene-1,2,13-tricarboxylic acid (**Figure 3**).

TABLE 3 | Cytotoxicity of test sample (IC_{50} in μg/ml).

Samples	Hep G2	MCF-7
Culture filtrate of HUST012	4.00 ± 0.10	3.00 ± 0.10
SP-E	0.31 ± 0.03	0.18 ± 0.02
SP-W1	>100	>100
SP-W2	>100	>100
SPE-B11.8	41.63 ± 0.61	29.54 ± 2.89
SPE-B 5.4	0.23 ± 0.05	0.18 ± 0.05
Ellipticine	0.51 ± 0.08	0.47 ± 0.05

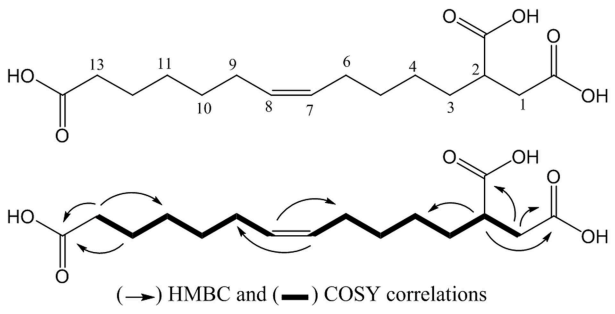

FIGURE 3 | Structure of the compound SPE-B11.8 (elucidated as (*Z*)-tridec-7-ene-1,2,13-tricarboxylic acid).

The compound **SPE-B5.4** was obtained as a red powder, soluble in methanol, ethyl acetate, ethanol, and DMSO, stable in aqueous solutions at 5–10°C. The HRESIMS spectrum revealed a peak at *m/z* 1255.6435 $[M+H]^+$, corresponding to the formula $C_{62}H_{87}N_{12}O_{16}$ (Figure S3). The ^{1}H-NMR, ^{13}C-NMR spectrum analysis data of the SPE-B5.4 compound is presented in Table S1. The spectral data was compared with the findings of Booth et al. (1976) and the compound SPEB-5.4 was identified as Actinomycin-D with molecular formula $C_{62}H_{86}N_{12}O_{16}$ (**Figure 4**).

Antibacterial and Cytotoxic Effects of the Dragon's Blood Extracted from Medicinal Plant *D. cochinchinensis*

The Dragon's blood extract was analyzed for its antibacterial and cytotoxic effects against MRSA, MRSE, *K. pneumoniae* and *E. coli,* and toward MCF-7 and Hep G2 cell lines. **Table 2** showed the MIC for the dragon's blood extracts in comparison with those of the crude metabolites extracts and the compounds SPE-B11.8 and SPE-B5.4.

Discussion

The antimicrobial resistance has been one of the most serious health threats. Infections from resistant bacteria are now too common, and some pathogens have even become resistant to multiple classes of antibiotics. The decline of effective antibiotics will undermine our ability to fight infectious diseases and manage the infectious complications common in vulnerable patients, especially those undergoing chemotherapy for cancer, dialysis for renal failure, and organ transplantation. When first- and second-line antibiotic treatment options are limited by resistance and/or unavailability, healthcare providers are forced to use toxic antibiotics which are frequently more expensive but less effective. Even when alternative treatments are available, research has shown that patients with resistant infections are often much more likely to result in death, and that survivors require longer hospital stays, delayed recuperation, and long-term disability. Hence, there is an urgent need for search of novel drugs against such pathogens. It has been envisaged that endophytic environment is an extreme source to provide exciting new bioactive compounds.

In the present study, an attempt was tried to identify the bioactive potential of the endophytic actinobacterium *Streptomyces* sp. HUST012. The strain was found to exhibit antimicrobial activities against a set of pathogenic bacteria and fungi (**Table 1**). The presence of antifungal activities is also an indication of probable biocontrol mechanisms against mycotoxin producing fungal strains. Similar findings have been reported in similar studies of *Streptomyces* strains (Rahman et al., 2010; Usha et al., 2010; Naine et al., 2015).

The cytotoxic ability of this strain was significant as compared to that reported in previous studies on *S. parvulus* strain VITJS11 (Naine et al., 2015). The compounds SPE-B11.8 and SPE-B5.4 had IC_{50}-values of 41.63 and 0.23 μg/ml on Hep G2 cells as compared to 500 μg/ml by *S. parvulus* strain VITJS11. Other reports showed that migrastatin, a secondary metabolite from *Streptomyces* inhibited the Hep G2 cells at the concentration of 6 and 10 μM after 24 and 48 h of treatment (Rambabu et al., 2014). The high bioactive effect of *Streptomyces* sp. HUST012 can be explained by the fact that endophytic actinomycetes live in close association with their host plants and that it could become a real possibility for exchange of genes involved in natural products biosynthesis between endophytic actinomycetes and host plants via horizontal gene transfer, resulting in synthesis of plant-derived compounds by a microbial endophyte (Chandra et al., 2013).

An important finding of this current study was the isolation of the new compound HPE-B11.8 which was elucidated as (*Z*)-tridec-7-ene-1,2,13-tricarboxylic acid, thereby underlying the importance of the source. The compound HPE-B11.8 possessed moderate antibacterial and anticancer activities against the test pathogenic microorganisms/cell lines. Another important finding was the isolation of Actinomycin D (compound SPE-B5.4). Actinomycin D was an antineoplastic antibiotic that inhibits cell proliferation. It finds wide range of applications, viz. as selective reagent in cell culture, studies in suppressing HIV-replication and programmed cell death of PC12 cells, and as an antibiotic in treatment of various malignant neoplasm including Wilm's tumor and the sarcomas. Actinomycin-D decreases Mcl-1 expression and acts synergistically with ABT-737 against small cell lung cancer cell lines (Aishan et al., 2010). According to the Internet bibliographic database-MEDLINE, actinomycins, especially Actinomycin-D, have been the subject of about 3300 research publications (Koba and Konopa, 2005). The isolation of Actinomycin-D is

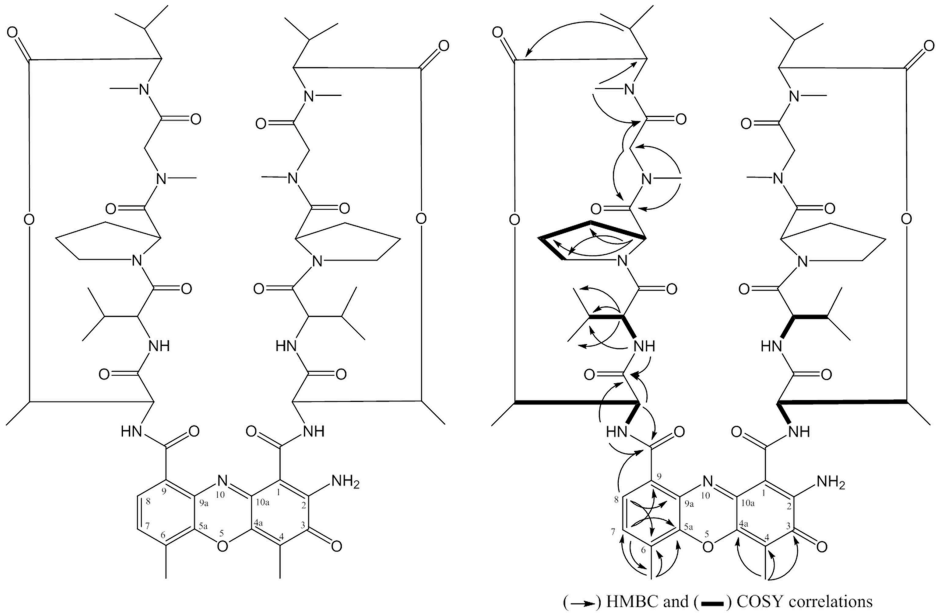

FIGURE 4 | Structure of the compound SPE-B3.4 (established as Actinomycin-D).

not a new discovery but our present study proved that the medicinal plant *D. cochinchinensis* Lour. was a rich source of endophytic actinomycetes producing the potent antibiotic agents.

Dragon's blood has been well documented for its antimicrobial, antioxidant, anti-antitumor and cytotoxic properties. However, the host plant *D. cochinchinensis* Lour. has no secretory tissue to release this useful metabolite, and therefore Dragon's blood remains in xylem parenchyma cells of the stem. The growth of the plant is extremely slow and has low yield of dragon's blood. To harvest a few pieces of resinous wood, a tree with hundreds of years old is often destroyed. This work aimed to evaluate the antimicrobial and cytotoxic effects of the natural Dragon's blood extracted from medicinal plant *D. cochinchinensis* Lour. in comparison with that secreted by the endophytic *Streptomyces* sp. HUST012 associated with the host plant. Our results were significant in comparison to the findings of other research groups (Al-Fatimi et al., 2005; Wang et al., 2010, 2011). This could give us a suggestion for the promotion of the application of secondary bioactive metabolites from endophytic actinomycetes associated with the medicinal plant *D. cochinchinensis* Lour. instead of destroying valuable endangered trees.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00574/abstract

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Presence of antioxidative agent, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- in newly isolated *Streptomyces mangrovisoli* sp. nov.

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A novel *Streptomyces*, strain MUSC 149^T was isolated from mangrove soil. A polyphasic approach was used to study the taxonomy of MUSC 149^T, which shows a range of phylogenetic and chemotaxonomic properties consistent with those of the members of the genus *Streptomyces*. The diamino acid of the cell wall peptidoglycan was LL-diaminopimelic acid. The predominant menaquinones were identified as MK9(H_8) and MK9(H_6). Phylogenetic analysis indicated that closely related strains include *Streptomyces rhizophilus* NBRC 108885^T (99.2% sequence similarity), *S. gramineus* NBRC 107863^T (98.7%) and *S. graminisoli* NBRC 108883^T (98.5%). The DNA–DNA relatedness values between MUSC 149^T and closely related type strains ranged from 12.4 ± 3.3% to 27.3 ± 1.9%. The DNA G + C content was determined to be 72.7 mol%. The extract of MUSC 149^T exhibited strong antioxidant activity and chemical analysis reported identification of an antioxidant agent, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-. These data showed that metabolites of MUSC 149^T shall be useful as preventive agent against free-radical associated diseases. Based on the polyphasic study of MUSC 149^T, the strain merits assignment to a novel species, for which the name *S. mangrovisoli* sp. nov. is proposed. The type strain is MUSC 149^T (=MCCC 1K00699^T=DSM 100438^T).

Keywords: *Streptomyces mangrovisoli*, novel taxa, antioxidant, DPPH, mangrove

Introduction

Oxidative stress has been implicated in physiological aging which may contribute to the development of chronic diseases. The disequilibrium of oxidation status has been associated with development of neurodegenerative diseases which includes Parkinson's disease and Alzheimer's disease (Floyd and Hensley, 2002; Farooqui and Farooqui, 2009). In fact, oxidative stress is recognized to play a critical role in carcinogenesis as well. It is plausible that the accumulation of free radicals results in various modifications or damages to biological macromolecules such as protein, lipid, and DNA (Reuter et al., 2010). These unwanted, harmful effects then expedite DNA mutation and increase cancer risks. Therefore, the discovery of the antioxidants from natural resources has always sparked great interest of researchers (Lee et al., 1998).

The mangrove is an exclusive woody plant area of intertidal coasts in tropical and subtropical coastal regions. This ecosystem is among the world's most prolific environments and produces commercial forest products, protects coastlines and supports coastal fisheries. Mangrove ecosystems are habitats of various flora and fauna of marine, freshwater and terrestrial species (Jennerjahn and Ittekkot, 2002). Recently, there has been increasing interest in exploitation of mangrove microorganism resources as the constant changes in factors such as salinity and tidal gradient in the mangrove ecosystems are consideration to be driving forces for metabolic pathway adaptations that could direct to the production of valuable metabolites (Hong et al., 2009; Lee et al., 2014a). Lately, numerous studies have discovered novel actinobacteria from the different mangrove environments globally, such as the isolation of *Streptomyces avicenniae* (Xiao et al., 2009), *S. xiamenensis* (Xu et al., 2009), *S. sanyensis* (Sui et al., 2011), *S. qinglanensis* (Hu et al., 2012), *S. pluripotens* (Lee et al., 2014b), and *S. gilvigriseus* (Ser et al., 2015).

Waksman and Henrici (1943) had proposed the genus *Streptomyces*; the genus *Streptomyces* is comprised of ca. 600 species with validly published names (http://www.bacterio.cict.fr/) at the time of writing (May 2015). Many members of this genus have made vital contributions to mankind due to their capabilities to produce various natural products (Berdy, 2005). These *Streptomyces*-derived secondary metabolites have attracted much attention from the community as they possess diverse bioactivities such as antibacterial, antifungal, antitumor, and antioxidant (Kaneko et al., 1989; Kim et al., 2008; Olano et al., 2009a; Saurav and Kannabiran, 2012; Thenmozhi and Kannabiran, 2012; Wang et al., 2013; Kumar et al., 2014; Khieu et al., 2015). Notably, some of the bioactivities described were associated with production of cyclic compounds such as cyclomarins and pyrrolizidines (Renner et al., 1999; Karanja et al., 2010; Fu and MacMillan, 2015).

In this study, this particular strain of *Streptomyces* was isolated from a mangrove soil located from the Tanjung Lumpur mangrove forest located in east coast of Peninsular Malaysia. With the polyphasic approach, it is revealed that MUSC 149^T represents a novel species of the *Streptomyces* genus, for which the name *S. mangrovisoli* sp. nov. is proposed. In our very initial attempt to explore the potential biological activity possessed by MUSC149^T, antioxidant activity was examined. The result indicated that MUSC149^T extract exhibited a significant antioxidant property. To the best of our knowledge, the antioxidant activity of MUSC149^T has hitherto not been reported. The chemical analysis was then conducted to identify the chemical constituents present in the extract of MUSC149^T. The outcomes derived from this research have provided a strong foundation for further in depth biological studies to be performed particularly focusing on free-radical associated diseases.

Materials and Methods

Isolation and Maintenance of Isolate

Strain MUSC 149^T was isolated from a soil sample collected at site MUSC-TLS1 (3° 48′ 3.2″ N 103° 20′ 11.0″ E), located in the mangrove forest of Tanjung Lumpur in the state of Pahang, Peninsular Malaysia, in December 2012. Topsoil samples of the upper 20-cm layer (after removing the top 2–3 cm) were collected and sampled into sterile plastic bags using an aseptic metal trowel, and stored at –20°C. Air-dried soil samples were ground with a mortar and pestle. Selective pretreatment of soil samples was performed using wet heat in sterilized water (15 min at 50°C; Takahashi et al., 1996). Five grams of the pretreated air-dried soil was mixed with 45 ml sterilized water and mill ground, spread onto the isolation medium ISP 2 (Shirling and Gottlieb, 1966) supplemented with cycloheximide (25 μg ml^{-1}) and nystatin (10 μg ml^{-1}), and incubated at 28°C for 14 days. Pure cultures of strain MUSC 149^T were isolated and maintained on slants of ISP 2 agar at 28°C and as glycerol suspensions (20%, v/v) at –20°C for long term preservation.

Genomic and Phylogenetic Analyses

The extraction of genomic DNA for PCR was performed as described by Hong et al. (2009). In short, approximately 0.5 g of each culture was suspended in TE buffer (0.5 ml) and ribolised for 30 s at a speed of 5.5 m/s following the addition of sterile glass beads (0.5 g, 100 mesh). The resultant preparations were extracted with an equal volume of chloroform: *iso*-amyl alcohol (24:1, v/v) and centrifuged at 15,000 g for 5 min at 4°C. The upper aqueous layers, which contained the DNA, were transferred to fresh tubes and used as template DNA. The amplification of 16S rRNA gene was performed according to Lee et al. (2014b). Briefly the PCR reactions were performed in a final volume of 50 μl according to protocol of SolGent^{TM} 2X Taq PLUS PCR Smart mix using the Kyratex PCR Supercycler (Kyratec, Australia) with the following cycling conditions: (i) 95°C for 5 min, (ii) 35 cycles of 94°C for 50 s, 55°C for 1 min and 72°C for 1 min 30 s; and (iii) 72°C for 8 min. The 16S rRNA gene sequence of strain MUSC 149^T was aligned with representative sequences of related type strains of the genus *Streptomyces* retrieved from the GenBank/EMBL/DDBJ databases using CLUSTAL-X software (Thompson et al., 1997). The alignment was verified manually and then used to generate phylogenetic tree. Phylogenetic trees were constructed with the maximum-likelihood (Felsenstein, 1981) (**Figure 1**) and neighbor-joining (Saitou and Nei, 1987) (Supplementary Figure S1) algorithms using MEGA version 5.2 (Tamura et al., 2011). Evolutionary distances for the neighbor-joining algorithm were computed using Kimura's two-parameter model (Kimura, 1980). The EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012) was used for calculations of sequence similarity. The stability of the resultant trees topologies were evaluated by using the bootstrap based on 1000 resampling method of Felsenstein (1985).

BOX-PCR fingerprint analysis was used to characterize strain MUSC 149^T and the closely related strains using the primer BOX-A1R (5′-CTACGGCAAGGCGACGCTGACG-3′) (Versalovic et al., 1991; Lee et al., 2014c). The BOX-PCR cycling parameters were 5 min at 94°C for pre-denaturation, 35 cycles each of 30 s at 94°C for denaturation, 30 s at 53°C for annealing, 7 min at 65°C for extension and a final extension at 65°C for 8 min (Lee et al., 2014d). The PCR products were visualized by 2% agarose gel electrophoresis.

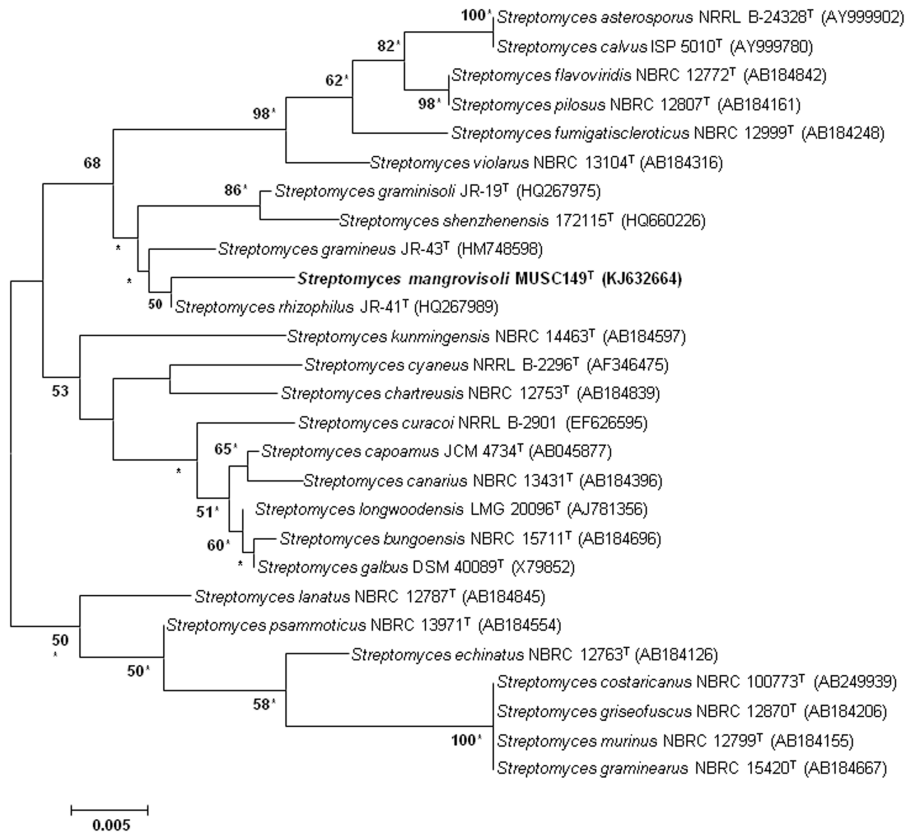

FIGURE 1 | Maximum-likelihood phylogenetic tree based on 1487 nucleotides of 16S rRNA gene sequence showing the relationship between strain MUSC 149^T and representatives of related taxa. Numbers at nodes indicate percentages of 1000 bootstrap re-samplings, only values above 50% are shown. Bar, 0.005 substitutions per site. Asterisks indicate that the corresponding nodes were also recovered using the neighbor-joining tree-making algorithm.

The protocol of Cashion et al. (1977) was used for the extraction of genomic DNA for DNA-DNA hybridization of strain MUSC 149^T, *S. graminisoli* NBRC 108883^T, *S. gramineus* NBRC 107863^T and *S. rhizophilus* NBRC 108885^T. DNA–DNA hybridization was carried out by the Identification Service of the DSMZ, Braunschweig, Germany following the protocol of De Ley et al. (1970) under consideration of the modifications described by Huss et al. (1983). The G + C content of strain MUSC 149^T was determined by HPLC (Mesbah et al., 1989).

Phenotypic Characteristics

The cultural characteristics of strain MUSC 149^T were determined following growth on ISP 2, ISP 3, ISP 4, ISP 5, ISP 6, ISP 7 (Shirling and Gottlieb, 1966), actinomycetes isolation agar (AIA; Atlas, 1993), *Streptomyces* agar (SA; Atlas, 1993), starch casein agar (SCA; Küster and Williams, 1964), and nutrient agar (Macfaddin, 2000) for 14 days at 28°C. Light microscopy (80i, Nikon) and scanning electron microscopy (JEOL-JSM 6400) were used to observe the morphology of the strain after incubation on ISP 2 agar at 28°C for 7–14 days (**Figure 2**). The designation of colony color was determined by using the *ISCC-NBS* color charts (Kelly, 1964). Gram staining was performed by standard Gram reaction and confirmed by using KOH lysis (Cerny, 1978). The growth temperature range was tested at 4-40 °C at intervals of 4

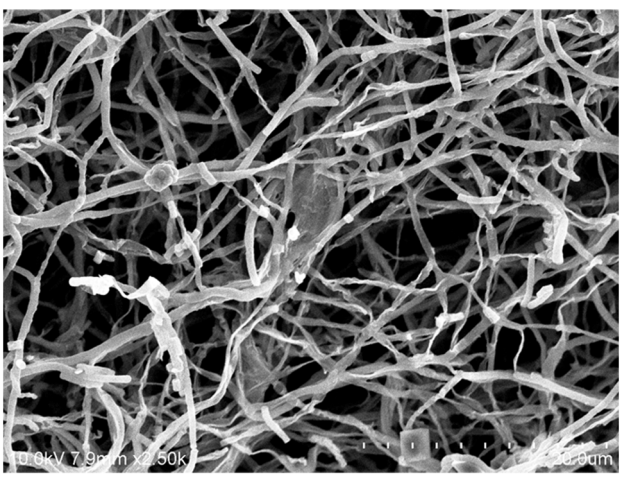

FIGURE 2 | Scanning electron microscope of *Streptomyces mangrovisoli* MUSC 149^T.

°C on ISP 2 agar. The pH range for growth was tested in tryptic soy broth (TSB) between pH 2.0 and 10.0 at intervals of 1 pH unit. The NaCl tolerance was tested in TSB and salt concentrations ranging from 0 to 10% (w/v) at intervals of 2%. The responses to temperature, pH and NaCl were observed for 14 days. Catalase activity and production of melanoid pigments were determined following protocols described by Lee et al. (2014e). The production of melanoid pigments was examined using ISP 7 medium. Hemolytic activity was assessed on blood agar medium containing 5% (w/v) peptone, 3% (w/v) yeast extract, 5% (w/v) NaCl, and 5% (v/v) horse blood (Carrillo et al., 1996). The plates were examined for hemolysis after incubation at 28°C for 7–14 days. Amylolytic, cellulase, chitinase, lipase, protease, and xylanase activities were determined by growing cells on ISP 2 agar and following protocols as described by Meena et al. (2013). The presence of clear zones around the colonies was taken to indicate the potential of isolates for surfactant production. Antibiotic susceptibility tests were performed by the disk diffusion method as described by Shieh et al. (2003). Antimicrobials used and their concentrations per disk (Oxoid, Basingstoke, UK) were as follows: ampicillin (10 μg), ampicillin sulbactam (30 μg), cefotaxime (30 μg), cefuroxime (30 μg), cephalosporin (30 μg), chloramphenicol (30 μg), ciprofloxacin (10 μg), erythromycin (15 μg), gentamicin (20 μg), nalidixic acid (30 μg), Penicillin G (10 μg), streptomycin (10 μg), tetracycline (30 μg), and vancomycin (30 μg). Carbon-source utilization and chemical sensitivity assays were determined using Biolog GenIII MicroPlates (Biolog, USA) according to the manufacturer's instructions. All of the phenotypic assays mentioned were performed concurrently for strain MUSC 149^T, *S. graminisoli* NBRC 108883^T, *S. gramineus* NBRC 107863^T, and *S. rhizophilus* NBRC 108885^T.

Chemotaxonomic Characteristics

The analyses of peptidoglycan amino acid composition and sugars of strain MUSC 149^T were carried out by the Identification Service of the DSMZ using protocols of Schumann (2011). Major diagnostic cell wall sugars of strain MUSC 149^T were obtained as described by Whiton et al. (1985) and analyzed by TLC on cellulose plates (Staneck and Roberts, 1974). Analysis of respiratory quinones, polar lipids (Kates, 1986) and fatty acids (Sasser, 1990) were carried out by the Identification Service of the DSMZ.

Extract preparation of MUSC 149^T

MUSC 149^T was grown in TSB for 14 days prior to fermentation process. The fermentation medium used was FM3 (Hong et al., 2009; Lee et al., 2012a). The medium was autoclaved at 121°C for 15 min prior to experiment. Fermentation was carried out in test tubes (30 mm × 200mm) containing 20 mL of FM3, at an angle of 45° for 7–10 days at 28°C. The resulting FM3 medium was recovered by centrifugation at 12000 *g* for 15 min. The supernatant was filtered and subjected to freeze dry process. Upon freeze-drying, the sample was extracted with methanol for 72 h and the methanol-containing extract was filtered and collected. The residue was re-extracted under the same condition twice at 24 h interval. Subsequently, the methanol-containing extract was evaporated using rotary vacuum evaporator at 40°C. The extract of MUSC 149^T was collected and suspended in dimethyl sulphoxide (DMSO) as vehicle reagent prior to assay.

Determination of Antioxidant Activity of MUSC 149^T Extract using 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Method

The stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma–Aldrich) was used to examine antioxidant activity by measuring its hydrogen donating or radical scavenging ability. Scavenging activity on DPPH free radicals by MUSC 149^T extract was accessed following previous method with minor modification (Ling et al., 2009). The decrease in radical is measured as decrease in the absorbance of 515 nm. Volume of 195 μL of 0.016% DPPH ethanolic solution was added to 5 μL of extract solution to make up final volume of 200 μL. Gallic acid was included as positive control. Reactions were carried out at room temperature in dark for 20 min before measurement with spectrophotometer at 515 nm. DPPH scavenging activity was calculated as follows:

$$\text{DPPH scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

Gas chromatography–mass spectrometry (GC–MS) analysis was performed in accordance with our previous developed method with slight modification (Supriady et al., 2015). The machine used was Agilent Technologies 6980N (GC) equipped with 5979 Mass Selective Detector (MS), HP-5MS (5% phenyl methyl siloxane) capillary column of dimensions 30.0 m × 250 μm × 0.25 μm and used helium as carrier gas at 1 mL/min. The column temperature was programmed initially at 40°C for 10 min, followed by an increase of 3°C/min to 250°C and was kept isothermally for 5 min. The MS was operating at 70 eV. The constituents were identified by comparison of their mass spectral data with those from NIST 05 Spectral Library.

Results and Discussion

Phenotypic, Phylogenetic, and Genomic Analyses

Strain MUSC 149^T was observed to grow well on ISP 2, ISP 3, ISP 5, ISP 6, ISP 7 agar, actinomycetes isolation agar, starch casein agar, and nutrient agar after 7–14 days at 28°C, and to grow poorly on *Streptomyces* agar, and did not grow on ISP 4 medium. The colors of the aerial and substrate mycelium were media-dependent (Supplementary Table S1). The morphological observation of a 15-day-old culture grown on ISP 2 agar revealed

TABLE 1 | Differentiation characteristics of strain MUSC 149^T and type strains of phylogenetically closely related species of the genus *Streptomyces*.

Characteristics	1	2	3	4
Morphology (on ISP 2)				
Color of aerial mycelium	Pale yellow	Grayish yellow	Yellowish white	Light greenish yellow
Color of substrate mycelium	Grayish yellow	Grayish yellow	Pale orange yellow	Grayish yellow
Growth at				
24°C	(+)	+	+	(+)
36°C	(+)	(+)	(+)	+
pH 5	(+)	(+)	(+)$^\gamma$	(+)
pH 8	(+)	+	(+)$^\gamma$	(+)
4% NaCl	(+)	+	(+)$^\gamma$	(+)
Catalase	+	+	+	−
Hemolytic	−	−	−	−
Hydrolysis of				
Casein (protease)	−	−	+	−
Tributyrin (lipase)	−	−	+	+
Starch (amylolytic)	−	+	+$^\gamma$	+
Carboxymethylcellulose (cellulase)	+	+	−	+
Xylan (xylanase)	−	−	−	+
Carbon source utilization				
D-trehalose	+	−	+	−
D-cellobiose	+	−	+	+
α-D-lactose	+	−	+	−
β-methyl-D-glucoside	+	−	−	+
N-acetyl-β-D-mannosamine	+	−	−	−
N-acetyl-D-galactosamine	+	−	−	−
N-acetyl-neuraminic acid	+	−	−	−
D-mannose	−	+	+	+
3-methyl glucose	−	+	−	−
Inosine	+	−	+	−
D-mannitol	+	−	+	+
D-serine	−	+	−	−
Glycyl-L-proline	−	+	+	+
L-alanine	+	−	+	+
L-arginine	−	+	+	+
L-pyroglutamic acid	−	+	+	+
D-gluconic acid	−	+	+	+
Mucic acid	+	−	+	−
Quinic acid	−	−	+	+
D-saccharic acid	−	+	+	−
D-lactic acid methyl ester	−	+	+	+
D-malic acid	+	−	+	+
Chemical sensitivity assays				
Troleandomycin	+	+	−	−
Lithium chloride	+	+	−	−

Strains: 1, S. mangrovisoli sp. nov. MUSC 149^T; 2, S. rhizophilus NBRC 108885^T; 3, S. gramineus NBRC 107863^T; 4, S. graminisoli NBRC 108883^T. All data were obtained concurrently in this study. +, Positive; –, negative; (+), weak. All strains are positive for utilization of Dextrin, D-*maltose, gentiobiose,* D-*melibiose,* α-D-*glucose,* D-*fructose,* D-*galactose,* L-*fucose,* L-*rhamnose, gelatine,* L-*serine, pectin, p-hydroxy-phenylacetic acid, methyl pyruvate,* L-*malic acid, bromo-succinic acid, tween 40,* γ*-amino-butyric acid,* α*- hydroxy-butyric acid,* β*-hydroxy-*D,L*-butyric acid and* α*-keto-butyric acid. S. graminisoli NBRC 108883^T.*
$^\gamma$*Results in accordance with that published for S. gramineus NBRC 107863^T by Lee et al. (2012b).*

a smooth spore surface and abundant growth of both aerial and vegetative hyphae, which were well developed and not fragmented. These morphological features are consistent with grouping of the strain to the genus *Streptomyces* (Williams et al., 1989). Growth occurred at pH 5.0–8.0 (optimum pH 6.0–7.0), with 0–4% NaCl tolerance (optimum 0–2%) and at 24–36°C (optimum 28–32°C). Cells were found to be positive for catalase but negative for both melanoid pigment production and hemolytic activity. Hydrolysis of carboxymethylcellulose was found to be positive, but negative for hydrolysis of casein, chitin,

soluble starch, tributyrin (lipase), and xylan. Strain MUSC 149^T can be differentiated from closely related members of the genus *Streptomyces* using a range of phenotypic properties (**Table 1**). In chemical sensitivity assays, cells are resistant to aztreonam, D-serine, fusidic acid, guanine HCl, lincomycin, lithium chloride, minocycline, nalidixic acid, niaproof 4, potassium tellurite, rifamycin RV, sodium bromate, sodium butyrate, 1% sodium lactate, tetrazolium blue, tetrazolium violet, troleandomycin, and vancomycin.

The nearly complete 16S rRNA gene sequence was obtained for strain MUSC 149^T (1487 bp; GenBank/EMBL/DDBJ accession number KJ632664) and phylogenetic trees were reconstructed to determine the phylogenetic position of this strain (**Figure 1**; Supplementary Figure S1). Phylogenetic analysis exhibited that strain MUSC 149^T is closely related to *S. rhizophilus* JR-41^T, as they formed a distinct clade (**Figure 1**; Supplementary Figure S1). The type strain *S. rhizophilus* JR-41^T was isolated from a bamboo (*Sasa borealis*) rhizosphere soil (Lee and Whang, 2014). The 16S rRNA gene sequence analysis of strain MUSC 149^T showed the highest similarity to that of *S. rhizophilus* NBRC 108885^T (99.2% sequence similarity), followed by *S. gramineus* NBRC 107863^T (98.7%) and *S. graminisoli* NBRC 108883^T (98.5%); sequences similarities of less than 98.3% were obtained with the type strains of other species of the genus *Streptomyces*. The DNA–DNA hybridization values between strain MUSC 149^T and *S. rhizophilus* NBRC 108885^T (12.4 ± 3.3%), followed by *S. gramineus* NBRC 107863^T (13.7 ± 0.5%) and *S. graminisoli* NBRC 108883^T (27.3 ± 1.9%) were significantly below 70%, the threshold value for the delineation of bacterial species (Wayne et al., 1987). The BOX-PCR results indicated that strain MUSC 149^T yielded a unique BOX-PCR fingerprint compared with the closely related type strains (Supplementary Figure S2). These results are in agreement with results of DNA–DNA hybridizations, which indicate that strain MUSC 149^T represents a novel species.

Chemotaxonomic Analyses

Chemotaxonomic analyses showed that the cell wall of strain MUSC 149^T is of cell-wall type I (Lechevalier and Lechevalier, 1970) as it contains LL-diaminopimelic. The presence of LL-diaminopimelic has been observed in many other species of the genus *Streptomyces* (Lee et al., 2005, 2014b; Xu et al., 2009; Hu et al., 2012; Ser et al., 2015). The predominant menaquinones of strain MUSC 149^T were identified as MK-9(H_8) (59%) and

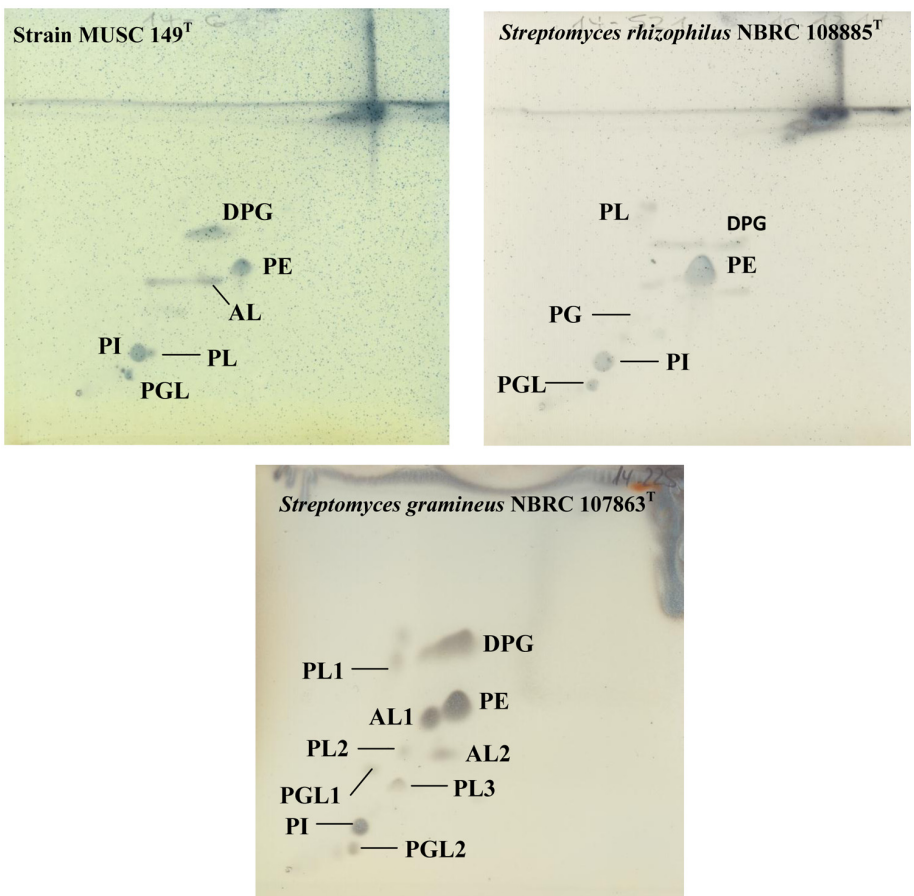

FIGURE 3 | Two dimensional total lipid profile of strain MUSC 149^T, *S. rhizophilus* NBRC 108885^T and *S. gramineus* NBRC 107863^T. AL, Aminolipid; DPG, Diphosphatidylglycerol; PL, Phospholipid; PI, Phosphatidylinositol; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PGL, Phosphoglycolipid.

MK-9(H_6) (15%). This is in agreement with Kim et al. (2003) that the predominant menaquinones of members of the genus *Streptomyces* are MK-9(H_6) and MK-9(H_8). The cell wall sugars detected were glucose, mannose and ribose. Strain MUSC149^T shared the same sugar profile with *S. gilvigriseus* (Ser et al., 2015). Furthermore the sugars glucose and ribose were detected in other members of the genus *Streptomyces* such as *S. rhizophilus* JR-41^T, *S. graminisoli* JR-19^T (Lee and Whang, 2014), *S. gramineus* JR-43^T (Lee et al., 2012b), *S. shenzhenensis* 172115^T (Hu et al., 2011), and *S. pluripotens* (Lee et al., 2014b). The G + C content of strain MUSC 149^T was determined to be 72.7 mol%; this is within the range of 67.0–78.0 mol% described for species of the genus *Streptomyces* (Kim et al., 2003).

The polar lipid analysis showed the presence of aminolipid, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphoglycolipid, and phospholipid (**Figure 3**). Differences in polar lipid profiles indicated that MUSC 149^T is different from related type strains (**Figure 3**); for example, strain MUSC 149^T was found to contain aminolipid, lipid that was not detected in *S. rhizophilus* NBRC 108885^T (**Figure 3**). The fatty acids profiles of strain MUSC 149^T and closely related type strains are given shown in **Table 2**.

The major cellular fatty acids in MUSC 149^T were identified as anteiso-$C_{15:0}$ (26.2%), iso-$C_{15:0}$ (17.7%), iso-$C_{16:0}$ (16.0%) and anteiso-$C_{17:0}$ (11.3%). The fatty acids profile of MUSC 149^T is consistent with those of closely related phylogenetic neighbors such as *S. rhizophilus* NBRC 108885^T, *S. gramineus* NBRC 107863^T, and *S. graminisoli* NBRC 108883^T, which contain anteiso-$C_{15:0}$ (26.5–17.5%), iso-$C_{16:0}$ (25.1–15.4%), and iso-$C_{15:0}$ (18.3–12.5%) as their major fatty acids (**Table 2**). However, the fatty acid profile of MUSC 149^T was quantitatively different from those of these type strains; for example, although anteiso-$C_{15:0}$ (26.2%) was found to be predominant in strain MUSC 149^T, the amount of anteiso-$C_{15:0}$ was significantly lesser (17.5%) in *S. graminisoli* NBRC 108883^T (**Table 2**).

Based on the results of DNA-DNA hybridization, phylogenetic analysis, chemotaxonomic, phenotypic and DNA fingerprinting, strain MUSC 149^T merits assignment to a novel species in the genus *Streptomyces*, for which the name *S. mangrovisoli* sp. nov. is proposed.

Antioxidant Activity of MUSC 149^T Extract

The antioxidant evaluation assay DPPH is based upon the reduction of DPPH free radical. It is widely used to determine free radical scavenging capacity of the tested samples (Blois, 1958; Molyneux, 2004). As a free radical, DPPH is observed as purple solution when dissolved in appropriate solvent. It is known to exhibit a high absorption at 515 nm when measured with visible spectroscopy. In the presence of free radical-scavenging agent(s) or hydrogen donor(s), the odd electron of DPPH will be paired off, it will subsequently result in discoloration of solution to become either yellowish or colorless. The strength of the radical scavenging or anti-oxidant activity can then be quantified by the difference of absorbance obtained with the samples when is comparing to control.

The DPPH scavenging assay was employed to examine the antioxidant activity of MUSC 149^T extract. The extract was tested for a dose-response study with five different concentrations (0.125, 0.25, 0.5, 1.0, and 2.0 mg/mL). Based on the results obtained, the extract of MUSC 149^T displayed a dose-dependent manner of antioxidant activity. It was inferred by a gradual increase in scavenging activity of MUSC 149^T extract with a low concentration of extract at 0.125 mg/mL to the highest concentration at 2.0 mg/mL. The scavenging activity of lowest concentration at 0.125 mg/mL and the highest concentration at 2.0 mg/mL was recorded at 1.1 ± 1.4% and 36.5 ± 3.0%, respectively (**Figure 4**). The ability of MUSC 149^T extract to scavenge DPPH free radicals indicates the possible presence of antioxidant agent(s) in the tested MUSC 149^T extract.

TABLE 2 | Cellular fatty acid composition of strain MUSC 149^T and its closely related *Streptomyces* species.

Fatty acid	1	2	3	4
iso-$C_{12:0}$	0.1	–	–	0.1
$C_{12:0}$	0.1	–	–	0.1
iso-$C_{13:0}$	0.4	0.6	0.2	0.4
anteiso-$C_{13:0}$	0.5	0.3	0.2	0.3
iso-$C_{14:0}$	2.9	3.5	4.3	5.7
$C_{14:0}$	0.6	0.5	0.2	1.1
iso-$C_{15:0}$	17.7	18.3	19.1	12.5
anteiso-$C_{15:0}$	26.2	26.5	21.4	17.5
$C_{15:0}$w6c	–	–	–	0.3
$C_{15:0}$	1.8	1.5	–	2.1
iso-$C_{16:1}$ H	1.9	–	1.3	1.7
iso-$C_{16:0}$	16.0	15.4	19.2	25.1
$C_{16:1}$Cis9	2.8	–	–	–
$C_{16:0}$	4.0	9.4	4.3	7.9
iso-$C_{17:1}$w9c	–	1.3	5.0	3.1
anteiso-$C_{17:1}$w9c	2.4	0.6	1.9	1.9
iso-$C_{17:0}$	6.1	10.3	10.4	5.0
anteiso-$C_{17:0}$	11.3	10.7	9.6	9.2
$C_{17:1}$w8c	–	–	0.4	0.4
$C_{17:0}$ CYCLO	0.4	–	0.5	0.6
$C_{17:0}$	0.3	0.7	0.7	0.5

Strains: 1, S. mangrovisoli sp. nov. MUSC 149^T; 2, S. rhizophilus NBRC 108885^T; 3, S. gramineus NBRC 107863^T; 4, S. graminisoli NBRC 108883^T. –, <0.1% or not detected. All data are obtained concurrently from this study.

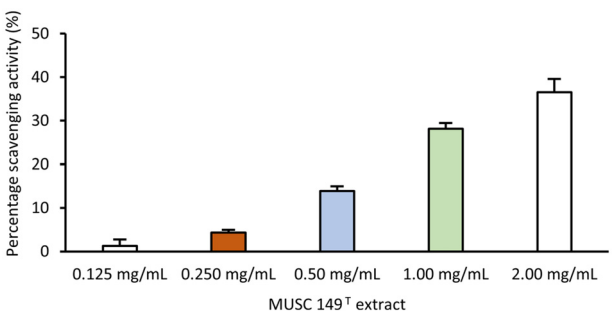

FIGURE 4 | Antioxidant activity of MUSC 149^T methanolic extract. Antioxidant activity of MUSC 149^T was evaluated at different concentration and values are SEM of four replicates.

TABLE 3 | Compounds identified from MUSC 149^T extract through Gas chromatography–mass spectrometry (GC–MS).

No.	Retention time	Compound	Formula	Molecular weight	Similarity (%)
1	5.913	Hexadecane, 1,1-bis(dodecyloxy)	$C_{40}H_{82}O_2$	595	64
2	9.753	Butanoic acid, 2-methyl-	$C_5H_{10}O_2$	102	74
3	33.499	Benzoic acid, 3-methyl-	$C_8H_8O_2$	136	90
4	51.535	(3R,8aS)-3-methyl-1,2,3,4,6,7,8,8a-octahydropyrrolo [1,2-a]pyrazine-1,4-dione	$C_8H_{12}N_2O_2$	168	90
5	52.994	Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-	$C_7H_{10}N_2O_2$	154	90

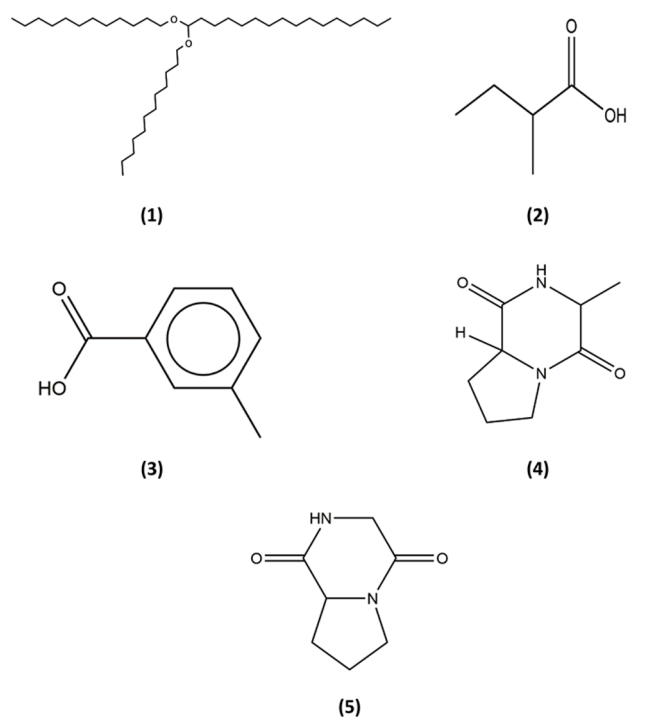

FIGURE 5 | Chemical structures of the identified compounds from MUSC 149^T. (1) Hexadecane, 1,1-bis(dodecyloxy); (2) Butanoic acid, 2-methyl-; (3) Benzoic acid, 3-methyl-; (4) (3R,8aS)-3-methyl-1,2,3,4,6,7,8,8a-octahydropyrrolo[1,2-a]pyrazine-1,4-dione; (5) Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-.

GC–MS Analysis of MUSC 149^T Methanolic Extract

Growing evidence implies that the accumulation of free radicals may contribute to pathogenesis of chronic diseases including Parkinson's disease and various types of cancers (Floyd and Hensley, 2002; Farooqui and Farooqui, 2009; Goldkorn et al., 2014; Mahalingaiah and Singh, 2014). Synthetic antioxidants may be able to scavenge these notorious free radicals, however, currently available antioxidants display low solubility and may promote negative health impacts (Barlow, 1990; Panicker et al., 2014). With this in mind, the search of the antioxidants from natural resources has always been one of the major focuses for many researchers (Lee et al., 1998; Harvey et al., 2015). In order to explore this premise, we examined the antioxidant activity of the extract of MUSC 149^T. The results obtained demonstrated that MUSC 149^T extract was posing significant antioxidant activity. This has prompted the necessities to further examine the chemical constituents which present in the extract of MUSC 149^T.

As *Streptomyces* are known to produce various secondary metabolites with diverse biological activity, numerous studies have incorporated powerful analytical techniques such as GC–MS to assist with the chemical analysis (Pollak and Berger, 1996; Karanja et al., 2010; Sudha and Masilamani, 2012; Ara et al., 2014; Jog et al., 2014). This robust technique produces reliable results as it combines separation power of GC and detection power of MS by generating characteristic mass spectral fragmentation patterns for each compounds present in mixture (Hites, 1997). For instance, recent study by Kim et al. (2008) has described detection of the bioactive compound (protocatechualdehyde) present in the extract of *S. lincolnensis* M-20 by using the GC–MS. With this intention, GC–MS analysis was performed in this study to explore the chemical constituents present in the extract of MUSC 149^T. Using this analytical technique, we have identified chemical constituents of the extract of MUSC 149^T (**Table 3**) and the chemical structures (**Figure 5**) as Hexadecane, 1,1-bis(dodecyloxy) (1), Butanoic acid, 2-methyl- (2), Benzoic acid, 3-methyl- (3) (3R,8aS)-3-methyl-1,2,3,4,6,7,8,8a-octahydropyrrolo[1,2-a]pyrazine-1,4-dione (4), and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (5).

The detection of heterocyclic organic compound in extract is deemed as one of the most important findings in current study. Pyrrolizidines are widely present or synthesized in several marine *Streptomyces* species (Olano et al., 2009b; Robertson and Stevens, 2014). Furthermore, pyrrolizidines are known to exhibit a wide range of bioactivities which including antitumor, anti-angiogenesis, and antioxidant activities. For instance, the detection of the compound known as pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (**Table 3**; **Figure 5**) in the extract has suggested the antioxidant activity could be contributed by this compound. Furthermore, other recent findings conducted on this compound suggested strong antioxidant activities as well (Gopi et al., 2014; Balakrishnan et al., 2015). These findings have demonstrated that pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- was able to scavenge or reduce amount of free radicals as evaluated by using reducing power assay. In short, an antioxidant is likely to play important roles in prevention and treatment of chronic diseases (Morales-González, 2013). The strong free radical scavenging effect possessed by the extract of MUSC 149^T warrants the future investigations into different type of biological activities.

Description of *S. mangrovisoli* sp. nov.

Streptomyces mangrovisoli sp. nov. (man.gro.vi.so′li. N.L. n. mangrovum, mangrove; L. gen. n. soli, of soil; N.L. gen. n. mangrovisoli, of mangrove soil, referring to the source of the inoculum).

Cells stain Gram-positive and form pale yellow aerial and grayish yellow substrate mycelium on ISP 2 agar. The colors of the aerial and substrate mycelium are media-dependent (Supplementary Table S1). Grows well on ISP 2, ISP 3, ISP 5, ISP 6, ISP 7 agar, actinomycetes isolation agar, starch casein agar, and nutrient agar after 1–2 weeks at 28°C; and to grow poorly on *Streptomyces* agar, whereas no growth on ISP 4 medium. Grows occur at pH 5.0–8.0 (optimum pH 6.0–7.0), with 0–4% NaCl tolerance (optimum 0–2%) and at 24–36°C (optimum 28–32°C). Cells are positive for catalase but negative for both melanoid pigment production and hemolytic activity. Carboxymethylcellulose is hydrolysed but negative for hydrolysis of casein, chitin, soluble starch, tributyrin (lipase), and xylan. The following compounds are utilized as sole carbon sources: acetic acid, acetoacetic acid, α-D-glucose, α-D-lactose, α-hydroxy-butyric acid, α-keto-butyric acid, α-keto-glutaric acid, β-hydroxyl-D,L-butyric acid, β-methyl-D-glucoside, bromo-succinic acid, citric acid, D-cellobiose, Dextrin, D-fructose, D-fructose-6-phosphate, D-fucose, D-galactose, D-galacturonic acid, D-gluconic acid, D-glucose-6-phosphate, D-glucuronic acid, D-lactic acid methyl ester, D-malic acid, D-maltose, D-mannitol, D-melibiose, D-raffinose, D-saccharic acid, D-sorbitol, D-trehalose, D-turanose, formic acid, gelatin, gentiobiose, glucuronamide, inosine, L-fucose, L-galactonic acid lactone, L-lactic acid, L-malic acid, L-rhamnose, methyl pyruvate, mucic acid, *N*-acetyl-β-D-mannosamine, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-neuraminic acid, pectin, *p*-hydroxyl-phenylacetic acid, propionic acid, quinic acid, stachyose, sucrose, Tween 40, and γ-amino-butyric acid. The following compounds are not utilized as sole carbon sources: D-salicin, D-mannose, D-arabitol, myo-inositol, glycerol, D-aspartic acid, D-serine, glycyl-L-proline, and 3-methyl glucose. L-alanine, L-histidine, and L-serine are utilized as sole nitrogen sources. L-arginine, L-aspartic acid, L-glutamic acid, and L-pyroglutamic acid are not utilized as sole nitrogen sources. Extract of the type strain exhibits strong antioxidant activity in a dose-dependent manner. The G + C content of the genomic DNA of the type strain is 72.7 mol%.

The type strain is MUSC 149^T (=MCCC 1K00699^T=DSM 100438^T), isolated from mangrove soil collected from the Tanjung Lumpur mangrove forest located in the state of Pahang, Peninsular Malaysia. The 16S rRNA gene sequence of strain MUSC 149^T has been deposited in GenBank/EMBL/DDBJ under the accession number KJ632664.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00854

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Investigation of Antioxidative and Anticancer Potentials of *Streptomyces* sp. MUM256 Isolated from Malaysia Mangrove Soil

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A *Streptomyces* strain, MUM256 was isolated from Tanjung Lumpur mangrove soil in Malaysia. Characterization of the strain showed that it has properties consistent with those of the members of the genus *Streptomyces*. In order to explore the potential bioactivities, extract of the fermented broth culture of MUM256 was prepared with organic solvent extraction method. DPPH and SOD activity were utilized to examine the antioxidant capacity and the results have revealed the potency of MUM256 in superoxide anion scavenging activity in dose-dependent manner. The cytotoxicity of MUM256 extract was determined using cell viability assay against 8 different panels of human cancer cell lines. Among all the tested cancer cells, HCT116 was the most sensitive toward the extract treatment. At the highest concentration of tested extract, the result showed 2.3-, 2.0-, and 1.8-folds higher inhibitory effect against HCT116, HT29, and Caco-2 respectively when compared to normal cell line. This result has demonstrated that MUM256 extract was selectively cytotoxic toward colon cancer cell lines. In order to determine the constituents responsible for its bioactivities, the extract was then subjected to chemical analysis using GC-MS. The analysis resulted in the identification of chemical constituents including phenolic and pyrrolopyrazine compounds which may responsible for antioxidant and anticancer activities observed. Based on the findings of this study, the presence of bioactive constituents in MUM256 extract could be a potential source for the development of antioxidative and chemopreventive agents.

Keywords: ***Streptomyces sp.*, antioxidant, anticancer, Malaysia, mangrove**

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INTRODUCTION

Cancer is a common cause of mortality in the world population. Recently, American Cancer Society has reported that cancer as the second leading cause of death is expected to surpass cardiovascular disease in a few year times (Siegel et al., 2015). Furthermore, the incidence of the development of resistance to chemotherapy has become a major health problem (Riganti et al., 2015). This issue is more serious in economically less developed countries due to the lack of accessibility to standard diagnostic facilities and high cost of treatment (Jemal et al., 2010). Thus, there is

an urgent need to search for alternative anticancer agents which may overcome the failure of chemotherapy. Free radicals are known to be the major etiology of a number of diseases such as coronary heart disease, degenerative diseases and cancer (Devasagayam et al., 2004). Although oxidation is an important biological process for energy generation in living organisms, the excessive free radical production and low antioxidant defense lead to oxidative stress which is detrimental to cells and also strongly associated with cancer development involving oxidative DNA damage. Due to the destructive role of free oxygen radicals, there are several cellular mechanisms involve in the eradication of the free radicals including the enzymatic conversion of reactive oxygen species (ROS, H_2O_2, $O_2^-\bullet$, and $\bullet OH^-$) into less reactive species, chelation by transition metal catalysts as well as detoxification of ROS by antioxidants (Valko et al., 2006). Many synthetic antioxidant such as butylated hydroxycanisole, butylated hydroxytoluene and propyl gallate have been developed in order to retard oxidation process and prevent the progression of diseases caused by ROS (Maxwell, 1995). However, these synthetic antioxidative compounds which exhibited strong radical scavenging activity have been reported to cause severe side effects (Baardseth, 1989; Tepe et al., 2005). Thus, alternative antioxidants from natural sources are more preferable and many recent studies have shown that besides plants as rich source of antioxidants (Wong et al., 2012; Tan et al., 2015), microorganisms can be used for the production of natural antioxidants. Recently, many studies reported that mangrove *Streptomyces* produced antioxidative agents (Rao and Rao, 2013; Ser et al., 2015a).

The intertidal coasts in the tropical and subtropical coastal regions consist of an exclusive woody plant area known as the mangrove area. The mangrove ecosystem is among the world's most prolific environments and produces commercial forest products, supports coastal fisheries and protects the coastlines. These ecosystems are favorable habitats of a variety of flora and fauna of marine, freshwater and terrestrial species (Jennerjahn and Ittekkot, 2002). Factors such as salinity and tidal gradient in the mangrove systems are considered as some of the driving forces for metabolic pathway adaptations that could direct to the production of valuable metabolites (Hong et al., 2009; Lee et al., 2014d). Therefore, in recent years, there has been increasing interest in exploitation of mangrove microorganism resources. Furthermore, many researchers have successfully discovered novel actinobacteria strains from mangrove environments across the earth, such as the isolation of *Streptomyces avicenniae* (Xiao et al., 2009), *Streptomyces xiamenensis* (Xu et al., 2009), *Streptomyces sanyensis* (Sui et al., 2011), *Streptomyces qinglanensis* (Hu et al., 2012), *Streptomyces pluripotens* (Lee et al., 2014c), *Streptomyces mangrovisoli* (Ser et al., 2015a), and *Streptomyces gilvigriseus* (Ser et al., 2015b).

The genus *Streptomyces* was proposed by Waksman and Henrici (1943) and this genus is comprised of ca. 600 species with validly published names (http://www.bacterio.cict.fr/) at the time of writing (August 2015). Many members of *Streptomyces* have made imperative contributions to human with their capabilities to produce various important natural products (Bérdy, 2005). To date, numerous bioactive compounds with profound impact on society have been reported from the genus *Streptomyces* whereby over 7000 bioactive compounds with diverse bioactivities including antimicrobial, antioxidant, anticancer and antifungals properties are identified from *Streptomyces*. Beyond the well-known antibiotics from *Streptomyces*, such as streptomycin (Schatz et al., 1944) and erythromycin (Weber et al., 1985), many other medically useful agents include anticancer drugs such as doxorubicin (Grimm et al., 1994) and bleomycin (Du et al., 2000), the antifungal nystatin (Brautaset et al., 2000) are derived from *Streptomyces* as well. The unique and highly dynamic mangrove ecosystem is believed to exert significant influence on bacterial speciation for metabolic and physiological adaptations, consequently leading to the production of unique secondary metabolites with interesting bioactivities (Duncan et al., 2014; Lee et al., 2014d). Several previous studies on secondary metabolites from mangrove *Streptomyces* have documented a number of unique bioactive compounds. For instance, seven azlomycin F analogs, macrocyclic lactones, with anticancer and antibacterial properties were discovered recently from *Streptomyces* sp. 211726 isolated from mangrove rhizosphere soil (Yuan et al., 2013). Furthermore, benzonaphthyridine alkaloid was isolated from a mangrove-derived *S. albogriseolus* (Li et al., 2010). Fu and colleagues also revealed two indolocarbazoles, streptocarbazoles A and B with antitumor properties from *Streptomyces* sp. isolated from mangrove soil in Sanya, China (Fu et al., 2012).

In this study, *Streptomyces* sp. MUM256, isolated from soil at the Tanjung Lumpur mangrove forest, Peninsular Malaysia, was studied in the search of antioxidant and anticancer biological activities. The chemical constituents present in the extract of MUM256 were further characterized. The outcomes derived from this research constitute important starting points for performing further in depth biological studies focusing on free-radical associated diseases such as cancer.

MATERIALS AND METHODS

Isolation and Maintenance of Isolate

Strain MUM256 was isolated from a soil sample collected at site MUM-KS1 (3° 21′ 45.8″ N 101° 18′ 4.5″ E), located in the mangrove forest of Kuala Selangor in the state of Selangor, Peninsular Malaysia, in Jan 2015. Topsoil samples of the upper 20-cm layer (after removing the top 2–3 cm) were collected and sampled into sterile plastic bags using an aseptic metal trowel, and stored at −20°C. Air-dried soil samples were ground with a mortar and pestle. Selective pretreatment of soil samples was performed using wet heat in sterilized water (15 min at 50°C) (Takahashi et al., 1996). Five grams of the pretreated air-dried soil was mixed with 45 ml sterilized water and mill ground, spread onto the isolation medium ISP 2 (Shirling and Gottlieb, 1966) supplemented with cycloheximide (25 μg ml^{-1}) and nystatin (10 μg ml^{-1}), and incubated at 28°C for 14 days. Pure cultures of strain MUM256 were isolated and maintained on slants of ISP 2 agar at 28°C and as glycerol suspensions (20%, v/v) at −20°C for long term preservation.

Genomic and Phylogenetic Analyses

The extraction of genomic DNA for PCR was performed as described by Hong et al. (2009). The amplification of 16S rRNA

gene was performed according to Lee et al. (2014c). Briefly the PCR reactions were performed in a final volume of 50 μl according to protocol of SolGent™ 2X Taq PLUS PCR Smart mix using the Kyratex PCR Supercycler (Kyratec, Australia) with the following cycling conditions: (i) 95°C for 5 min, (ii) 35 cycles of 94°C for 50 s, 55°C for 1 min and 72°C for 1 min 30 s; and (iii) 72°C for 8 min. The 16S rRNA gene sequence of strain MUM256 was aligned with representative sequences of related type strains of the genus *Streptomyces* retrieved from the GenBank/EMBL/DDBJ databases using CLUSTAL-X software (Thompson et al., 1997). Phylogenetic trees were constructed with the neighbor-joining (Saitou and Nei, 1987; **Figure 1**) and maximum-likelihood (Felsenstein, 1981) and (Figure S1) algorithms using MEGA version 6.0 (Tamura et al., 2013). Evolutionary distances for the neighbor-joining algorithm were computed using Kimura's two-parameter model (Kimura, 1980). The EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012) was used for calculations of sequence similarity. The stability of the resultant trees topologies were evaluated by using the bootstrap based on 1000 resampling method of Felsenstein (1985).

Phenotypic Characteristics

The cultural characteristics of strain MUM256 were determined following growth on ISP 2, ISP 3, ISP 4, ISP 5, ISP 6, ISP 7 (Shirling and Gottlieb, 1966), actinomycetes isolation agar (AIA) (Atlas, 2010), starch casein agar (SCA) (Küster and Williams, 1964), and nutrient agar (Mac Faddin, 1976) for 14 days at 28°C. The light microscopy (80i, Nikon) was used to observe the morphology of the strain after incubation on ISP 2 agar at 28°C for 7–14 days. The Gram staining was performed by standard Gram reaction and confirmed by using KOH lysis (Cerny, 1978). The determination of colony color was done by using the ISCC-NBS color charts (Kelly, 1964). The growth temperature range was tested at 4–40°C at intervals of 4°C on ISP 2 agar. The NaCl tolerance was tested in tryptic soy broth (TSB) and salt concentrations ranging from 0 to 10% (w/v) at intervals of 2%. The pH range for growth was tested in TSB between pH 2.0 and 10.0 at intervals of 1 pH unit. The responses to temperature, pH and NaCl were observed for 14 days. The production of melanoid pigments and catalase activity were determined following protocols described by Lee et al. (2014b). The production of melanoid pigments was examined using ISP 7 medium. Hemolytic activity was assessed on blood agar medium containing 5% (w/v) peptone, 3% (w/v) yeast extract, 5% (w/v) NaCl, and 5% (v/v) horse blood (Carrillo et al., 1996). The plates were examined for hemolysis after incubation at 28°C for 7–14 days. Amylolytic, cellulase, chitinase, lipase, protease, and xylanase activities were determined by growing cells on ISP 2 agar and following protocols as described by Meena et al. (2013). The presence of clear zones around the colonies was taken to indicate the potential of isolates for surfactant production. Antibiotic susceptibility tests were performed by the disc diffusion method as described by Shieh et al. (2003). Antimicrobials used and their concentrations per disc (Oxoid, Basingstoke, UK) were as follows: ampicillin (10 μg), ampicillin sulbactam (30 μg), cefotaxime (30 μg), cefuroxime (30 μg), cephalosporin (30 μg), chloramphenicol (30 μg), ciprofloxacin (10 μg), erythromycin (15 μg), gentamicin (20 μg), nalidixic acid (30 μg), Penicillin G (10 μg), streptomycin (10 μg), tetracycline (30 μg), and vancomycin (30 μg).

Extract Preparation of MUM256

MUM256 was grown in TSB for 14 days prior to fermentation process. The fermentation medium used was FM3 (Hong et al., 2009; Lee et al., 2012). The medium was autoclaved at 121°C for 15 min prior to experiment. Fermentation was carried out in test tubes (30 × 200 mm) containing 20 mL of FM3, at an angle of 45° for 7–10 days at 28°C. The resulting FM3 medium was recovered by centrifugation at 12,000 g for 15 min. The supernatant was filtered and subjected to freeze dry process. Upon freeze-drying, the sample was extracted with methanol for 72 h and the methanol-containing extract was filtered and collected. The residue was re-extracted under the same condition twice at 24 h interval. Subsequently, the methanol-containing extract was evaporated using rotary vacuum evaporator at 40°C. The extract of MUM256 was collected and suspended in dimethyl sulphoxide (DMSO) as vehicle reagent prior to assay.

Antioxidant Activity

Free Radical Scavenging Activity Determination

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay was performed to determine the antioxidant activity by measuring the hydrogen donating or radical scavenging ability. The DPPH radical scavenging activity of extract of MUM256 was measured according to the previously described method with minor modifications (Ser et al., 2015a). A volume of 5 μL of sample at different concentrations was mixed with 195 μL of freshly prepared 0.016% DPPH in 95% ethanol. The mixture was kept at room temperature in the dark for 20 min before measuring the reduction of DPPH radical at 515 nm with microplate reader. Gallic acid was used as a positive control. The percentage inhibition of DPPH radical or scavenging activity was calculated according to the formula expressed below:

$$\%\ \text{DPPH scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

Superoxide Anion Scavenging Activity Determination

Superoxide anion scavenging activity/superoxide dismutase (SOD) activity was determined using a commercially available colorimetric microtiter plate method (19160 SOD Assay Kit-WST, Sigma Aldrich) according to the manufacturer's protocol. The SOD activity of MUM256 extract was assayed colorimetrically at 450 nm as the reduction of the Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) by superoxide anion, O_2^-. Twenty microliter of MUM256 extract at different concentrations were loaded into respective well of the 96-wellplate. The plate was incubated at 37°C for 20 min after the addition of respective reaction solution as the described protocol and prior to measurement of absorbance at 450 nm using a microplate reader. The SOD activity (percentage of inhibition

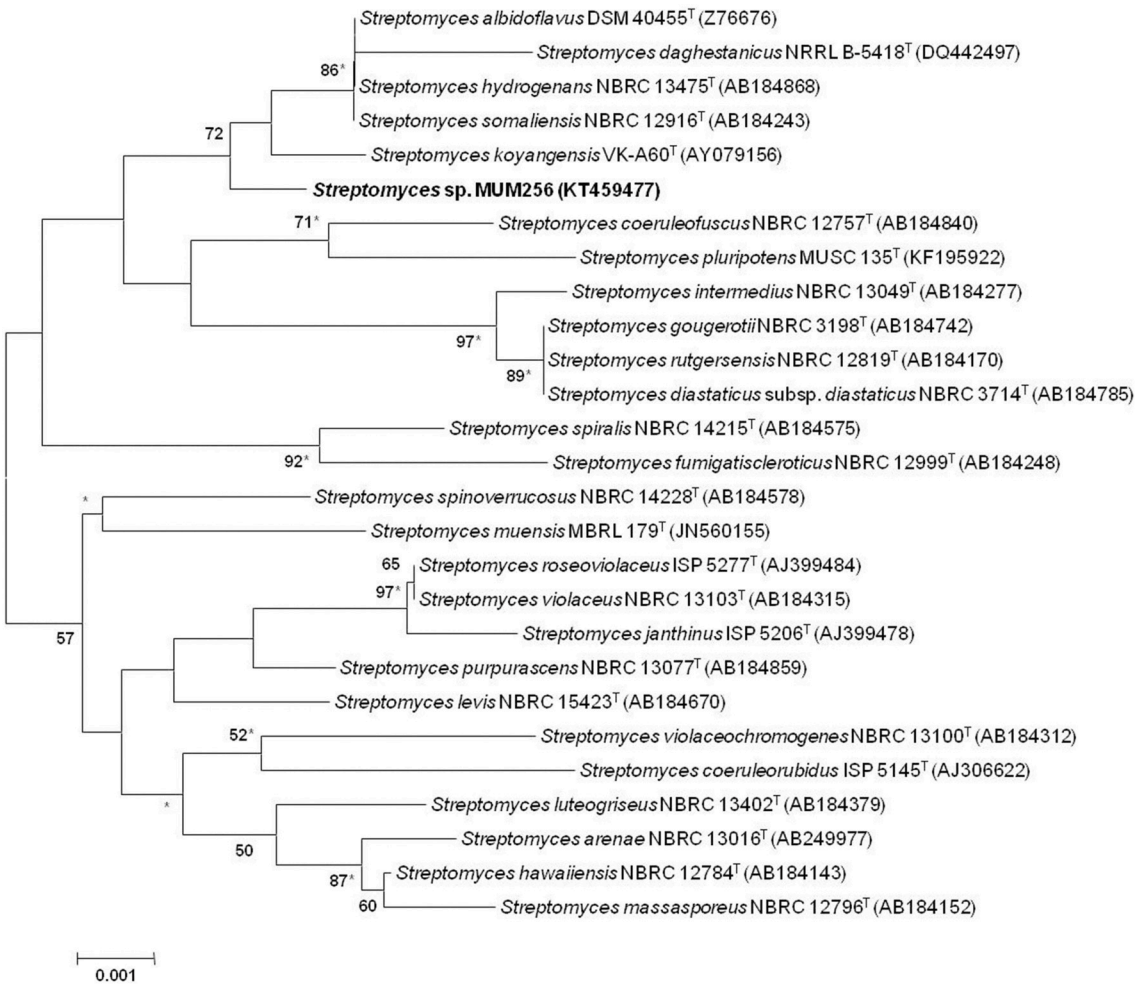


FIGURE 1 | Neighbor-joining phylogenetic tree based on 1487 nucleotides of 16S rRNA gene sequence showing the relationship between strain MUSC 149^T and representatives of related taxa. Numbers at nodes indicate percentages of 1000 bootstrap re-samplings, only values above 50% are shown. Bar, 0.001 substitutions per site. Asterisks indicate that the corresponding nodes were also recovered using the maximum-likelihood tree-making algorithm.

of WST-1 reduction) was determined according to the formula expressed below:

$$\%\ \text{SOD activity} = \frac{\left(\text{Abs control blank} - \text{Abs buffer blank}\right) - \left(\text{Abs sample} - \text{Abs sample blank}\right)}{\text{Abs control blank} - \text{Abs buffer blank}} \times 100\%$$

Abs = absorbance measured at 450 nm

Anti-cancer Activity

Cell Lines Maintenance and Growth Condition

All the human cancer and normal cell lines involved in this study was maintained in RPMI (Roswell Park Memorial Institute)-1640 (Gibco) supplemented with 10% fetal bovine serum and 1x antibiotic-antimycotic (Gibco) at 37°C humidified incubator containing 5% $CO_2$and 95% air. The cancer cell lines involved were HCT116, HT29, SW480, Caco-2, A549, DU145, CaSki, and MCF-7 while BEAS-2B was used as the normal cell lines in this study (Wong et al., 2012; Goh et al., 2014). The cultures were viewed using an inverted microscope to assess the degree of confluency and to confirm the absence of bacterial and fungal contamination.

Anticancer Activity Determination Using MTT Assay

The effect of *Streptomyces* sp. MUM256 on cell viability of human cancer cell lines was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the established method with minor modifications (Supriady et al., 2015). Cells were seeded into a sterile flat bottom 96-well plate at a density of 5×10^3 cells/well and allowed to adhere overnight. Twenty microliter of the MUM256 extract was added into each well with the final concentration ranging from 25 to 400 µg/mL. The concentration of DMSO used as the solvent was maintained at 0.05% (v/v) and also incorporated as negative control in all the experiments. Cells were further incubated with the extract for 72 h before performing MTT assay. Twenty microliter of 5 mg/mL of MTT (Sigma) was then added to each well and the

plates were incubated at 37°C in a humid atmosphere with 5% CO_2, 95% air for 4 h. The medium was then gently aspirated, and 100 μL of (DMSO) was added to dissolve the formazan crystals. The absorbance of dissolved formazan product was determined spectrophotometrically at 570 nm (with 650 nm as reference wavelength) using a microplate reader. The percentage of cell viability was calculated as follows:

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells (0.05\% DMSO only)}} \times 100\%$$

Gas Chromatography-mass Spectrometry (GC-MS) Analysis

GC-MS analysis was performed in accordance with our previous developed method with minor modification (Supriady et al., 2015). The machine used was Agilent Technologies 6980N (GC) equipped with 5979 Mass Selective Detector (MS), HP-5MS (5% phenyl methyl siloxane) capillary column of dimensions 30.0 m × 250 μm × 0.25 μm and used helium as carrier gas at 1 mL/ min. The column temperature was programmed initially at 40°C for 10 min, followed by an increase of 3C/min to 250°C and was kept isothermally for 5 min. The column temperature was programmed initially at 40°C for 10 min, followed by an increase of 3°C/min to 250°C and was kept isothermally for 5 min. The MS was operating at 70 eV. The constituents were identified by comparison of their mass spectral data with those standard compounds from NIST 05 Spectral Library (Figure S2).

Statistical Analysis

All the experiments on the antioxidant and cytotoxic properties were performed in quadruplicates. The results were expressed as mean ± standard deviation (SD) and analyzed using SPSS statistical analysis software. One-way analysis of variance (ANOVA) and Tukey's *post-hoc* analysis were performed to determine the significance of difference between the treated and control groups. An independent *t*-test analysis was also conducted to compare between the effect of the extract against cancer and normal cell line. A difference was considered statistically significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

Phenotypic Analyses of Strain *Streptomyces* sp. MUM256

Strain MUM256 was Gram-positive and aerobic. The strain grew well on ISP 2, ISP 3, ISP 5, ISP 6, ISP 7 agar, AIA, nutrient agar, and starch casein agar after 1 to 2 weeks at 28°C, whereas it grew poorly on ISP 4 agar. The morphological observation of the 15-day-old culture grown on ISP2 medium revealed an abundance growth of both aerial and vegetative hyphae which was well developed and not fragmented. These morphological characteristics were consistent with its assignment to the genus *Streptomyces* (Williams et al., 1989). The colors of the aerial and substrate mycelium were light yellow and pale yellow on ISP 2 agar. Growth occurred at pH 6.0–10.0 (optimum pH 7.0), with 0–6% NaCl tolerance (optimum 4%) and at 20–40°C (optimum 32°C). Cells were positive for catalase and hemolytic activitiy but negative for melanoid pigment production. Hydrolysis of soluble starch was positive; but negative for hydrolysis of carboxymethylcellulose, tributyrin (lipase), casein, chitin, and xylan. Cells are sensitive to cefuroxime, cephalosporin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, streptomycin, tetracycline, and vancomycin. Cells are resistant to ampicilin, ampicillin sulbactam, cefotaxime, nalidixic acid, and Penicillin G.

Phylogenetic and Genomic Analyses

The almost-complete 16S rRNA gene sequences were determined for strain MUM256 (1343 bp). The 16S rRNA gene sequences of strain MUM256 was aligned with the corresponding partial 16S rRNA gene sequences of the type strains of representative members of the genus *Streptomyces* retrieved from GenBank/EMBL/DDBJ databases. Phylogenetic tree was constructed based on the 16S rRNA gene sequences showed that strain MUM256 (**Figure 1**) formed a distinct clade with type strains *Streptomyces albidoflavus* DSM 40455^T, *Streptomyces hydrogenans* NBRC 13475^T, *Streptomyces somaliensis* NBRC 12916^T, *Streptomyces koyangensis* VK-A60^T, and *Streptomyces daghestanicus* NRRL B-5418^T at bootstrap value of 72%, indicating the high confidence level of the association (**Figure 1**). Strain MUM256 exhibited highest 16S rRNA gene sequence similarity to *Streptomyces albidoflavus* DSM 40455^T (99.7%), *Streptomyces hydrogenans* NBRC 13475^T (99.7%), *Streptomyces somaliensis* NBRC 12916^T (99.7%), followed by *Streptomyces koyangensis* VK-A60^T (99.5%) and *Streptomyces daghestanicus* NRRL B-5418^T (99.5%).

Antioxidant Activity

During metabolism process, organism produces reactive oxygen species as by-products (Cruz De Carvalho, 2008). The accumulation of excess free radicals can result in oxidative stress. It has been associated with many detrimental effects including food deterioration, aging in organisms and cancer promotion (Ames et al., 1993). With the knowledge about the critical role of free oxygen radicals as the etiology of various multifactor diseases such as cancer, neurodegenerative and cardiovascular diseases has prompted investigations on novel and potent antioxidant discovery. Literatures show that plants rich in antioxidants have been extensively studied and reviewed for their protection effects against oxidative stress related disease such as cancer (Wang et al., 2012).

Extensive studies revealed that many potent antioxidative chemical constituents can be derived from microbial origin. The investigation also has evidenced that the microbes derived from extreme environment possess high antioxidant capacity. It was believed that these microbes may have acquired the ability to synthesize specific antioxidative agent or develop specific defense mechanisms after long-term evolutionary processes for survival against oxidative stress (Hong et al., 2009). Likewise, the antioxidant activity of MUM256 extract was investigated by assessing its radical scavenging abilities on both DPPH radicals and superoxide anions. The results are presented in **Table 1**.

TABLE 1 | The antioxidant activities demonstrated by MUM256 extract in both DPPH assay and SOD activity assay.

Concentration of extract *Streptomyces* sp. MUM256 (μg/mL)	Antioxidant activities	
	DPPH radical scavenging activity (%)	Superoxide dismutase activity (%)
125	ND	16.33 ± 2.89
250	ND	21.35 ± 2.41
500	ND	31.56 ± 4.25
1000	ND	46.45 ± 5.72
2000	6.69 ± 0.83	67.25 ± 8.82
4000	12.08 ± 1.05	NT

ND, not detected; NT, not tested.

There are many reports available on the use of DPPH assay in determining the antioxidant activity of *Streptomyces* sp. (Karthik et al., 2013; Lee et al., 2014a), showing that DPPH is widely accepted and well established method for antioxidant activity assessment. DPPH is a discoloration assay using a stable free DPPH radical to assess the free radical scavenging ability of the hydrogen donating antioxidant, which can transfer hydrogen atoms or electron to DPPH radicals. In this study, the color change observed from the purple DPPH radical solution into yellow-colored diphenylpicrylhydrazine suggested that MUM256 extract exhibited hydrogen donating ability at high concentration. The extract MUM265 demonstrated significant ($P < 0.05$) 6.69 ± 0.83% and 12.08 ± 1.05% inhibitions of DPPH activity at both 2 and 4 mg/mL respectively. This result also indicated that the presence of potential antioxidative compounds in the MUM256 extract that can terminate the chain reaction of free radicals.

Furthermore, the dose-dependent manner of superoxide dismutase like activity demonstrated in SOD activity assay further confirming the antioxidant potential of MUM256 extract. It is also important to investigate the ability of the extract to scavenge *in vitro* oxygen-derived species such as superoxide anion (O_2^-) because O_2^- is a powerful oxidants capable to generate more notorious reactive oxygen species, including singlet oxygen, peroxynitrite and hydroxyl radicals (Stadtman and Berlett, 1997) which can result in more serious disease induced by oxidative stress. In this study, the superoxide radical was produced from the hypoxanthine-xanthine oxidase reaction coupled with WST. The MUM256 extract exhibited a potent superoxide anion scavenging activity with significantly strong inhibitory activity ($P < 0.05$) on the formation of yellow water-soluble WST formazan upon reduction with superoxide anion, measured IC_{50} at 1.26 ± 0.17 mg/mL. Strong correlation was reported in previous study between the SOD activity and total phenolic content (Reddy et al., 2012), suggesting that the presence of phenolic compounds in the MUM256 extract.

Anti-cancer of MUM256 Extract

In order to examine the growth inhibitory activity of the MUM256 extract in several human cancer cell lines, MTT assay was employed in this study to measure the cell viability after being treated with the extracts at different concentrations. Furthermore, it has been widely known that genetic background of cell lines could influence the efficacy and sensitivity of anticancer agent. Thus, four human colon cancer cell lines with different molecular characteristics (HCT116, HT-29, Caco-2, and SW480), one human breast cancer cell line (MCF7), one androgen-independent prostatic cancer cell (DU145), one human lung cancer cell line (A549), a human cervical cancer cell line (CaSki) were used as the panel for the anticancer activity screening of the extract. Besides that, the human bronchial epithelium cell line (BEAS-2B) was used to determine the toxicity of the extract against non-cancerous cells in which could reflect the specificity and selectivity of the extract against cancer cells.

MTT assay is used to measure the mitochondrial activity in viable cells based on the activity of mitochondrial dehydrogenase enzyme that reduces the yellow tetrazolium MTT into purple formazan crystal. The amount of the purple formazan formed

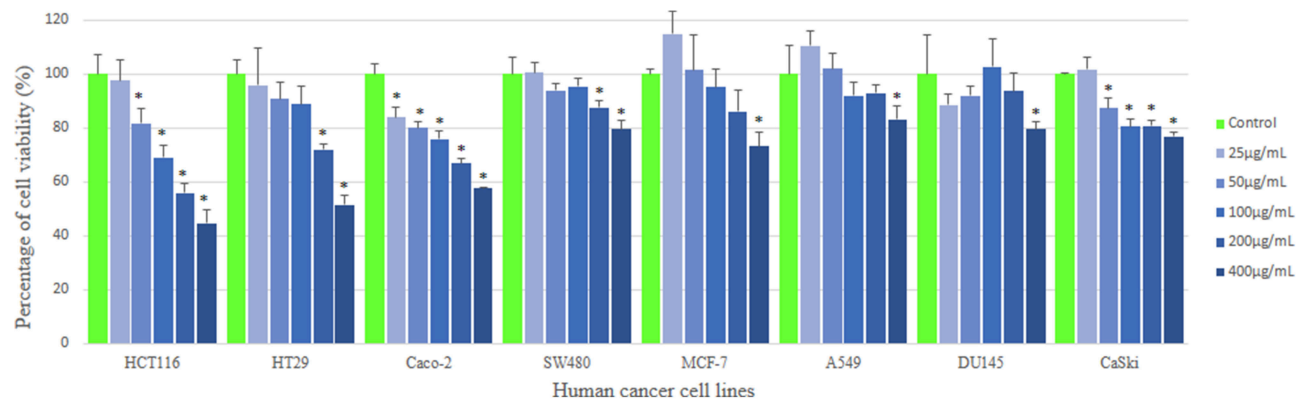
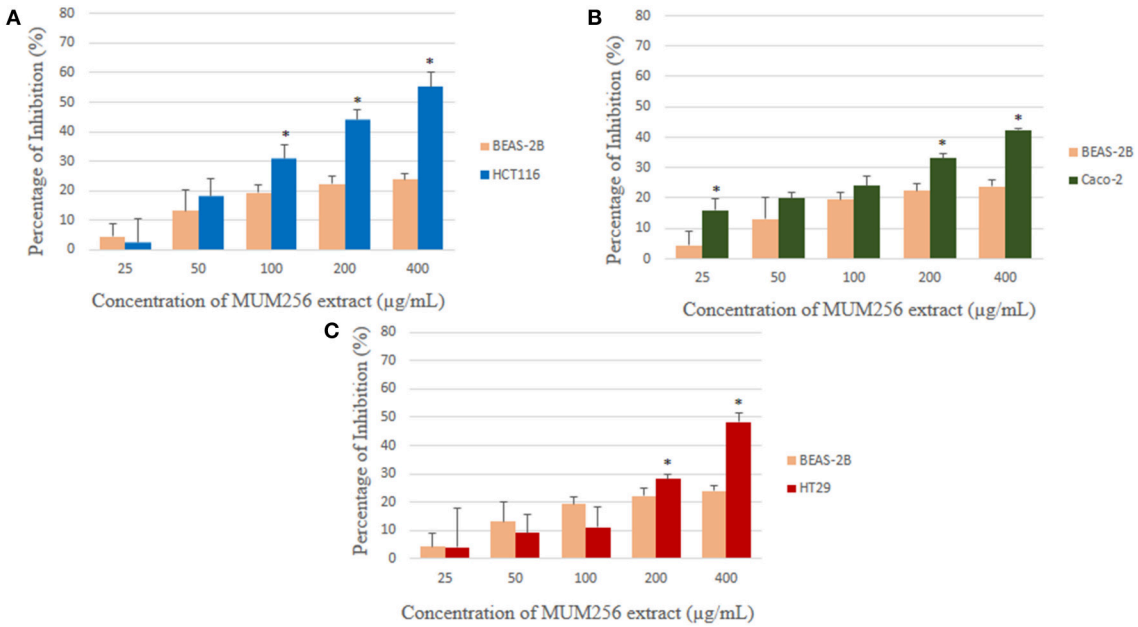


FIGURE 2 | Anticancer activity of MUM256 extract against human cancer cell lines. The anticancer activity of streptomyces sp. Mum256 extract against all the cancer cell lines measured using mtt assay. Each bar represents the mean of the cell viability of the cell lines after treatment with extract at respective concentrations tested ($n = 5$). The vertical lines associated with the bars represent the standard deviation of the mean. Symbol (*) indicates $p < 0.05$ significant difference compared to control.



D The table shows the selective cytotoxicity of extract toward cancer cells with the reference to normal cell.

Selective cytotoxicity index	Cell lines		
Concentration of extract (µg/mL)	HCT116	HT29	Caco-2
400	2.3	2.0	1.8

* Selective cytotoxicity index: < 1 denotes extract is toxic to normal cells and not selective to cancer cells
= 1 denotes no difference in toxicity towards both normal and cancer cells
> 1 denotes selective cytotoxic toward cancer with less effect on normal cells

FIGURE 3 | Comparison of the percentage of inhibition exerted by the extract at respective concentrations between normal cell line (BEAS-2B) and colon cancer cell lines [HCT116 (A), HT29 (B), and Caco-2 (C)]. Selective cytotoxicity index determined for the extract against the colon cancer cells **(D)**. Each bar represents the mean of inhibition (%) of the extract at respective concentrations tested ($n = 5$) against respective cell lines. The vertical lines associated with the bars represent the standard deviation of the mean. Symbol (*) indicates $p < 0.05$ significant difference between the normal cell line and the colon cancer cell line.

indicates the number of metabolically active viable cells (Twentyman and Luscombe, 1987). The results of the inhibitory effect of MUM256 extract were illustrated in (**Figure 2**), showing the cell viability of each cell line after 72 h treatment with different concentration of the extracts. Furthermore, the results were also expressed in term of the selective toxicity of the extract toward HCT116, HT29, and Caco-2 cancer cell lines with the reference to the normal cell BEAS-2B (**Figure 3**).

Collectively, the MUM256 extract exhibited significant growth inhibitory activity ($P < 0.05$) against all the cell lines tested at the highest concentration (400 µg/mL) when compared to the control. It can be observed that the MUM256 extract exhibited varying levels of inhibitory effect against HCT116, HT29. SW480, Caco-2, A549, DU145, CaSki, and MCF-7 cancer cell lines. Despite that, the extract showed minimal toxic effect on BEAS-2B normal lung cell line with 23.87 ± 2.11% inhibition at 400 µg/mL concentration. In fact, the toxic effect reached a plateau at 100 µg/mL with no significant difference ($P > 0.05$) observed when increased dose to 200 and 400 µg/mL (**Figure 3**). This result also suggested that the MUM256 extract exhibited a preferential or specific cytoxicity against colon cancer cell line in which HCT116, HT29, and Caco-2 were significantly ($P < 0.05$) inhibited by increased concentration of the extract.

Among the tested panel of cancer cells, HCT116 was the most sensitive cell toward the extract treatment with the IC_{50} measured at 292.33 ± 31.98 µg/mL. With the comparison to the toxic level of the extract determined on BEAS-2B, approximately 2.3-fold significantly stronger cytotoxic effect ($P < 0.05$) against HCT116 was observed at 400 µg/mL (**Figure 3**). It was then followed by 2.0 and 1.8-fold significant stronger cytotoxic effect ($P < 0.05$) against HT29 and Caco-2 respectively with the reference to BEAS-2B at 400 µg/mL. However, SW480 colon cancer cell appeared less sensitive toward this extract with low cytotoxic effect observed. This could be due to the difference in genetic makeup between those colon cancer cells. The previous investigation demonstrated that SW480 which is a mismatch repair (MMR)-wild type cell line was shown to be more resistant to cytotoxic methylating agent than other colon cancer cells with MMR-deficient cell line such as HCT116 (Liu et al., 1999). Another study also revealed that KRAS G12V mutation conferred resistance in SW480 to chemotherapy with both cetuximab and panitumumab (Kumar et al., 2014). Thus, it was speculated

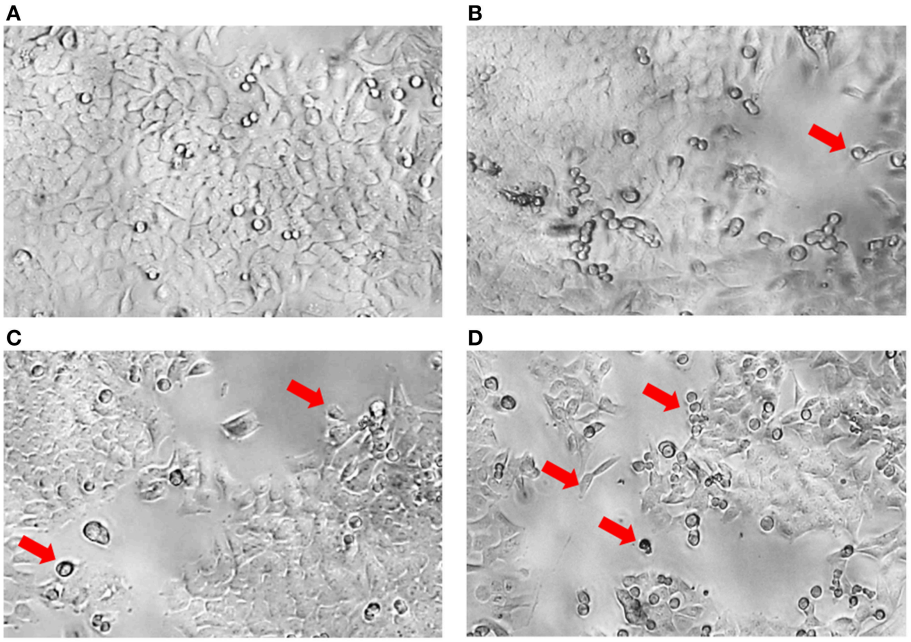


FIGURE 4 | Morphology of HCT116 after treatment with MUM256 extract at different concentrations. Comparison of the morphological features of HCT116 after the 72 h with MUM256 extract at respective concentrations [control **(A)**, 100 μg/mL **(B)**, 200 μg/mL **(C)**, 400 μg/mL **(D)**] observed under an inverted microscope with objective lens x40. Arrow indicates the abnormal morphological features resulted from the anticancer effect of MUM256 extract.

that the cytotoxic effect of the extract may be mediated by MMR-deficiency and wild-type KRAS of colon cancer cell lines.

Although significant results were demonstrated in this study indicating that the MUM256 extract exhibited certain extent of cytotoxic effect on colon cancer cell line, it should be noted that using MTT assay is not possible to differentiate between cell growth inhibition and an increase in cell death. In **Figure 4**, most of the HCT116 appeared as normal angular and spindle shapes in control (a), but most of the cells lost these features after treated with increasing concentrations of the extract (b, c, and d). For instance, cell shrinkage with lesser cytoplasm mass and even apoptotic bodies can be observed (indicated by arrows) in **Figures 4B–D**. These morphological changes of the cells observed after treated with the extract has provided some insight on the effect of the extract against the HCT116. However, data from studies focusing on elucidation of the molecular basis is essential in order to determine the putative anticancer activity of the extract against colon cancer cells.

GC-MS Analysis of MUM256 Extract

In the present investigation, the MUM256 extract has shown significantly antioxidant capacities in SOD activity and DPPH assays and anticancer properties against human colon cell lines. In this regards, it has prompted the necessities to perform chemical constituents profiling of the MUM256 extract. Hence, GC/MS analysis was employed to identify the chemical constituents present in the extract. The analysis revealed that the presence of phenolic, pyrrolopyrazine, β-carboline and dicarboxylic acid ester compounds in the MUM256 extract. The detailed information about the identified chemical constituents were listed in **Table 2** and the chemical structures were illustrated in **Figure 5**. Furthermore, the mass spectrum of the constituents identified by GC/MS in MUM256 extract is also provided in Figure S2.

Phenolic compounds have been widely known as potent antioxidant agents or free radical terminators which they possess hydrogen-donating ability to reduce free radicals (Sulaiman et al., 2011; Yogeswari et al., 2012). Phenol,2,4-bis(1,1-dimethylethyl)- (1), phenol,2,2′-methylenebis[6-(1,1-dimethylethyl)-4-methyl- (6) were the two phenolic compounds identified from the extract. Similarly, a recent study showed the detection phenol,2,4-bis(1,1-dimethylethyl)- (1) with GC/MS in *Streptomyces cavouresis* KUV39 isolated from vermicompost samples in India and demonstrated that this compound exhibited potent antioxidant properties and cytotoxicity against Hela cells (Narendhran et al., 2014). Thus, *Streptomyces* sp. MUM256 could be also a potential source of phenolic compounds to be used as preventive agent for oxidative-stress related diseases.

In the present study, the detected three pyrrolopyrazine compounds include, pyrrolo[1,2a]pyrazine-1,4-dione, hexahydro- (2), pyrrolo[1,2a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (3), and pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)- (5) were also present in previously isolated *Streptomyces* sp. (Narasaiah et al., 2014; Manimaran et al., 2015; Ser et al., 2015a). Both of the

TABLE 2 | Chemical constituents identified in of *Streptomyces* sp. MUM256 extract.

No.	Constituents	Class	Retention time (min)	Molecular formula	Molecular Weight	Similarity (%)	References
1	Phenol,2,4-bis(1,1-dimethylethyl)-	Phenolic compound	44.445	$C_{14}H_{22}O$	206	96	Narendhran et al., 2014
2	Pyrrolo[1,2a]pyrazine-1,4-dione,hexahydro-	Pyrrolopyrazine	53.297	$C_7H_{10}N_2O_2$	154	98	Narasaiah et al., 2014; Ser et al., 2015a
3	Pyrrolo[1,2a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-	Pyrrolopyrazine	58.510	$C_{11}H_{18}N_2O_2$	210	64	Narasaiah et al., 2014; Abdullah et al., 2015; Manimaran et al., 2015; Ser et al., 2015a
4	9*H*-Pyrido[3,4-b]indole	β-carboline alkaloid	60.381	$C_{11}H_8N_2$	168	96	Zheng et al., 2006
5	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)-	Pyrrolopyrazine	72.071	$C_{14}H_{16}N_2O_2$	244	97	Narasaiah et al., 2014
6	Phenol,2,2′-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	Phenolic compound	73.507	$C_{23}H_{32}O_2$	340	96	Narendhran et al., 2014
7	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	Dicarboxylic acid ester	76.883	$C_{16}H_{22}O_4$	278	91	Krishnan et al., 2014

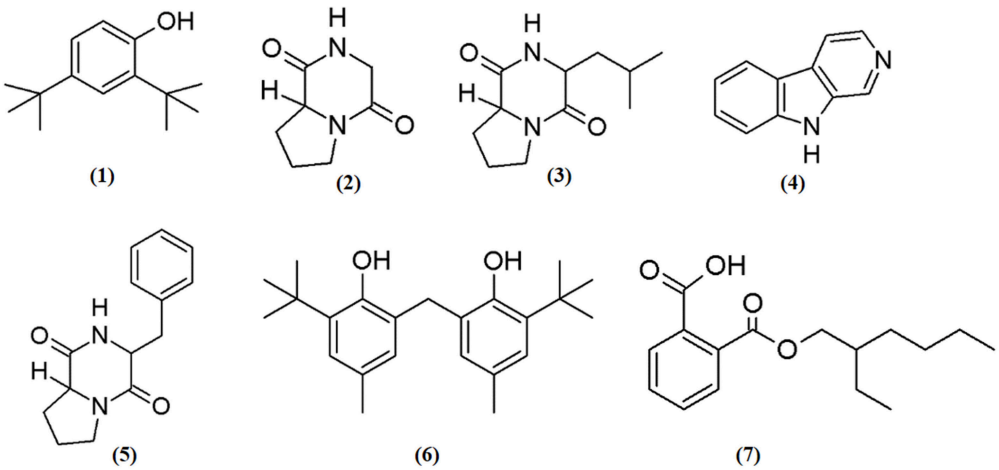


FIGURE 5 | Chemical structures of constituents detected in MUM256 extract.

pyrrolopyrazine compounds identified had been suggested to possess potent antioxidant activity (Ser et al., 2015a). Besides the detection of pyrrolopyrazine in *Streptomyces*, Gopi et al. (2014) also reported that the structure of pyrrolo[1,2a]pyrazine-1,4-dione, hexahydro- (2) isolated from sponge associated *Bacillus* sp. has the ability to reduce oxidative damages by radicals. Furthermore, another study revealed that the extract of *Micrococcus lutues* containing hexahydro- (2) and pyrrolo[1,2a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (3) exhibited promising cytotoxic effect on HCT15 with (Abdullah et al., 2015). Thus, it was suggested that both of the identified pyrrolopyrazine could have contributed the antioxidant and anticancer activities observed in MUM256 extract.

Furthermore, a tricyclic indole β-carboline alkaloid, 9*H*-pyrido[3,4-b]indole (4) was detected in MUM256 extract. Previous study by Zheng et al. (2006) demonstrated that this compound which is also known as norharman extracted from a marine bacterium, *Pseudoalteromonas piscicida*, exhibited cytotoxicity toward both HeLa cervical cancer and stomach cancer cells with an IC_{50} of 5 μg/mL. It was shown that norharman caused HeLa cells death via apoptotic process, specifically through the perturbation of cell cycle at G_2M phase of the cancer cell (Zheng et al., 2006).

Lastly, 1,2-benzene dicarboxylic acid, mono 2-ethylhexyl ester (7) has been detected in various sources ranging from plant extracts (Akpuaka et al., 2012; Sivasubramanian and Brindha, 2013), endophytic fungal (Verma et al., 2014), and also microbial origin including *Streptomyces* sp. (Krishnan et al., 2014). In previous study, the cytotoxicity of 1,2-benzene dicarboxylic acid, mono 2-ethylhexyl ester (7) extracted from *Streptomyces* sp. was evaluated against liver cancer cell line HepG2 and also breast cancer cell line MCF7 with IC_{50} at 42 and 100 μg/mL respectively (Krishnan et al., 2014).

According to the GC/MS analysis, the identified chemical constituents are well recognized for their antioxidant and anticancer activity and we postulate that these constituents could be the major contributing factor for both antioxidant capacity and anticancer activities of MUM256 extract.

CONCLUSION

In summary, the findings demonstrates that MUM256 extract exhibits antioxidant and anticancer activities. The extract is able to scavenge superoxide anion radicals in dose dependent manner and show a selective cytotoxic effect toward colon cancer cells. The phenolic compounds, pyrrolopyrazine, β-carboline and dicarboxylic acid ester present in the extract could be responsible for the antioxidant and anticancer activities observed. Those findings suggest that *Streptomyces* sp. MUM256 could be potential source for antioxidative agents and hence merit further studies concerning the development of chemopreventive drugs against cancer.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01316

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Ketide Synthase (KS) Domain Prediction and Analysis of Iterative Type II PKS Gene in Marine Sponge-Associated Actinobacteria Producing Biosurfactants and Antimicrobial Agents

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The important biological macromolecules, such as lipopeptide and glycolipid biosurfactant producing marine actinobacteria were analyzed and their potential linkage between type II polyketide synthase (PKS) genes was explored. A unique feature of type II PKS genes is their high amino acid (AA) sequence homology and conserved gene organization. These enzymes mediate the biosynthesis of polyketide natural products with enormous structural complexity and chemical nature by combinatorial use of various domains. Therefore, deciphering the order of AA sequence encoded by PKS domains tailored the chemical structure of polyketide analogs still remains a great challenge. The present work deals with an *in vitro* and *in silico* analysis of PKS type II genes from five actinobacterial species to correlate KS domain architecture and structural features. Our present analysis reveals the unique protein domain organization of iterative type II PKS and KS domain of marine actinobacteria. The findings of this study would have implications in metabolic pathway reconstruction and design of semi-synthetic genomes to achieve rational design of novel natural products.

Keywords: glycolipid, lipopeptide, biosurfactant, polyketide synthases, actinobacteria, three-dimensional structure

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INTRODUCTION

Natural products of microorganisms are potential source of bioactives that have been extensively exploited to develop next generation anti-infective drugs proposed by pharmaceutical companies (De Carvalho and Fernandes, 2010). But in recent years, the exploration of marine microorganisms received greater attention due to their complex biosynthetic pathways and potential implications on the development of anti-cancer agents and anti-infectives to combat multi-resistant strains (De Carvalho and Fernandes, 2010). Past few decades the bioprospecting of natural resources

and microbial isolates were tremendously increased, however, the leads transformed to drugs are very few (Watve et al., 2001). Perhaps this trend might have led to the exploration of pristine and unexplored bioresources including hydrothermal vents and extreme niches. Marine sponges are sedentary animals harboring more than 40% of microorganisms by volume. Among the marine fauna and flora, marine sponges are potential source of bioactive natural products (Faulkner and Ghiselin, 1983, 1994; Matsunaga and Fusetani, 2003). However, recent deliberations envisage that the sponge derived secondary metabolites are biosynthesized by the associated microorganisms. However, this hypothesis is being remained unproven as sponge-specific bacteria are uncultivable with conventional approaches. Exploration of sponge associated microbial diversity and symbiont-assisted complex biosynthetic pathway of bioactive leads have increased the scope of natural product discovery from marine sponges (Faulkner and Ghiselin, 1983, 1994; Hentschel et al., 2002). Recent developments in genome mining and metagenomics have widely used in the exploitation of such complex biosynthetic pathways of marine natural products. By and large the biosynthetic pathways of polyketides, non-ribosomal peptides, and their derivatives are useful to integrate sponges and their symbiotic biosynthetic machineries. Marine sponges are richest source of polyketide and peptide bioactive molecules. Unlike terrestrial counterparts, sponge-derived bioactive molecules are unique and having specific targeted activities expected for drug leads (Li et al., 2002; Matsunaga and Fusetani, 2003; Montalvo et al., 2005; Montalvo and Hill, 2011). The sponge-derived bioactive peptides are non-ribosomal origin and are modified with unusual amino acids (AAs; Matsunaga et al., 1985).

Polyketide synthases (PKSs) are modular proteins involved in the biosynthesis of complex bioactive molecules through sequential catalytic activities. These enzymes mediate biosynthesis of bioactive molecules with diverse structural complexities by combinatorial use of a specific sequential order of catalytic domains. The tailoring of catalytic domains and AA sequence of these domains are drastically changes with natural bioresources and therefore, the nature and chemical structure of end product is varied between/within the species (Yadav et al., 2009). The mechanism of sequential order and/or selection of catalytic domains remains a major challenge in chemical ecology of secondary metabolite synthesis. The fully dissociable complex of small, discrete mono-functional proteins that catalyze combinatorial synthesis of aromatic polyketides, which is in general termed as type II PKS. In the iterative PKSs, the active site of each catalytic module for tailoring of type II PKS is encoded by a single gene. There is only one set of a hetero-dimeric ketosynthase (KSα–KSβ) and an acyl carrier protein (ACP) that tailored the synthesis of polyketide molecule in a specific order and defined number of cycles to build a polyketide chain (He and Hertweck, 2003). The chain length is maintained through sequential iterative process including cyclisation, reduction, and aromatization steps which are catalyzed by cyclase (CYC), KR, and aromatase (ARO), respectively. In certain group of type II PKSs, the malonyl-CoA ACP acyl transferase (MAT), which catalyzes condensation of acyl transfer between malonyl-CoA and the ACP (Revill et al., 1995). The type II PKSs in general catalyze the biosynthesis of diverse range of multi-functional aromatic polyketides and are mostly restricted among bacteria (Shen et al., 2000). The type II PKSs, such as those responsible for the biosynthesis of the aromatic polyketides actinorhodin (ACT; Fernández-Moreno et al., 1992) and tetracenomycin (TCM), (Bibb et al., 1989; Summers et al., 1992) are composed of three to seven separate mono- or bi-functional proteins, the active sites of which are used iteratively for the assembly and early modification of the polyketide chain.

The KS domain of PKS gene was retrieved from marine actinobacteria producing biosurfactants and antimicrobial compounds. Therefore, this study was aimed to integrate PKS gene in biosurfactant production. Based on the literature, PKS gene can be expected from actinobacteria producing antimicrobial compounds, but PKS gene was not linked with biosurfactant production. A PKS gene possibly encodes biosynthesis of some biosurfactants, being considered as smart biomolecules having the ability to reduce surface and interfacial tension, wider bioactivities and possibly involved in bacterial quorum sensing (Kiran et al., 2015). Biosurfactant production has been reported by our research group in several actinobacteria (Selvin et al., 2009b; Kiran et al., 2010) and they were linked with non-ribosomal peptide synthases (NRPS), PKS (Kiran et al., 2010), and large multifunctional proteins with a modular organization. Biosynthetic pathway of biosurfactants in *Bacillus* and *Pseudomonas* was well-established. However, biosynthetic pathway of biosurfactants produced by marine actinobacteria, in general remains undisclosed. The biosurfactants invariably showed antibiofilm activity without inhibiting the biomass of pathogens tested. Based on *in vitro* experiments, it was found that the biosurfactants produced by marine actinobacteria is having antimicrobial and antibiofilm activity. The PCR amplified KS domain from these actinobacteria envisages the biosynthetic pathway of biosurfactants might have mediated through PKS biosynthetic gene clusters. Therefore, in this study, the *in vitro* findings are integrated with *in silico* analysis to substantiate the hypothesis that the biosynthesis of biosurfactants produced by marine actinobacteria might have mediated by PKS gene. To date, there are few reports about the interaction between PKS type II gene clusters and biosurfactant production (Kiran et al., 2010). There is no report on marine actinobacteria and their PKS structural diversity related with biosurfactant production. Hence we decided to focus on this aspect with three biosurfactants (MSA10, MSA13, and MSA21; Gandhimathi et al., 2009; Kiran et al., 2010, 2014) and two antagonistic compounds producing (MAD01 and MSI051; Selvin et al., 2009a,b) actinobacterial strains and they were isolated from marine sponges, *Fasciospongia cavernosa* and *Dendrilla nigra*, respectively. *In silico* analysis of PKS gene clusters and modular structure of iterative type II PKS are important tool for designing various experimental approaches toward the combinatorial synthesis of diverse aromatic polyketides. Therefore, present study was aimed to analyze and evaluate the KS domains of iterative PKS gene type II and ketosynthase genes retrieved from marine sponge-associated actinobacteria and their biosurfactant producing ability related to iterative type II PKS gene.

MATERIALS AND METHODS

Microorganisms and PKS Type II Gene Amplification

The actinobacterial strains used in this study were already been isolated from marine sponges, such as *F. cavernosa* (MSA10) and *D. nigra* (MSA13, MSA21, MAD01, and MSI051) collected from southwest cost of India. The 16S rRNA GenBank accession numbers as follows *Nocardiopsis alba* MSA10: EU563352 (Gandhimathi et al., 2009), *Brevibacterium aureum* MSA13: GQ153943 (Kiran et al., 2010), *Brachybacterium paraconglomeratum* MSA21: GQ153945 (Kiran et al., 2014), *Streptomyces* sp. MAD01: GQ246755 (Selvin et al., 2009b), and *Streptomyces dendra* MS1051: EF417875 (Selvin, 2009), respectively. The PKS type II gene was amplified from five actinobacterial strains (MSA10, MSA13, MSA21, MAD01, and MSI051) according to Selvin (2009). The genes encoding PKS were amplified using degenerate primers (**Table 1**). The PCR temperature profile used was 95°C for 3 min, and then followed by 30 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 60 s and finally an extension step at 72°C for 10 min. The resultant amplified PCR products were purified and cloned using the TOPO TA cloning kit (Invitrogen) for sequencing.

Evaluation of Antibiofilm Effect

The culture supernatant obtained from actinobacterial strains were evaluated for biofilm inhibitory effect against *Vibrio harveyi*. The biofilm was allowed to develop on cover slips and treated with the actinobacterial extracts and incubated for 48 h at 37°C. After incubation the planktonic and spent media were discarded. The cells were washed twice with deionized water air dried and stained with 0.1% acridine orange and examined under confocal laser scanning microscopy (CLSM).

Determination of Bacterial Cell Viability in Biofilm

Cell viability of the bacteria in the biofilm was assessed using MTT assay as described by Traba and Liang (2011) with necessary modifications. Biofilm of *V. harveyi* was allowed to develop on 96-well plate and treated with 50 μl culture filtrates of the five actinobacterial strains and incubated for 24 h at 37°C. Untreated wells were set as control. After 24 h the bacterial suspension was collected and then treated with 100 μl of phosphate buffered saline and 50 μl of MTT at concentration of 0.3% were added and then incubated for 2 h at 37°C. The MTT solutions were removed and formazan crystals formed were dissolved in 150 μl of DMSO and 25 μl of 0.1 M glycine buffer of pH 10.2. The absorbance was recorded in a microplate reader at 550 nm.

KS Domain Protein Data Set, Phylogeny Construction, and Domain Structural Analysis

Type II KS domain sequences and ketosynthase gene sequences were translated using sequence manipulation suite[1] and these deduced AA sequences of type PKS II and ketosynthase were deposited to NCBI-GenBank with the accession numbers of ACS45380–ACS45382 (type II PKS), and ketosynthase bearing following accession numbers ACV31767 and ABP57802. KS domain of type II PKS gene sequences and ketosynthase (Cds) sequences of sponge-associated actinobacteria were retrieved from National center for Biotechnology Information[2]. GenBank accession numbers of these KS domains and ketosynthase sequences were given as GQ153947 (*N. alba* MSA10), GQ153948 (*B. aureum* MSA13), GQ153949 (*Brachybacterium* sp. MSA21), and GQ246762 (*Streptomyces* sp. MAD01), EF520724 (*Streptomyces dendra* MS1051), respectively. The predicted KS domains of all retrieved actinobacterial gene sequences and the PKS type II protein sequences from reference actinobacteria were aligned by CLUSTAL W2[3] and translated deduced AA sequences were verified using the NCBI-BLAST[4] search with expected value set to the default value of 10 was performed using the protein sequences of *N. alba, B. aureum, Brachybacterium* sp. MSA21, *Streptomyces* MAD01 and *S. dendra*, respectively, and the various sequences against 138 complete eubacterial and 20 complete archaebacterial genomes. Phylogenetic tree of the deduced AA sequences of PKS II segments and ketosynthase genes were generated using neighbor-joining method through MEGA programs (Kumar et al., 2004). KS domain phylogeny was based in the prediction of putative enzymes of identical or nearly identical biochemical function. The type of KS was identified based on the top BLAST match in the reference data set. NCBI CDD search, SEARCPKS and Motif scan were performed to derive the existence of significant domains and their organization. Comparative analyses of KS domains of five subject organisms were performed with known polyketide producers and with the structure of polyketides using NCBI

[1]http://www.bioinformatics.org/sms2/

[2]www.ncbi.nlm.nih.gov/GenBank

[3]http://www.ebi.ac.uk/Tools/msa/clustalw2

[4]www.ncbi.nlm.nih.gov/BLAST

TABLE 1 | PKS type II gene retrieved from marine sponge-associated actinobacteria.

Protein GenBank accession number	Primers	PKS-II amplicon size	Source organism
ACS45380	GCIATGGAYCCICARCARMGIVTGTICCIGTICCRTGISCYTCIAC	579bp	*Nocardiopsis alba* MSA10
ACS45381	GCIATGGAYCCICARCARMGIVTGTICCIGTICCRTGISCYTCIAC	639bp	*Brevibacterium aureum* MSA13
ACS45382	GCIATGGAYCCICARCARMGIVTGTICCIGTICCRTGISCYTCIAC	662bp	*Brachybacterium paraconglomeratum* MSA21
ACV31767	GGIAAYGGITAYGCIMGIGGGTICCIGTICCRTAIGCYTC	519bp	*Streptomyces* sp. MAD01
ABP57802	GGIAAYGGITAYGCIMGIGGGTICCIGTICCRTAIGCYTC	504bp	*Streptomyces dendra* MSI051

CDD and SEARCHPKS, respectively. The AA composition was also predicted to substantiate the function of type II PKS and ketosynthase of our interest.

Profile Hidden Morkov Model (HMM) analysis was carried out by HMMER package. The available three (*Nocardiopsis*, *Brevibacterium, Brachybacterium*) actinobacterial KS dataset was analyzed, whether these domains are modular or iterative KS domains. All these three iterative KS domains of PKS type II gene clusters of actinomycetes were modeled using comparative modeling approach. Threading analysis was carried out using a local version of threader package[5] to identify the structural templates for modeling of actinobacterial KS domains. The remaining two KS domains (from *Streptomyces* MAD01 and *S. dendra* MSI051) have been modeled using fatty acid KAS structure as template (*Escherichia coli* KAS I), which show only about 40% sequence identity with polyketide KS domains. Even the sequence identity was lesser between the target and template, the two KS proteins structures can be reliable and they adopt similar structure. The secondary structures of type II PKS and ketosynthase domains of 3D models were created using a $(PS)^2$ is an automated homology modeling server (Chen et al., 2006). The $(PS)^2$ combines PSI-BLAST, IMPALA, and T-Coffee in both template selection and target-template alignment. The final three dimensional structures were built using the modeling package MODELLER.

RESULTS AND DISCUSSION

The Nature of KS Domains of Type II PKS and Ketosynthase

The actinobacterial isolates from marine sponges were screened for biosurfactant activity using emulsification index (E_{24}) as per Kiran et al. (2010). Among the five actinobacteria MSA10, MSA13, and MSA21 were potential producer of biosurfactants (**Figure 1**). The active moieties were identified from GCMS data. The active moieties of MSA10, MSA13, and MSA21 were evidenced as biosurfactant molecules, but the moieties of MAD01 and MSI051 were not related with biosurfactants (**Table 2**). The antimicrobial moiety of MAD801 was identified as cyclohexane carboxylic acid hexyl ester. It was reported that cyclohexane carboxylic acid is a moiety of the antifungal polyketide ansatrienin A (Patton et al., 2000).

[5]http://bioinf.cs.ucl.ac.uk/psipred/

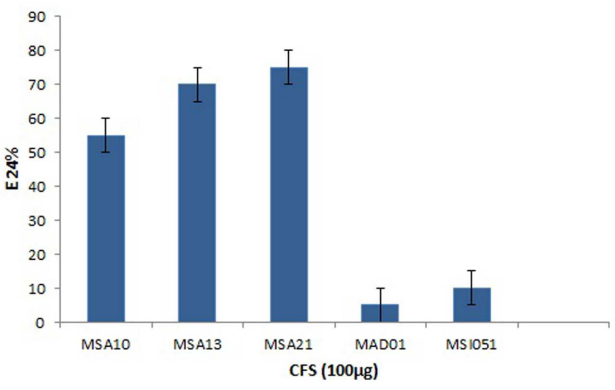
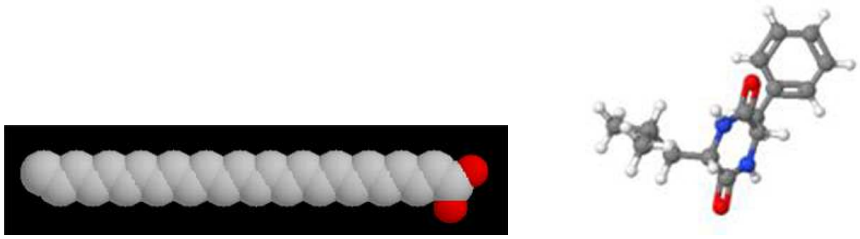
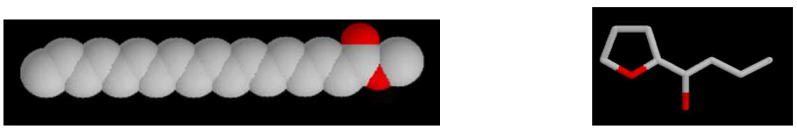
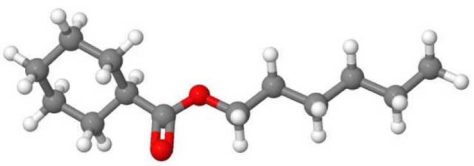
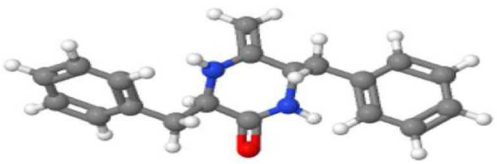


FIGURE 1 | Emulsification index of biosurfactants produced by marine actinobacteria.

Sponge-associated actinobacteria: i.e., *N. alba, B. aureum,* and *Brachybacterium paraconglomeratum* beared the type II PKS (GQ153947, GQ153948, and GQ153949, **Table 1**). The KS domain of these gene segments were translated into AAs counts, viz; 191, 212 and 220, respectively. All these KS domains encodes the condensation enzymes (cds), which catalyzes (decarboxylation or non-decarboxylation) Claisen-like condensation reaction, and the KS domains sharing the strong structural similarities are involved in the synthesis and degradation of fatty acids.

KS domain of PKS gene is the most conserved catalytic domain and is involved in the tailoring PKS molecule by catalyzing the chain condensation step. We have performed *in silico* analysis to identify KS domain counterparts from modular and iterative PKSs and other PKS families. The analyzed domains are separated into distinct clusters in a phylogenetic tree (**Figure 2**). Based on HMM by the HMMEP package, three actinobacterial KS domain of type II PKS genes were analyzed and the results show that these three isolates contains iterative PKS gene and this outcome provides potential in genome sequencing efforts for the identification of novel PKS genes. Iterative condensation steps play a vital role in biosynthesis by PKS proteins and phylogenetic analysis of iterative KS domains inferred that the clustering of iterative PKS gene sequence is highly correlated with the number of iterations they perform. From this study, we suggest that marine sponge associated actinobacterial community predominantly possesses the iterative KS domain of type II PKS rather than modular type I PKS or NRPS-PKS hybrids. The type II PKS from three different genera is characterized to study and understand their function and diversity. The isolation and identification of PKS with different enzymatic activity in marine actinobacteria has been reported, as well as the occurrence of PKS gene families in a community (Kim and Fuerst, 2006). This is the first report on the *in silico* analysis of iterative type II PKS of sponge-associated actinobacteria. Recent literature (Kiran et al., 2010, 2015) evidenced that these actinobacteria are potent biosurfactant producers with antimicrobial activity (lipopeptide and glycolipid derivatives). The present *in silico* analysis revealed that these isolates possessing iterative domains (**Figure 2**) type II PKS genes and it can be hypothesized that the antimicrobial biosurfactants synthesis might be mediated by iterative type II PKS genes. Another group of actinobacterial antibiotics producers from the marine sponge *D. nigra* such as *S. dendra* MSI051 (Selvin, 2009) and *Streptomyces* sp. MAD01 (Selvin et al., 2009b) were included in the analysis. Their partial ketosynthase genes were retrieved from GenBank (GQ246762 and EF520724) with 519 and 504 bp encoding 173 and 168 aa, respectively (**Table 1**).

TABLE 2 | 3D structures of active moieties identified from GC-MS data.

Strain name	Compound	3D structures of active moieties detected from GS-MS data
MSA10	Lipopeptide	
MSA13	Lipopeptide	 Gly–Gly–Leu–Pro–
MSA21	Glycolipid	
MAD01	Cyclohexanecarboxylic acid	
MSI051	2,5-Piperazinedione, 3,6-Bis Phenylmethyl	

Antibiofilm Effect Against *Vibrio*

Antibiofilm effect of the culture supernatant was well noticed by CLSM. The culture supernatant inhibits the biofilm formation *of V. harveyi*. Among the extract used the lipopeptide producer MSA10 and MSA 13 inhibit the biofilm formation by 80% compared to the other actinobacterial extracts (**Figure 3**). The antibiofilm effect may be due to the biosurfactant production mediated by PKS gene.

Cell Viability in Biofilm

The viability of the cells were reduced by adding the biosurfactants as shown in **Figure 4**. When compared to the control the extracts from MSA 10 and MSA 13 inhibits the viability of *Vibrio* cells by more than 80%, followed by MSA 21 by 70%.

Domain Architecture and Homology Modeling of Iterative Type II PKS

In silico analysis of Type II PKS and ketosynthase unveiled an unprecedented organization of various domains encoding discrete ketoacyl synthase (KAS) and thiolase, PKC, CK2, and ACP some are lacking an ACP. Certain polyketides undergoes non-iterative biosynthesis which involves a novel type II PKS that acts directly on acyl CoA substrates. These results demonstrate the capability of nature's in designing complex bioactive compounds and suggest new methods for PKS design and engineering through synthetic biology approaches to expand the scope and diversity of polyketide library. The structural diversity of PKS would ultimately help in searching for PKS with novel chemistry for combinatorial biosynthesis (Shen and Kwon, 2002). All the proteins studied here are found to have potential KS domains which catalyze the polyketide chain elongation step. In the beginning of chain elongation, an enzyme intermediate is formed between the growing polyketide chain and the thiol of its active site Cys. Then condensation reaction occurs with the methylmalonyl-ACP or malonyl-ACP co-substrate (Shen, 2003).

Analysis evidenced that the PKS sequence retrieved from *N. alba* and *B. aureum* are having ACP domain, i.e., beta-ketoacyl-ACP synthase and beta-KAS (**Figure 5**). KASs are involved in the elongation steps in the pathway of fatty acid biosynthesis. KAS III is involved in the catalysis of the initial condensation and KAS I and II are responsible for elongation steps by Claisen condensation of malonyl-ACP with acyl-ACP. Remaining three protein sequences lack ACP, some non iterative

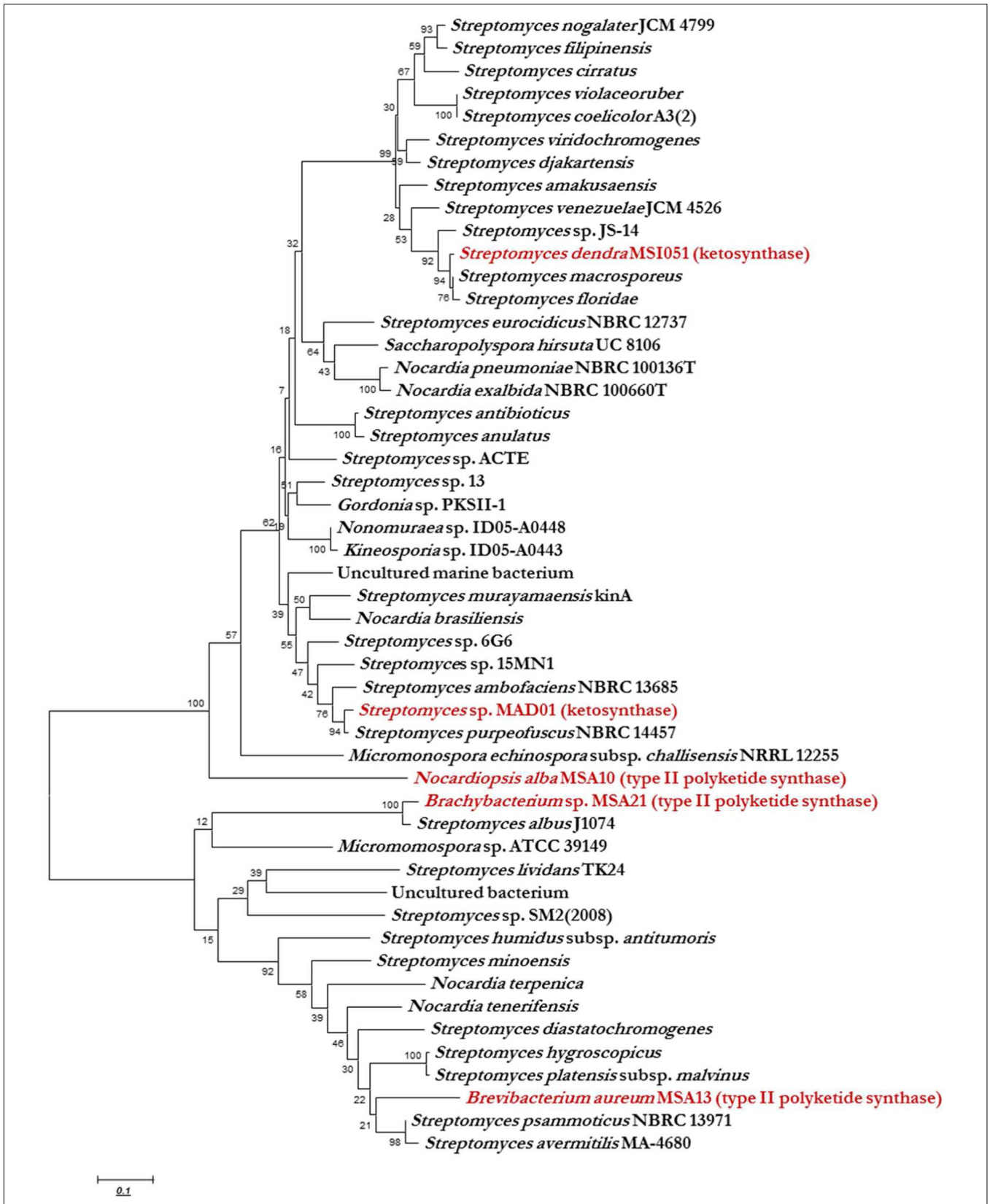


FIGURE 2 | Phylogenetic analysis (MEGA 5.0) of ketosynthase regions with respect to the diverse range of ketosynthase domains, including iterative types II, modular PKS, and KS domain. The phylogenetic trees were constructed using bootstrapping and the neighbor-joining rules.

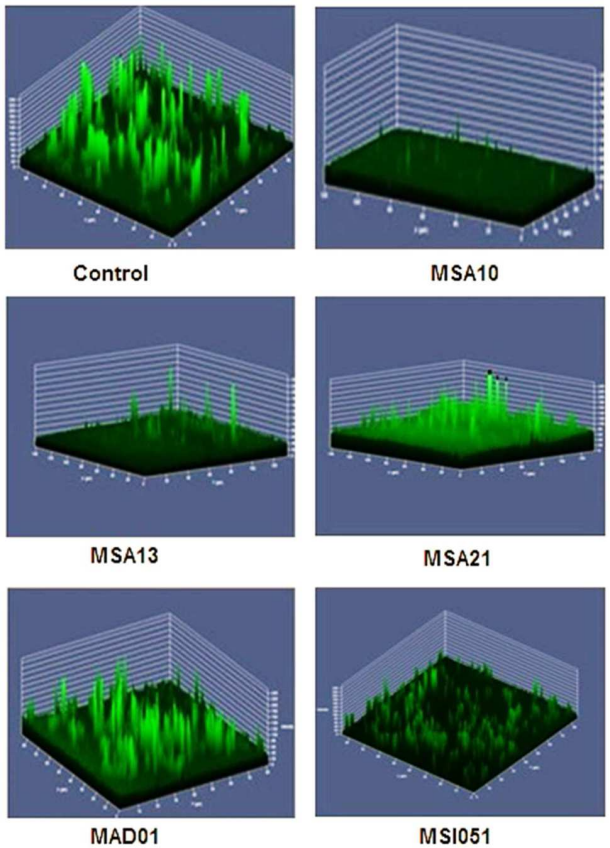


FIGURE 3 | Antibiofilm activity of cell free supernatants from marine actinobacteria. The pre-formed biofilm on glass slides was treated for 24 h with 150 μg each of freeze-dried cell free supernatant (CFS). Untreated biofilm (control) and remaining biofilm of *V. harveyi* treated after CFS on glass slides were stained with 0.1% acridine orange and observed in CLSM (LSM 710, Carl Zeiss).

type II PKSs lack ACP, utilize acyl CoAs as substrates for macrotetrolide biosynthesis. It was reported that the PKSs are using ACP to activate the acyl CoA substrate and channel the polyketide intermediates (Shen, 2003).

Outside of the module, the beta-KAS domains are dimeric. However, the number of domains within the module is dimeric still remains to be established (Tsai et al., 2001, 2002; Broadhurst et al., 2003). Perhaps every enzyme within the module made contacts across the *ser* and *cys*, ACP suppose to diffuse farther than the peptide linkers on each side would permit (Keatinge-Clay and Stroud, 2006). The deduced quaternary structure of the proteins indicates a surprising configuration which is homologous to many PKS genes that are capable of synthesizing active polyketides. Even alignment of KS domains of our sequence of interest shows 53–62% of similarity with structures like amphotericin, ACT, epothilone, meagalomycin, myxalomycin, and rifamycin.

Most of the KS are dimeric with active site at the interface of dimer and type II PKS probably functions by making contact across the twofold axis and the active sites of KS are accessible to ACP (Keatinge-Clay and Stroud, 2006). In the present findings, we observed the PKC domain is common in all the protein sequences and lack AT domain. The analysis showed the chances of inactive enzymes within the modules may perform some important functions. The ACP module is bound by peptide linkers on both ends, and this module can pass between each enzyme in the module as well as the next KS or thiolase C and N terminal (Perham, 2000). The linkers helps to prevent a polyketide from interacting with enzymes and contribute little translational freedom to the polyketide compared to the peptide linkers on both ends of ACP. Thus helps in the biosynthesis of polyketides (Keatinge-Clay and Stroud, 2006). The interaction of ACP with the KS domain facilitate to docks in a deep groove which is formed by the interaction of the KS, PKC, and the other linker, thereby implicating both the PKC and the thioesterase linker in functional KS-ACP recognition (Liu et al., 2002). The KS domain of type II PKS (*N. alba*) ACS45380.1 was closely related to those of ACT PKS. Type II PKS (*N. alba*) consists of seven structural domains includes Asn Glycosylation, CK2 Phospho site, PKC, Tyr Phosphosite, ACP and KAS (49–161 AA residues) which shares 59% similarity with ACT polyketide putative beta-KAS 2 which contains eight chains, out of which two chains are homologous to our PKS protein which are chain A: beta-KAS/acyl transferase and chain B: ACT polyketide putative beta-KAS 2. The synthesis of aromatic polyketides are mostly begins with the formation of a polyketide chain (Keatinge-Clay and Stroud, 2006). The polymeric chains of type II PKS are tailored by the heterodimeric ketosynthase-chain length factor (KS-CLF). KS-CLF is the homolog of KS domain of type II PKS of *N. alba* which regulates chain length by catalyzing both chain initiation and elongation. Exploration of the mechanistic details of this central PKS polymerase may support designing and reconstruction of pathways being invented on synthetic biology platforms. This protein was structurally elucidated with four alpha helix and seven beta sheets. And it is slightly acidic composed of 39.79% of aliphatic (G,A,V,L,I), 18.32% of Acidic (B,D,E,N,Q,Z), 15.18% of basic (K,R,H), 3.66% of sulfur (C,M), 3.66% of aromatic (F,W,Y), and 12.04% of aliphatic hydroxyl (S,T).

Type II PKS of (*B. aureum)* ACS45381.1 is sequentially identical to type I ketosynthase (*Streptomyces* sp. T12-208) ACR61389.1. Structurally it is similar to the human fatty acid synthase (FAS), a modular enzyme involved in the metabolism of fatty acids and a drug target of antineoplastic and anti-obesity agents. Detailed structural study on human FAS has been limited due to its size and flexibility. Large part of human FAS that encompasses the tandem domain of beta-KAS is closely related to the KS domain of *B. aureum.* The KS domains are appear as the canonical dimer, and its substrate-binding site differs from that of bacterial homologs but is similar to type II PKS of *B. aureum.*

According to domain analysis, the PKS is a multi-domain protein consists of 14 domains includes ASN glycosylation, PKC, CK2, beta KAS, ACP synthase III, thiolase C and N terminal, and KAS C terminal domains. The position of KAS domain is 1–151 and 159–212 AA residues. The AA composition of the protein is predicted with 48.58% of aliphatic (G,A,V,L,I), 5.19% of aromatic (F,W,Y), 2.83% of sulfur (C,M), 9.43% of basic (K,R,H),

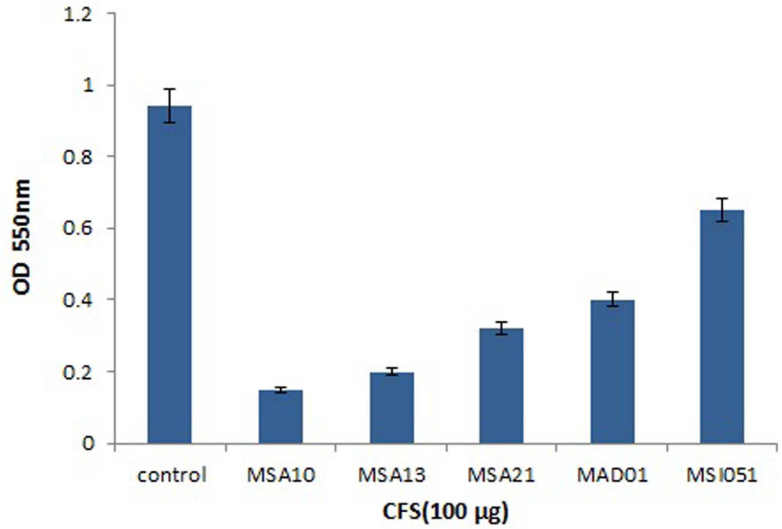


FIGURE 4 | Cell viability of *V. harveyi* treated with CFS of marine actinobacterial cultures.

16.51% of acidic (B,D,E,N,Q,Z), and 14.15% of aliphatic hydroxyl (S,T). Type II PKS (*Brachybacterium* sp. MSA21) ACS45382.1 is identical to type I PKS of *Streptomyces* sp. and structurally proposed to contain five domains as follows KAS C and KAS N, myristyl site, PKC, and thiolase. The tertiary structure of the protein depicts six alpha helix and six beta sheets and composed of 50.45% of aliphatic (G,A,V,L,I), 5% of aromatic (F,W,Y), 2.73% of sulfur (C,M), 11.36% of basic (K,R,H), 13.18% of acidic (B,D,E,N,Q,Z), and 13.18% of aliphatic hydroxyl (S,T) AAs.

The position of the KS domain is 1–159 and 167–220 AA residues. In PSI- BLAST, PKS is predicted to have a structure similar to chain A, the ACT ketosynthase chain length factor since 73% identity, the *E*-value: 6.61*e* – 67, bit-score: 256, aligned-length: 173, this protein is structurally related to ACT ketosynthase and proposed to be rich in acidic and aliphatic AA residues, since the ligands may be acetyl group/magnesium ion/sodium ion. Six alpha helix and five beta sheets., and the composition is 41.04% of aliphatic (G,A,V,L,I), 6.94% of aromatic (F,W,Y), 3.47% of sulfur (C,M), 13.87% of basic (K,R,H), 19.08% of acidic (B,D,E,N,Q,Z), and 10.40% of aliphatic hydroxyl (S,T). The ketosynthase (*Streptomyces dendra*) ABP57802.1 found to have seven domains includes CKII phosphorylation site, PKC, KS C terminal and N terminal, phage tail fiber repeat and the AA composition is 42.26% of aliphatic (G,A,V,L,I), 4.76% of aromatic (F,W,Y), 4.17% of (sulfur C,M), 13.69% of basic (K,R,H), 18.45% of acidic (B,D,E,N,Q,Z) and 11.90% of aliphatic hydroxyl (S,T). The structural configuration presents six alpha helix and four beta sheets and mimics the structure of chain A. The ACT ketosynthase CLF with the values as follows, *E*-value: 1.00*e* – 72, bit-score: 258, aligned-length: 191, and identity to query: 67%. The PSI-BLAST shares 99% of similarity with doxorubicin PKS (*E*-value 4*e* – 75), 3-oxoacyl-ACP synthase I (*Streptomyces avermitilis* MA-4680), putative ketosynthase of *Streptomyces antibioticus* with (*E*-value 4*e* – 60), and granaticin polyketide putative beta-KAS 1 of *Streptomyces hygroscopicus* ATCC 53653 (*E*-value 3*e* – 60). KS domain analysis of type II PKS and ketosynthase was performed using PSI BLAST and MEGA (CLUSTAL W2) to highlight the unique conservative motif of each protein. The strain *B. aureum* shares specific motif with BAH67362.1 (PKS *Streptomyces minoensis*) denoted as "VDTACSSSLVALHLAAQALRSG." Comparative analysis of KS domain of *Brachybacterium* sp. MSA21 exhibit the presence of unique motif PQQR(H)L in all the reference sequences which are capable of synthesizing cirramycin (BAH67190), minomycin (BAH67362), maridomycin (BAH67036), an anticancer compound (BAH67464), and platiomycin (BAH67144). This is the first report on the possible structural diversity mediated by type II PKS in *Brachybacterium* sp. MSA21.

KS Domain Phylogeny

Ketosynthase domain phylogeny was used to infer the phylogeny of type II PKS and ketosynthase. Phylogenetic analysis showed that the sponge associated actinobacterial sequences of PKS II genes and KS fragments were matched to conserved regions of previously characterized functional domains of other PKS I, II, and ketosynthase proteins. The KS domain of *Brachybacterium* and *Brevibacterium* showed a unique clustering, found KS domain of *Brevibacterium* clustering between two *Streptomyces* group and each group having two isolates and they possess high similarities among them like 100 and 98, respectively, but having less homology with *B. aureum* (**Figure 2**). The *Brachybacterium* was closely clustering with *S. albus* J1074 with 100% similarity. *N. alba* was not clustered with any actinobacterial KS domain since it was having the unique identity with KS domain of *Streptomyces* MAD01 and *S. dendra*. KS domain of *Streptomyces* MAD01 showing 97% of similarities with *S. purpeofuces* NBRC 14457 and *S. dendra* was clustering between *Streptomyces* sp. JS-14 and *S. macrosporeus* with 98 and 97% similarity, respectively. From the cluster analysis, we observed that two different marine

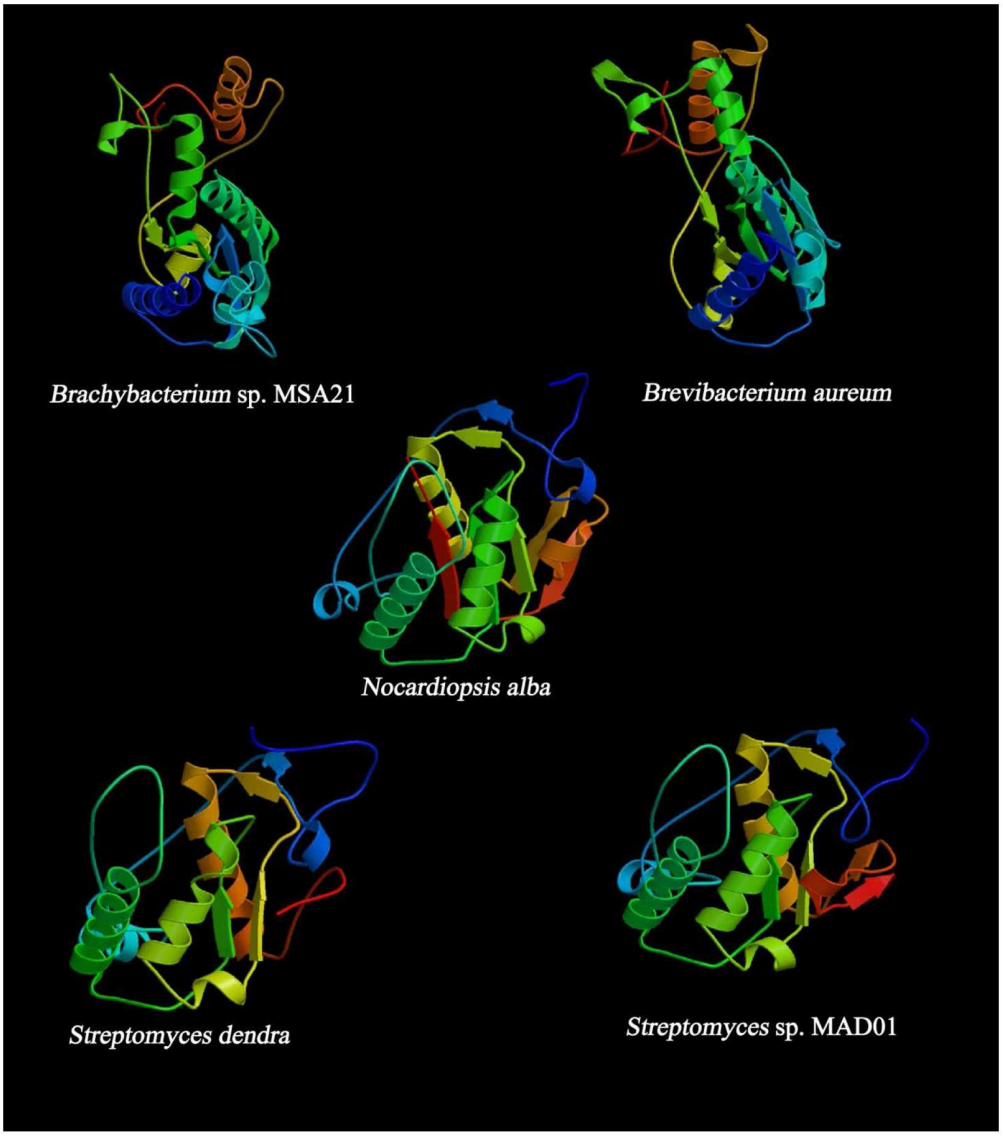


FIGURE 5 | Predicted secondary structure of KS domain using $(PS)^2$: Protein Structure Prediction Server.

sponge-associated actinobacteria possessing the identical KS domains of iterative PKS.

The major aim of this study was to find out the gene diversity of the PKS II and ketosynthase in two marine sponge associated actinobacterial polulation. The KS gene diversity could be useful to understand the evolution pattern of actinobacteria in the marine sponges, mode of interaction between sponge and associated microbes (Selvin, 2009) and chemical diversity of PKS II in marine sponge. Phylogenetic analysis of iterative PKS sequences is highly correlated with the number of iterations they performs. The PKS gene analysis provide a new insights that the poorly studied genera, such as *Brevibacterium* and *Brachybacterium* represent the KS genes which proves the unexplored resource for natural-product discovery. Conversely, the nearly ubiquitous detection of PKS genes in *Streptomyces* and *Nocardiopsis* envisages the possibility similar kind of natural products, but in reality the compounds are expected to be highly complex with diverse bioactivities (Selvin, 2009; Selvin et al., 2009b). To overcome these challenges, KS domain of PKS genes retrieved and analyzed in this study. KS domains tend to cluster phylogenetically based on the secondary metabolites of the actinobacterium from which the gene was retrieved. The active KS domains predictions could be based simply on the analysis of around 500 bp regions of KS domain from single PKS gene. The level of KS sequence domain in the iterative biosynthesis of natural products needs to be determined. The level of KS domain in strains may differ as it depends on the rate of sequence evolution, niche selectivity, host evolution pattern, and the time of pathways have been isolated in the respective genomes. It is also of interest that the three KS sequences associated

with iterative type II PKS pathways were widely distributed among diverse taxonomic groups. The fact that all these KS domain sequences display relatively low levels of identity to the ketosynthase domain of *Streptomyces* MAD01 and *S. dendra* suggests that they are not associated with the production of iterative type of PKS domains. The mixed clustering of different sponge associated KS domains already been documented here for the first time we are reporting the evolutionary relatedness of KS domains of type II PKS and ketosynthase from *D. nigra* and *F. cavernosa* isolates. According to recent literatures, the PKS genes and their products exhibit novel insights in antimicrobial drug discovery (Selvin, 2009; Sasso et al., 2014; Wang et al., 2014). KS domain of type II PKS phylogeny is also highly need to know their relationship and structural diversity.

Potential Linkage Between Iterative Type II PKS Gene and Lipopeptide and Glycolipid Biosurfactant Production in Marine Sponge-Associated Actinobacteria

The marine sponge-associated bacteria have been recognized as rich source of biological macromolecules that are of potential interest to various industrial sectors (Kiran et al., 2015). Study reports evidenced that marine actinobacteria are unexplored resource for biosurfactant production. In this study, three actinobacterial strains (MSA10, MSA13, and MSA21) isolated from marine sponge were able to produce lipopeptide and glycolipid biosurfactants, respectively and showed positive for type II PKS gene. Two actinobacterial strains (MAD01 and MSI051) from *D. nigra* failed to produce biosurfactants but has the capability to synthesis polyketide based antagonistic compounds. There is an existing evidence for the synthesis of lipopeptide biosurfactant in *Bacillus subtilis* by NRPSs or hybrid PKS/NRPSs (Ongena and Jacques, 2008). These modular proteins in marine sponge associated microbes are responsible for the biosynthesis of several bioactive metabolites. They are mega-enzymes structured by iterative functional units called modules catalyzes various condensation, reduction, transferase reactions leading to polyketide and peptide transformation for the synthesis of biosurfactant. The positive strains display biosurfactant activity and, significantly, iterative type II PKS domain gene fragments, indicating the existence of a PKS gene cluster associated with biosurfactive compound biosynthesis. The present study reveals that the actinobacteria are a rich source of bioactive compounds and biosurfactant, and also represent the unrecognized group of organisms having type II PKS systems for polyketide biosynthesis. In this study, the bacterial motility was also checked for the surfactive compound production (data not shown). Bacterial motility mechanisms, comprising swimming, swarming, and twitching, are known to have significant roles in biofilm formation, colonization, and the subsequent expansion into complete organized surface populations. All the actinobacterial strains showed positive in swimming, swarming, and twitching motility assays which indicate that these strains possess biofilm forming ability. The strains with increased swimming motility also possess good swarming ability. Current research evidenced that the strain *Streptomyces* sp. MAD01 possess good biofilm forming capacity as well as antimicrobial activity against test organisms. It also proves that a ketosynthase type II PKS system is responsible for the biosynthesis of the antagonistic compounds in marine actinobacteria. Based on the present findings, the production of biosurfactants might be linked with type II iterative PKS gene cluster and the synthesis of biosurfactant by the sponge-associated actinobacteria might have significant role in the chemical ecology of host sponge (Kiran et al., 2015). However, the hypothesis has not been tested in controlled *in vitro* and *in vivo* experiments. Based on the functions of biosurfactants including antibacterial/antibiofilm activity, the biosurfactants may play a role in host defense fouling processes (Gandhimathi et al., 2009; Kiran et al., 2014). Therefore, explorations of marine sponge-associated actinobacteria for the lipopeptide and glycolipid biosurfactant production will have wider applications in industrial processes, bioremediation, and enhanced oil recovery.

CONCLUSION

In the current study, the iterative nature of actinobacterial type II PKS was proved by HMM profile. The domain architecture of *N. alba* and *B. aureum* have the potential of constructing "minimal PKS" and the later species share the specific motif "VDTACSSS" with *S. minoensis*. Both strains displayed PKS domains structurally similar with encoding ACT. *S. dendra* is found to have a unique repeat called phage tail fiber repeat which is responsible for altering the host specificity of secondary metabolites through protein–protein interaction. The other three actinobacterial strains *Brachybacterium* sp., *Streptomyces* sp., and *S. dendra* lack ACP results in inactive minimal PKS or may act non-iteratively. This study also provides a new insight on the KS genes of *Brevibacterium* and *Brachybacterium* proving that marine resources are still largely unexplored for natural-product discovery. In these regards, *in silico* gene mining is quite useful for prospecting novel metabolites produced by marine sponge endosymbionts. Further *in vitro* studies are needed to design novel natural products using a biosynthetic engineering approach.

AUTHOR CONTRIBUTIONS

JS and GSK designed the experiments, GS performed *in silico* analysis, ANL performed *in vitro* assays, NAA and MV performed data analysis and interpretation, GSK, GS, and JS written the manuscript.

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REVIEW
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Quorum Sensing: An Under-Explored Phenomenon in the Phylum *Actinobacteria*

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Quorum sensing is known to play a major role in the regulation of secondary metabolite production, especially, antibiotics, and morphogenesis in the phylum *Actinobacteria*. Although it is one of the largest bacterial phylum, only 25 of the 342 genera have been reported to use quorum sensing. Of these, only nine have accompanying experimental evidence; the rest are only known through bioinformatic analysis of gene/genome sequences. It is evident that this important communication mechanism is not extensively explored in Actinobacteria. In this review, we summarize the different quorum sensing systems while identifying the limitations of the existing screening strategies and addressing the improvements that have taken place in this field in recent years. The γ-butyrolactone system turned out to be almost exclusively limited to this phylum. In addition, methylenomycin furans, AI-2 and other putative AHL-like signaling molecules are also reported in Actinobacteria. The lack of existing screening systems in detecting minute quantities and of a wider range of signaling molecules was a major reason behind the limited information available on quorum sensing in this phylum. However, recent improvements in screening strategies hold a promising future and are likely to increase the discovery of new signaling molecules. Further, the quorum quenching ability in many Actinobacteria has a great potential in controlling the spread of plant and animal pathogens. A systematic and coordinated effort is required to screen and exploit the enormous potential that quorum sensing in the phylum *Actinobacteria* has to offer for human benefit.

Keywords: Actinobacteria, *Streptomyces*, *Mycobacterium*, quorum sensing, GBL, MMFs, c-di-GMP, quorum quenching

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INTRODUCTION

Cell-to-cell communication in bacteria via quorum sensing is a density-dependent regulation of gene expression. The system relies on two major components, a signaling molecule and a transcriptional activator protein. In many Gram-negative bacteria, a member of the N-acylhomoserine lactone (AHL) family acts as a diffusible signal molecule, the synthesis of which is controlled by the members of the LuxI family of synthases (**Figure 1**). Above a threshold concentration, this signal molecule activates target genes in conjunction with a member of the LuxR family of transcriptional activators (Fuqua et al., 1996). The AHL-based quorum sensing system plays major role in regulating multiple functions such as bioluminescence (Nealson and Hastings, 1979), synthesis of antibiotics (Bainton et al., 1992), the production of virulence

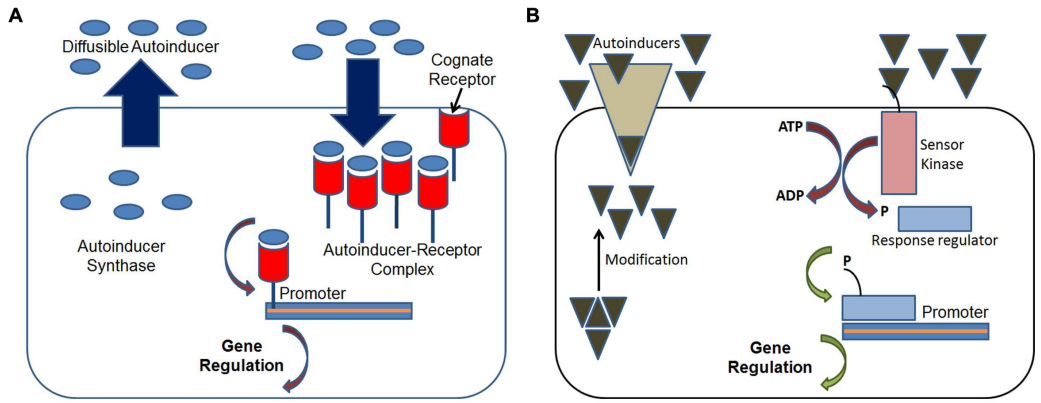

FIGURE 1 | Schematics of quorum sensing systems in bacteria. (A) Gram-negative bacteria: at threshold concentrations the diffusible autoinducer signaling molecule, typically a homoserine lactone, binds to its cognate receptor inside the cell forming the autoinducer-receptor complex which then regulates the expression of target genes through its binding to the target promoter; and **(B)** Gram-positive bacteria: at threshold concentrations the autoinducer peptide molecule which is actively transported activates the sensor kinase protein inside the cell which phosphorylates the response regulator protein, which then regulates the expression of target genes through its binding to the target promoter. Adapted from Koh et al. (2013).

factors (Barber et al., 1997), exopolysaccharide biosynthesis (Beck von Bodman and Farrand, 1995), bacterial swarming (Eberl et al., 1996), and plasmid conjugal transfer (Fuqua and Winans, 1994). In contrast, most Gram-positive bacteria use processed oligo-peptides for signaling and communication (Kleerebezem et al., 1997; Sturme et al., 2002). These signals, referred to as autoinducing polypeptides (AIPs) are produced in the cytoplasm as precursor peptides and are subsequently cleaved, modified, and exported. The AIP-based quorum-sensing systems are known to regulate the expression of many factors such as genetic competence (Solomon et al., 1995), sporulation (Magnuson et al., 1994), and virulence factor expression (Qin et al., 2000). While it may seem that the differentiation in the type of signaling compound is a consequence of the structural differences in the cell wall between the two bacterial types; however, this is not the case. For instance, certain Actinobacteria (Gram-positive) are known to use γ-butyrolactones for signaling, whereas most Gram-negative bacteria are known to possess signaling peptides as part of their genome (Lyon and Novick, 2004). Regardless of the cell type, quorum sensing is a near universal mode of cell-to-cell communication amongst pathogenic bacteria. Hence, it is now considered an important target for controlling their spread, especially antibiotic resistant bacteria.

Despite the diversity and importance of the phenotypes that are regulated by the quorum sensing network, the information on their environmental distribution is very limited. Further, those that are available, only focus on the AHL-mediated gene expression systems. A survey by Manefield and Turner (2002) showed that merely 2.2% (21 bacterial genera) of the total number of bacterial genera listed in the *Bergey's Manual of Systematic Bacteriology* (Garrity et al., 2001) are known to harbor the AHL producing species, and all of which belong to the alpha, beta and gamma proteobacteria only. At the species level, the percentage of AHL producers drops to a fraction of a percent. Although the estimate is more than a decade old, it still reflects on the state of the information available on quorum sensing in bacteria. Motivated by this lack of information, our screening for *luxRI* homologs and AHL production in the genus *Aeromonas* not only revealed that the homologs are universally present in this genus, but also reported that a wide diversity of AHLs are secreted by the species in the genus (Jangid et al., 2007, 2012). This study only points to the fact that quorum sensing is indeed a widespread phenomenon among bacteria, however, a systematic evidence is lacking. Thus, there is a need to survey the existence and study the taxonomic distribution of the quorum sensing systems amongst bacterial taxa.

The phylum *Actinobacteria* is one of the largest phyla within domain *Bacteria* and consists of six classes, 23 orders including one *Incerta sedis* and 53 families (Ludwig et al., 2012). As of December 2015, there were 342 genera in this phylum with standing in nomenclature as determined from the LPSN database (Parte, 2015). Actinobacteria are typically Gram-positive but at times stain-variable and have a rigid cell wall that contains muramic acid with some containing wall teichoic acids. The phylum comprises of a plethora of phenotypically diverse organisms, with widespread distribution in nature and exhibiting varied oxygen, nutritional, temperature, and pH requirements for growth, making it an important phylum.

Their diverse physiological potential makes Actinobacteria a dominant role player in the biotechnology industry. Their applications are widespread and vary from agroindustry, pharmaceuticals, bioremediation among numerous others. They play a key role in natural geochemical cycles, especially through their ability to decompose organic matter. Actinobacteria are also abundant in the rhizosphere and produce a wide range of biologically active metabolites, thereby influencing plant development (Selvakumar et al., 2014). Many Actinobacteria are also known pathogens of plants and animals. However, amongst the most important potential of Actinobacteria, it is the production of a significant number of secondary metabolites like antibiotics and other compounds of biotechnological interest that has been exploited most. For instance, among

the polyene macrolides, a class of polyketides which are antifungal compounds, are synthesized by more than 100 different species of actinomycetes (Recio et al., 2004). In addition, members of the genus *Streptomyces* are known to produce more than 70% of commercially available antibiotics (Weber et al., 2003). The expression of virulence determinants, production of secondary metabolites, and morphogenesis is associated with high cell densities and typically controlled by diffusible low molecular weight chemical substances, similar to the Gram-negative autoinducer, suggesting a role of quorum sensing in regulating these mechanisms (Takano, 2006; Santos et al., 2012). Further exploration of novel phenotypes under quorum sensing regulations is likely to contribute to the advancement in medical, biotechnological and ecological fields. Hence, there is a need of studying quorum sensing in *Actinobacteria*.

Most of what is known about quorum sensing in Actinobacteria, comes from the study of antibiotic production in this taxa. While it is indeed the most important phenomenon, the aim of this review is not to present an overview on the quorum sensing regulation of antibiotic production. The reader is therefore directed to read Takano (2006), Liu et al. (2013) and references within. Further, for clarity Actinobacteria means all species within the phylum *Actinobacteria*, unless otherwise stated as class *Actinobacteria*.

In this review, we present an overview of quorum sensing systems described so far for the phylum *Actinobacteria*, indicating the limitations of existing screening strategies and addressing improvements in newer technologies for the discovery of quorum sensing in more taxa. In addition, we summarize the current status of known quorum quenching activity in this phylum.

QUORUM SENSING IN THE PHYLUM *Actinobacteria*

Although Actinobacteria is one of the largest groups of organisms in the bacterial domain, very few reports were available for known quorum sensing regulation in the phylum. An analysis of literature for quorum sensing in Actinobacteria revealed that only 25 actinobacterial genera have some sort of quorum sensing regulation (**Figure 2**). This number represents a mere 7.3% of the 342 genera reported in the latest update of LPSN (Parte, 2015). Of these, only nine genera (2.6%) have known quorum sensing regulation, whereas remaining 16 genera (4.7%) are known to only harbor the homologs of LuxR based on the analysis of available gene/genome sequences. It is noteworthy that 24 of the 25 genera belonged to the single class *Actinobacteria* whereas only a single genus *Rubrobacter* belonged to the class *Rubrobacteria*. No reports were available for the other four actinobacterial classes: *Acidimicrobiia*, *Coriobacteriia*, *Nutriliruploria*, and *Thermoleophilia*. This short list in fact suggests that an enormous scope exists for screening more taxa for further exploration of quorum sensing in Actinobacteria.

One quorum sensing system that seems to be limited to Actinobacteria is the γ-butyrolactone (GBL) system. The GBL system is quite similar to the AHL-based system in Gram negative bacteria due to the structural similarity between GBL and AHL, as well as that it is a one-component system where the communication molecule sensing protein is also the response regulator (Takano, 2006). Most reports on the GBL-system come from the genus *Streptomyces* which produces numerous important secondary metabolites and undergoes a sophisticated morphological differentiation program (Horinouchi and Beppu, 1993; Takano et al., 2000) (**Table 1**). In most cases, these processes are under the direct control of GBL autoregulator in tandem with specific cognate GBL receptors (Healy et al., 2009). The membrane-diffusible GBL autoregulator controls the expression of structural genes encoding secondary metabolite pathway enzymes. The GBL receptors are transcriptional regulators belonging to the TetR superfamily of transcription factors (Nishida et al., 2007). Given the large number of species in the genus *Streptomyces* and the very few GBL regulatory systems known, lot more work on the signaling cascade and receptor proteins is required.

With the exception of the well characterized GBL-based system of *Streptomyces* sp. (Takano, 2006), communication in this phylum has been scarcely explored. Based mostly on indirect evidence, Santos et al. (2012) made a significant contribution toward increasing the number of genera known to harbor LuxR homologs. A diversified and stereoscopic organization of LuxR proteins among members of this phylum was reported through an extensive *in silico* analysis of the phylogenomic distribution and functional diversity of the LuxR proteins. The authors identified a total of 991 protein sequences from 53 species that contained at least one LuxR domain. The distribution of these sequences was not even among species and ranged from organisms with a single sequence (e.g., *Mycobacterium leprae*) to others with over 50 (e.g., *Streptomyces* sp.). Using a domain-based strategy, the LuxR family of proteins in Actinobacteria was shown to include two major subfamilies: one that resembled the classical LuxR transcriptional regulators and another in which the LuxR domain is associated with N-terminal REC domain (receiver). In a third and smaller group of sequences, LuxR domain appears associated with a series of signal transduction-related domains other than REC, forming multidomain proteins (Santos et al., 2012). From the evolutionary perspective, it was shown that the ancestor gene sequence codified for a protein with a single LuxR domain. The original LuxR-encoding genes then suffered a series of duplications presumably followed by functional specification, but they also acquired different domains, originating new subfamilies with implications in a wide range of functionalities. The phylogenetic results described suggested a conspicuous promiscuity of the LuxR domain among Actinobacteria. For details on the distribution of the LuxR proteins within the phylum, the reader is suggested to refer to the original study (Santos et al., 2012).

Selective Actinobacteria with Known Quorum Sensing Systems

Streptomyces

The genus *Streptomyces* with 778 species (Parte, 2015) is the largest genus of *Actinobacteria* and is a natural inhabitant of

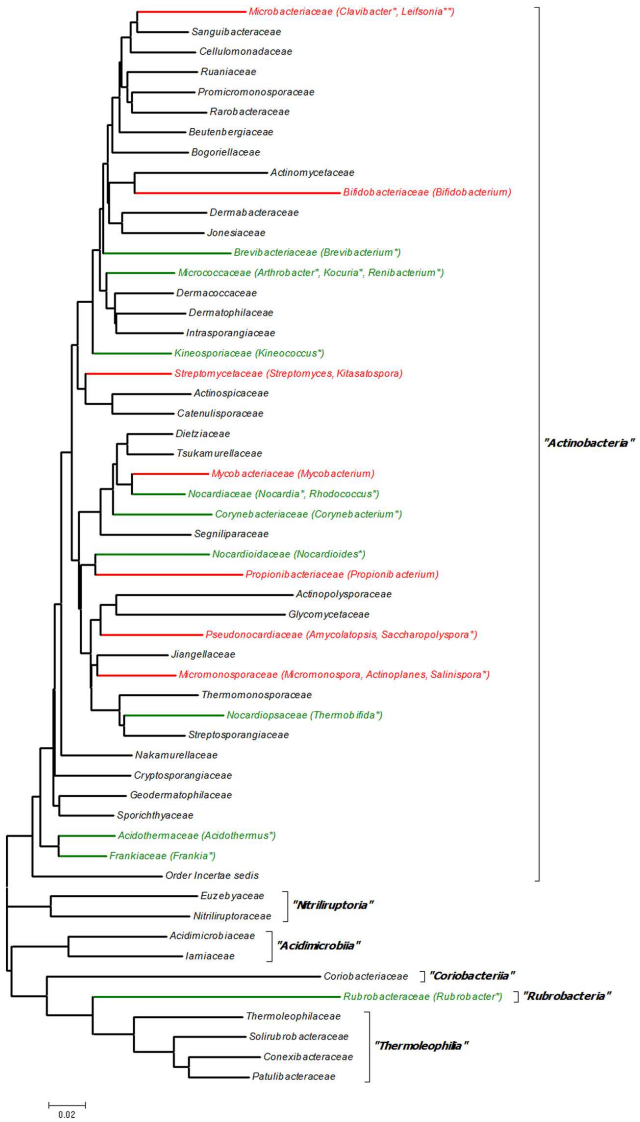

FIGURE 2 | 16S rRNA gene sequence based family tree of the phylum *Actinobacteria* depicting the genera with known quorum sensing systems. The evolutionary history was inferred using the Neighbor–Joining method (Saitou and Nei, 1987) using the bootstrap test of phylogeny (1000 replicates) (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). A total of 54 sequences were used for constructing the tree and belonged to the type species of the type genera of each family as described in Ludwig et al. (2012) and are submitted as Supplementary Data in fasta format. Black color branch denotes family with no evidence of quorum sensing; red color denotes family with experimental evidence of quorum sensing; green color denotes family for which only gene homologs are known; '*' indicates genus for which only gene homologs are known; and '**' denotes genus for which putative AHL-like signal molecules are involved in quorum sensing.

soils and decaying vegetation. *Streptomyces* are characterized by its complex morphological differentiation and their ability to produce a variety of secondary metabolites, contributing to two-thirds of naturally occurring antibiotics. The synchronized behavior of these species in producing antibiotics and modulation of gene expression is governed by quorum sensing through a spectrum of small chemical signaling molecules, called GBLs (Bhukya et al., 2014). GBLs diffuse freely through the cell membrane and regulate these pathways when the intra and extracellular concentrations of GBLs reaches a threshold. In this sense, they behave very similar to the AHL-based quorum sensing in Gram-negative bacteria.

Much of what is known in actinobacterial quorum sensing could be attributed to the information gained from GBL-based quorum sensing in *Streptomyces*. In fact, the first signaling molecules, the GBLs, were already known from *Streptomyces* in the 1960s (Khokhlov et al., 1967) much before the term 'quorum sensing' was coined by Fuqua et al. (1994). Today, at least 60% of *Streptomyces* species appear to produce GBLs regulating multiple phenotypes even in nano Molar concentrations (Takano et al.,

TABLE 1 | Status of quorum sensing systems in *Actinobacteria*.

S. No.	Genus	Signal type	Proteins/Homologs Involved/Domain Architecture	Phenotypes regulated	Reference
Actinobacterial genera with experimental evidence of quorum sensing					
1	*Actinoplanes*	VB-type		Medically important secondary metabolite	Choi et al., 2003
2	*Amycolatopsis*	IM2-type		Medically important secondary metabolite	Choi et al., 2003
3	*Bifidobacterium*	Autoinducer AI-2	LuxS, LuxR_C_Like, REC	Biofilm formation	Santos et al., 2012; Sun et al., 2014
4	*Kitasatospora*	GBL	KsbA	Bafilomycin production	Choi et al., 2004
5	*Leifsonia*	Putative AHL signal	LuxR_C_Like, REC		Santos et al., 2012; This study
6	*Micromonospora*	IM2-type with long C2 chain		Medically important secondary metabolite	Choi et al., 2003
7	*Mycobacterium**	cAMP and cGMP, ppGpp, c-di-GMP and c-di-AMP	AAA, CHD, HDc, LuxR_C_Like, MAP0928, REC, WhiB3	Biofilm formation and pathogenicity	Banaiee et al., 2006; Takano, 2006; Chen and Xie, 2011; Santos et al., 2012; Sharma et al., 2014
8	*Propionibacterium*	Autoinducer AI-2	LuxR_C_Like, REC	Biofilm formation and upregulation of virulence factors	Coenye et al., 2007; Santos et al., 2012; Lwin et al., 2014
9	*Streptomyces**	GBLs, MMFs, Factor-A, Factor-I, IM-2, VB, PI factor	AAA, AlpZ, AplW, ArpA, Aur1R, AvrA, BarA, BarB, Brp, CprA, FarA, JadR2, LuxR_C_Like, MmfR, NcsR2, Orf74, Orf79, Orf82, REC, SabR, SAV2268, SAV2270, SAV3702, ScbA, ScaR, ScbR, SCO6286, SCO6323, Sng, SpbR, TarA, TPR, TylP, TylQ	Production of antibiotics (Act, Clavulanic acid, Cephamycin, D-cycloserine, Kas, Methylenomycin, Natamycin, Nikkomycin, Nucleoside, Pristinamycin, Red, Streptomycin, Tylosin, Virginiamycin), morphogenesis and sporulation	Recio et al., 2004; Takano, 2006; Gottelt et al., 2012; Santos et al., 2012 (and references within); Willey and Gaskell, 2011
Actinobacterial genera with only bioinformatic evidence of quorum sensing					
10	*Acidothermus*		LuxR_C_Like, REC		Santos et al., 2012
11	*Arthrobacter**		AAA, LuxR_C_Like, REC		Santos et al., 2012
12	*Brevibacterium*		Transcriptional regulator (GenBank: ZP_00378009)		Takano, 2006
13	*Clavibacter*		LuxR_C_Like, REC		Santos et al., 2012
14	*Corynebacterium*		LuxR_C_Like, REC		Santos et al., 2012
15	*Frankia*		AAA, LuxR_C_Like, REC		Santos et al., 2012
16	*Kineococcus*		AAA, LuxR_C_Like, REC		Santos et al., 2012
17	*Kocuria*		LuxR_C_Like, REC		Santos et al., 2012
18	*Nocardia*		FHA, LuxR_C_Like, REC, Transcriptional regulator (GenBank: BAD59728, BAD55455)		Takano, 2006; Santos et al., 2012
19	*Nocardioides**		HDc, LuxR_C_Like, REC		Santos et al., 2012
20	*Renibacterium*		LuxR_C_Like, REC		Santos et al., 2012
21	*Rhodococcus**		AfsA, ArpA, CSP_CDS, FHA, HDc, LuxR_C_Like, PBD2.026, PKC, REC, TPR, Similar to VB-R (Genbank: AAR90230), Transcriptional regulator (GenBank: AAR90151)	Plant pathogenesis, Biocontrol agent	Takano, 2006; Wuster and Babu, 2008; Santos et al., 2012; Latour et al., 2013
22	*Rubrobacter*		LuxR_C_Like, PAS, REC		Santos et al., 2012
23	*Saccharopolyspora*		LuxR_C_Like, REC, SeaR, TPR		Takano, 2006; Santos et al., 2012
24	*Salinispora*		LuxR_C_Like, REC		Takano, 2006; Santos et al., 2012
25	*Thermobifida*		LuxR_C_Like, REC, TPR		Santos et al., 2012

Information used in the table was derived from the references cited here and some taxa may have been missed. '' denotes genera with known quorum quenching ability that also includes Microbacterium which is not shown in here as no known quorum sensing evidence exists for it.*

2000). Most GBLs are structurally similar but chemically distinct. They are extractable in organic solvents and are heat, protease, and acid resistant. Although they share structural similarity with AHLs (except for the carbon side-chain, **Figure 3**), the GBL receptors do not bind to AHL or vice-versa. At the genomic level, a lineage-specific LuxR protein homolog with a very limited diversity of associated domains is known to exist in *Streptomyces* (Santos et al., 2012).

Their molecular mechanism reveals a diverse and complex system (Choi et al., 2003). The most intensively studied GBL is A-factor or the autoregulatory-factor (2-isocapryloyl-3*R*-hydroxymethyl-g-butyrolactone), which is known to control the expression of more than a dozen genes, amongst which streptomycin production and sporulation in *Streptomyces griseus* are the most extensively studied (Takano et al., 2000) (**Figure 4**). It is known to exert its effects on both clonal hyphae in a single mycelium as well as genetically distinct *S. griseus* hyphae. A-factor likely diffuses between filaments and acts by biding with the A-factor receptor, ArpA which is a transcriptional repressor that targets *adpA*. Upon binding, the A-factor-ArpA complex activates *adpA* expression (Willey and Gaskell, 2011). A suit of genes are under the AdpA-dependent activation, such as *strR* whose expression regulates the streptomycin biosynthetic gene cluster, and genes that are involved in morphological differentiation. All the characteristics of A-factor tell us that A-factor is a microbial hormone comparable to eukaryotic hormones such as the sex pheromones controlling differentiation in fungi (Horinouchi and Beppu, 1993). However, it is neither the most abundant nor the most stable GBL. The *S. coelicolor* butanolide 1 (SCB1), reported previously to stimulate blue-pigmented polyketide actinorhodin (Act) and the red-pigmented tri-pyrolle undecylprodigiosin (Red) production in a growth phase-dependent manner, is known to be most abundant and more stable than A-factor (Takano, 2006). The genes involved in the synthesis of SCB1 (*scbA*) and binding (*scbR*) have been identified (Gottelt et al., 2012). ScbR regulates transcription of both scbA and itself by binding to the divergent promoter region controlling both genes, and the GBL SCB1 inhibits this binding. *S. coelicolor* is known to produce multiple GBLs with distinct biological activities. Similarly, *S. virginiae* produces at least five virginiae butanolides (VB-A, B, C, D, and E) that stimulate virginiamycin production, each with a different minimum effective concentration. In contrast, both *S. griseus* and *S. lavendulae* produce a single GBL, the A-factor and IM-2, respectively. While A-factor regulates streptomycin production in *S. griseus*, IM-2 regulates the production of nucleoside antibiotics showdomycin and minimycin in *S. lavendulae* (Gottelt et al., 2012). The presence of multiple GBLs in *Streptomyces* is an indication of the complex communication mechanisms that exist in this genus and have still not been explored in great details.

A new class of water soluble autoinducer different from the GBLs was reported by Recio et al. (2004). This factor, called the PI Factor was identified as 2,3-diamino-2,3-bis(hydroxymethyl)-1,4-butanediol (**Figure 3**). It was isolated from *S. natalensis* and regulates Pimaricin biosynthesis in the organism. By using complementation assay, pimaricin production was restored in the presence of the A-factor in a pimaricin-impaired mutant. Similar to other GBLs, the PI factor is also active at nano Molar concentrations. However, the restoration of pimaricin production in the presence of both A-factor and PI factor suggests that *S. natalensis* has an integration of multiple quorum signals from actinomycetes. Interestingly, the PI factor has not been reported in the microbial world and has an entirely novel chemical structure that is only distantly related to the homoserine lactone and furanosyl diester inducer families. These unique properties of PI factor only point to the fact that this taxa holds a great potential for further exploration of quorum sensing.

In addition to GBLs, methylenomycin furans (MMFs) have recently been shown to regulate antibiotic production in *S. coelicolor* via quorum sensing (Willey and Gaskell, 2011). Different *S. coelicolor* mutants that were deficient in methylenomycin production, would co-synthesize the antibiotic when grown in close proximity of each other, suggesting that a diffusible signal was involved in its biosynthesis. Between the two, the mutant strain that rescued the non-producer is called the 'secretor', whereas the one that regained the capacity to produce the antibiotic when grown near secretor is called the 'convertor'. Studies have shown that the secretor strains possess the ability to synthesize small signaling molecules similar to GBLs, called MMFs, but themselves lack the methylenomycin biosynthetic genes, while the opposite is true of converters. While being very similar to GBLs in chemical properties, the MMFs are structurally distinct with a common 2-alkyl-4-hydroxymethylfuran-3-carboxylic acid core but a different C2 alkyl group (**Figure 3**). The discovery of MMFs only points to the fact that the exploration of quorum sensing in Actinobacteria is very limited and the possibility of discovering such novel homologs is not farfetched, it just needs a systematic approach (Willey and Gaskell, 2011).

Mycobacterium

Mycobacteria hold an extreme medical importance worldwide. *Mycobacterium tuberculosis* is a successful human pathogen, with $\sim 2 \times 10^9$ individuals; nearly one-third of the world's population infected globally (Banaiee et al., 2006). The distinguishing feature of mycobacteria is the presence of thicker cell wall which is rich in mycolic acids and a very slow growth rate. With the emergence of drug-resistance, treating mycobacterial infections is becoming increasingly difficult and hence, looking for newer drug targets, especially those involving quorum sensing is an essential component of mycobacterial research. However, the Gram positive mycobacteria remain a mystery with no clear evidence known about their quorum sensing mechanism (Sharma et al., 2014). Bioinformatics analysis has revealed the presence of LuxR homologs in *M. tuberculosis*, but the experimental supports are lacking (Chen and Xie, 2011; Santos et al., 2012). Some of these homologs are ubiquitous across the multiple mycobacterial species and are involved in mycobacterial biofilm formation or persistence, suggesting a possible existence of similar quorum sensing mechanisms. Given the fact that biofilm formation is mostly linked with quorum sensing regulation and with many non-tuberculous

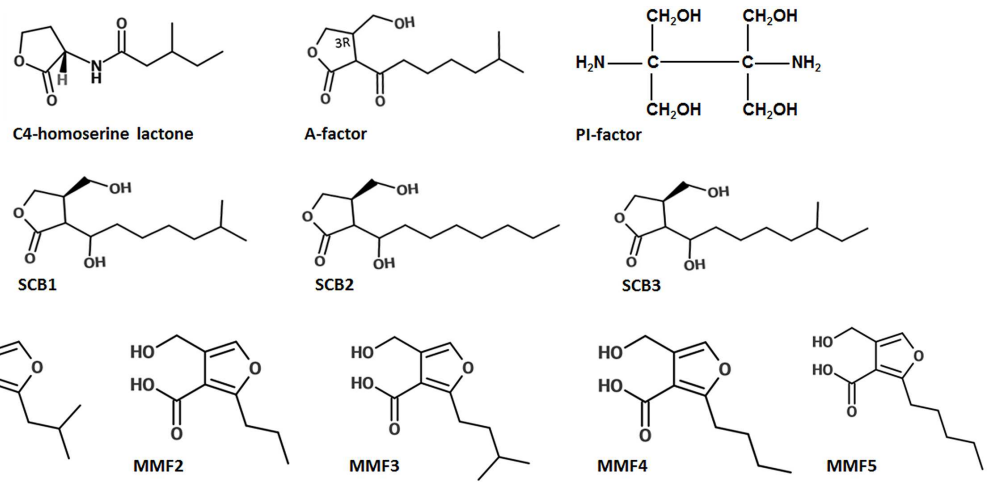

FIGURE 3 | Structures of representative signaling molecules in *Actinobacteria*. The A-factor of *Streptomyces griseus*, the GBLs of *S. coelicolor* (SCB1, SCB2, and SCB3), MMFs of *S. coelicolor* which are structurally distinct sharing a common 2-alkyl-4-hydroxymethylfuran-3-carboxylic acid core structure but differ in the identity of the C2 alkyl group. The C4-homoserine lactone of *Pseudomonas aeruginosa* is shown for comparison. Adapted from Willey and Gaskell (2011).

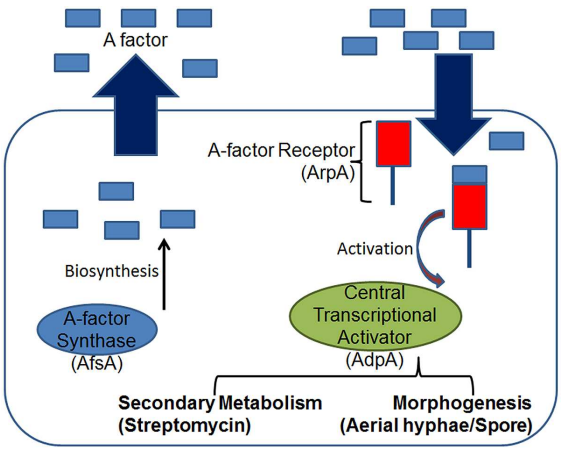

FIGURE 4 | The *S. griseus* A-factor regulon. Similar to Gram-negative bacteria, at threshold concentrations the diffusible A-factor (a γ-butyrolactone) binds the intracellular receptor ArpA and activates expression of the transcriptional activator AdpA which in-turn regulates multiple phenotypes either indirectly via a multi-step cascade, such as the development of aerial hyphae and sporulation, or directly, such as the production of secondary metabolites like streptomycin.

mycobacteria known to form biofilms, such as *M. smegmatis, M. marinum, M. fortuitum, M. chelonae, M. ulcerans, M. abscessus, M. avium,* and *M. bovis* (Sharma et al., 2014), the existence of quorum sensing in these organisms cannot be ruled out. However, this hypothesis needs experimental validation.

The evidence of quorum sensing in Mycobacteria is mostly indirect. The *M. tuberculosis whiB3* gene, a putative transcriptional regulator that was recently implicated in causing gross and microscopic lesions, is likely to be under quorum sensing regulation (Banaiee et al., 2006). Although no evidence was presented, the expression pattern of *whiB3* was shown to reflect changes in bacterial density thereby suggesting a role for quorum sensing in its regulation. A survey of 22 *M. tuberculosis* genes showed that *whiB3* was induced maximally during the early phase of infection in the mouse lung and cultured macrophages. The expression of *whiB3* inversely correlated with bacterial density in the mouse lung, BMMϕ medium, and broth culture (Banaiee et al., 2006). Since this pattern of expression is consistent with quorum sensing, further studies are warranted to study this system in *M. tuberculosis*.

Another indirect evidence of the involvement of quorum sensing regulation in mycobacteria is known through the studies on second messengers. Second messengers are those compounds that are involved in the signal transduction phosphorelay cascade enabling the 'decoding' of the 'coded' information received in the form of quorum sensing molecules (autoinducers) to sense and bring appropriate changes in their environment by expression of target genes (Bharati and Chatterji, 2013). Thus, inter- and intra-cellular signaling must be integrated. A variety of small molecules, such as, mono (cAMP and cGMP) and di-cyclic or modified nucleotide (ppGpp, c-di-GMP, and c-di-AMP), are important intracellular signaling molecules in mycobacteria and play a key role in relaying the signals received from the receptor (on the surface) to the target molecule in the cell (Sharma et al., 2014). These nucleotide-based second messengers regulate different processes in various bacterial systems. Of these, c-di-GMP is a ubiquitous bacterial second messenger and in effective concentrations it is known to facilitate phenotypes, such as virulence and biofilm formation. The involvement of these second messengers indirectly implies the existence of quorum sensing systems in both the pathogenic and non-pathogenic mycobacteria (Sharma et al., 2014).

Propionibacterium

Propionibacterium acnes is an anaerobic Gram-positive rod shaped bacterium which is a natural inhabitant of human skin. It plays an important role in the pathogenesis of acne vulgaris, a common disorder of the pilosebaceous follicles. However, as the infection progresses the organisms shows resistance to antibiotics. In fact, there has been a gradual decrease in the efficacy of topically applied erythromycin, most likely due to the development of resistance via biofilm formation. Indeed, genomic analysis of *P. acnes* shows that the organism has three separate gene clusters that code for enzymes involved in extracellular polysaccharide biosynthesis, suggesting that it is capable of forming the necessary extracellular biofilm matrix (Coenye et al., 2007). Further experimentation revealed that the organism is able to form biofilms as well as showed increased production of the autoinducer AI-2 by sessile cells of *P. acnes* and the upregulation of its virulent activity, such as hydrolysis of sebum triglycerides by its bacterial lipases, secreting free fatty acids (FFAs) such as oleic, palmitic, and lauric acids. The increased concentration of such irritant fatty acids is thought to contribute to the inflammation and thereby plays an important role in the pathogenesis of acne. While the discovery of AI-2 suggested the presence of quorum sensing in this organism, the mechanisms under its regulation are still not clear.

In an interesting hypothesis, Lwin et al. (2014) proposed that quorum sensing indirectly plays a role in the pathogenesis of acne. Based on the danger model of immunity by Matzinger (1994) which states that responses to antigens are not dependent solely upon the recognition of 'non-self' by the immune system, initiation of the optimal immune response requires a sense of tissue damage or evidence of a pathogenic micro-organism via so called 'danger signals'. In case of acne, the FFAs act as danger-associated molecular patterns. In its controlled growth as a skin commensal, *P. acnes* sends no or only 'safety' signals, but sends 'danger' signals via quorum sensing in the form of excess FFA production during pathogenic state, thereby stimulating inflammation. As of today, there is no *in vivo* evidence of quorum sensing by *P. acnes* even though a known quorum sensing signal, AI-2, is produced by the organism. However, experimental validation of this hypothesis is likely to offer novel therapeutic targets as well as open new possibilities of quorum sensing in this organism.

Rhodococcus

Actinobacteria in the genus *Rhodococcus* are aerobic, Gram-positive to variable and non-motile. They represent a group with remarkable metabolic diversity making them an ideal candidate for use in the bioremediation of contaminated sites, and as biocatalysts during biotransformations. Hence, they are of interest to the chemical, environmental, energy, and pharmaceutical sectors (Jones and Goodfellow, 2012). With a high economic value, further research into the exploration of physiological potential of this actinobacterial group is of increasing importance.

The presence of quorum sensing in *Rhodococcus* is only known through bioinformatic evidence based on genomic sequences of a few strains. Although GBL was detected in *Rhodococcus rhodochrous* NCIMB 13064 culture medium, it was shown that GBL accumulated due to chemical oxidation of haloalkane in high cell density cultures (Curragh et al., 1994). *In silico* analysis of the *Rhodococcus erythropolis* PR4 genome revealed the presence of genes encoding a communication molecule synthase, AfsA, and a communication molecule response regulator, ArpA with 31 and 36% amino acid sequence identity, respectively, suggesting the possible presence of a functional GBL-based quorum sensing system in this strain (Latour et al., 2013). A similar analysis of the genome of *Rhodococcus* strain RHA1showed the presence of homologs for protein domains of both the GBL synthase and the receptor which suggests that GBL might play a role in this organism too (Wuster and Babu, 2008). The fact that both the synthase genes and the response regulator genes are in close proximity of each other as in the case of AHL-based quorum sensing systems, suggests that these homologs may act as a quorum sensing system. This suggests that such a system is present in *Rhodococcus*.

Bifidobacteria

With a substantial effort in categorizing the human microbiome, new information has revealed that members of the genus *Bifidobacteria* represent one of the dominant groups of normal human gastrointestinal microbiota. They are also among the first colonizers of the gastrointestinal tract after birth. At the genomic level, all publically available genome sequences of bifidobacteria harbor putative *luxS* genes, and their corresponding amino acid sequences are well conserved in the genus with >82% sequence similarity to the LuxS protein of *Vibrio harveyi* (Sun et al., 2014). Using this information, Sun et al. (2014) experimentally confirmed that *Bifidobacteria* sp. exhibit LuxS-dependent AI-2 activity and biofilm formation. In this context, AI-2- dependent biofilm formation, e.g., on food particles or host-derived mucus, could be an important mechanism for early colonization of the host by commensal strains or persistence for prolonged periods by probiotic strains (Sun et al., 2014). With major implications in human health due to their use as probiotics, the advantages of exploiting the biofilm formation capability in bifidobacteria are enormous.

Other Actinobacteria

The evidence of quorum sensing in other actinobacterial genera is very meager. At least three closely related non-*Streptomyces* genera are known to produce GBL autoregulators and their receptor proteins based on specific ligand-binding assay (Choi et al., 2003). Using the binding assay with tritium-labeled autoregulator analogs as ligands, the authors screened crude cell-free lysates of five different non-*Streptomyces* strains with intermittent samplings during cultivation up to 96 h. The authors concluded that the teicoplanin-producer *Actinoplanes teichomyceticus* IFO13999 produced a VB type autoregulator, whereas both the rifamycin-producer *Amycolatopsis mediterranei* IFO13415 and the gentamicin-producer *Micromonospora echinospora* IFO13150

produced IM-2-type autoregulators. However, the IM-2 autoregulator produced by *M. echinospora* was likely with a longer C2 side chain as the biosensor strain *S. lavendulae* FRI-5 only recognizes IM-2-type autoregulators having a C2 side-chain length of 4–5 carbons (Choi et al., 2003). Moreover, the production of the autoregulators roughly corresponded to the late exponential growth phase and reached a plateau between 48 and 60 h, at the early stationary phase. The inability to detect autoregulator(s) in the other two strains, *Actinoplanes* sp. ATCC 31044 and *Amycolatopsis orientalis* IFO12806, does not exclude the possibility that, under different conditions, these strains might produce autoregulator(s). Hence, screening for autoregulators in different conditions using multiple biosensors is the key to go ahead in future.

Among other Actinobacteria, evidence exists for *Frankia* and *Nocardia* from genome analysis that they possess homologs of AfsA and ArpA, respectively (Wuster and Babu, 2008). Similarly, a LuxR system including a putative two-component system response regulator of the LuxR family of protein together with 23 transcriptional regulators is reportedly present in the *Nocardia brasiliensis* HUJEG-1 as determined based on its complete genome sequence (Vera-Cabrera et al., 2013). Further, members of the genera *Acidothermus, Arthrobacter, Brevibacterium, Clavibacter, Corynebacterium, Kineococcus, Kocuria, Nocardoides, Renibacterium, Rubrobacter, Saccharopolyspora, Salinispora,* and *Thermobifida* are known to harbor homologs of the LuxR regulators (Takano, 2006; Santos et al., 2012). Although LuxR regulators may also be involved in intracellular signaling, the presence of LuxR proteins is intriguing since no AHLs are known to act on the actinobacterial quorum sensing systems, where signaling is generally assured by cyclic or modified peptides and GBLs. However, this does not exclude the possibility that AHL-mediated quorum sensing does not exist in Actinobacteria because none of the screening strategies reported till date have used the conventional AHL-responsive biosensors. While our preliminary screening for AHL-mediated quorum sensing in Actinobacteria using AHL-responsive biosensors yields support for this hypothesis (personal observation), further investigation would help in ascertaining whether certain actinobacterial strains release AHLs or if there are other as-yet unknown compounds to which these AHL-responsive biosensors respond. In either case, AHL-production by Actinobacteria or AHL-responsive biosensors responding to the non-AHL signals produced by Actinobacteria is interesting. In this context, Yang et al. (2009) noted that *N*-hexanoyl-DL-homoserine lactone (C_6-HSL) interacts with the *S. coelicolor* GBL receptor (ScbR) activating the expression of *gfp* suggesting that such cross-phylum interactions are not impossible. However, in our case it is the contrary observation that does not find support in existing literature. It is an interesting and important observation for both the biosensor strains as well as Actinobacteria, and therefore needs further validation. In our opinion, we believe that such cross-taxa screening strategies might lead to discovery of newer molecules.

SCREENING FOR QUORUM SENSING IN ACTINOBACTERIA: LIMITATIONS AND IMPROVEMENTS

The lack of good biosensor system(s) which can respond to a very low quantity and a range of signaling molecules is a major limitation. Quorum sensing can be conclusively demonstrated only upon the isolation of the signaling molecule, followed by its structural determination and its ability to regulate phenotypes when added externally in the medium. However, Actinobacteria, such as *Streptomyces* cultures generally produce very low quantity of GBLs and its purification typically requires organic extraction of large (e.g., >400 L) volumes of spent culture medium. The existing sensor strains neither respond to such low quantity nor the range of GBLs produced, especially with longer C2 side chains. It is probably the main reason why the structure of only a few GBLs are known. In fact for these technical and economic reasons, Healy et al. (2009) did not determine the structure of GBL from *S. acidiscabies* and instead used an alternative strategy to indirectly prove the interaction of GBL with its cognate receptor (see below). In stark contrast, the AHL-responsive biosensor strains, such as *Chromobacterium violaceum* CV026 (McClean et al., 1997) and *gfp*-based recombinant *Escherichia coli* biosensor strains containing plasmids pJBA89, pJBA130, and pJBA132 (Andersen et al., 2001) respond to a wide range of AHL compounds even in nano Molar quantities. Their availability has significantly increased the detection of AHL-mediated quorum sensing in Gram-negative bacteria. Given this, there is an immediate requirement for efforts to create a similar biosensor system that can detect a wide variety of GBLs. In order to move forward, the priority should be to generate more data from the known GBLs and the mechanisms they regulate. This new information will significantly add toward developing such wide-range GBL-responsive biosensors.

Not many Actinobacteria exhibit AI-2-mediated quorum sensing which is typical of many other Gram-positive organisms. However, this could be attributed to its sensitivity to high glucose and acidic pH in the culture medium both of which have a strong inhibitory effect. While screening for AI-2 activity in bifidobacterial culture supernatants, Sun et al. (2014) could not detect any activity in MRSc, i.e., the standard culture medium for bifidobacteria. MRSc contains 20 g/L glucose and the end products of the bifidobacterial metabolism on hexoses are mainly acetic and lactic acid. By testing *V. harveyi* BB170, a known AI-2 producer at different pH values, the authors concluded that acidic pH negatively affected detection. AI-2 activity was reduced to approximately 40% at pH 4, i.e., the pH observed in bifidobacterial supernatants, and at 0.25 g/L of glucose (Sun et al., 2014). In contrast, during assays for the signaling molecule response regulator, ArpA, only those that are acidic (pH ∼5) bind the autoregulator when tested; basic proteins did not (Willey and Gaskell, 2011). Hence, information on the sensitivity of existing signaling molecules is warranted. Once this information is generated, it likely to improve the existing screening strategies.

A more feasible approach is to search for homologs of the autoregulator receptor gene (Willey and Gaskell, 2011).

As shown by Santos et al. (2012), these genes share a high degree of similarity within a given taxon and designing of degenerate primers to PCR amplify and sequence them is a better strategy. Using a similar strategy, we were able to sequence majority of quorum sensing gene homologs from the genus *Aeromonas* and show that the system is ubiquitously present across all the species in this genus (Jangid et al., 2007). With the advancements in sequencing technology and reduced costs, conducting metagenomic studies using a similar approach would be very easy and is likely to generate an enormous depth of information that is still hidden and untapped. However, such novel strategies must be followed with caution and utmost planning. Further, the mere presence of the gene is by no means an evidence of a functional signaling system. Hence, cloning of such structural genes in an expression system is the only way to confirm its activity. However, it is imperative that for such a strategy the intact functional protein much be obtained and later-on use the purified proteins for further investigation.

Recently, some new receptor-based methodologies have been described. To circumvent the issue of requiring large amounts of cultures, Yang et al. (2005) reported an alternative detection system using ScbR, the receptor protein from *S. coelicolor* and electrospray tandem mass spectrometry (ESI-MS/MS). Using the success of affinity capture technology in proteomics studies, the authors developed recombinant receptors of butanolides, such as such as ArpA from *S. griseus*, FarA from *S. lavendulae*, BarA from *S. virginiae*, and SpbR from *S. pristinaespiralis* and used them as affinity capture molecules to trap butanolides, followed by MS analysis for identification. This method allows the isolation of butanolides from as low as 100 ml of *S. coelicolor* culture broth. In addition, it enables the detection of quorum sensing system in cases where the interaction between the signaling molecule and its cognate receptor is inhibited in acidic pH and high glucose. For instance, Healy et al. (2009) detected fragment ions bound by the purified GBL receptors from *S. acidiscabies*. These ions showed masses that were consistent with molecules possessing lactone functional groups such as those found in GBL compounds. This strategy might therefore be useful for strains with identified GBL receptors but where the interactions could not be proven.

The availability of a diverse set of biosensor plasmids is likely to increase the frequency of detection of Actinobacterial quorum sensing systems. Recently, Yang et al. (2009) developed a *gfp*-containing *E. coli*-based cell-free system for detecting GBL in *Streptomyces*. In this ScbR quorum sensing system, the *gfp* is fused downstream of the DNA binding site for the *S. coelicolor* GBL receptor, ScbR. The presence of purified His-tagged ScbR and cognate GBL results in fluorescence. This system allows to circumvent the issues of cell wall penetration and can be used for high-throughput screening as it allows assays to be completed within 4 h. Further, the protein–ligand interaction can easily be monitored without the use of radioisotopes and acrylamide gels. Similarly, Hsiao et al. (2009) used ScbR and its target DNA to control the expression of a kanamycin resistance gene in the presence of its cognate GBL. This new sensitive reporter system also allows detection of small quantities of GBLs and those that are difficult to detect. The authors propose that by altering the timing for extract preparation from cells, the detection of other GBLs could be enhanced from different strains. The kanamycin bioassay is likely to facilitate large-scale screening of GBL producers due to its higher sensitivity toward wide range of GBLs than the commonly used bioassay.

While these new approaches are likely to facilitate the discovery of additional GBLs, one important limitation is that most are targeted to detecting GBLs from Actinobacteria, especially *Streptomyces*. Hence, detailed investigation of other non-GBL mediated quorum sensing systems is required to gain insight into the mechanisms involved and thereby develop strategies for expanding the array of signaling molecule detection.

QUORUM QUENCHING ACTIVITY IN ACTINOBACTERIA

With constant rise in the number of antibiotic-resistant bacteria, there is a need to look for alternative strategies to control their spread. Since most pathogens regulate their virulence by quorum sensing, it has become the most sought-after alternative target to control their spread. Chemical inactivation of the Gram-negative AHLs via alkaline hydrolysis is known for quite some time. However, the enzymatic degradation of signaling molecules is now the most researched field in quorum quenching to limit the growth of many animal and plant pathogens. Quorum quenching enzymes act in either of the two ways: (1) analogous to the chemical ring hydrolysis, acyl-homoserine is generated by AHL lactonases; and (2) the amide bond is degraded by AHL acylases. Screening for these enzymes in different ecosystems have shown great potential. For instance, AHL-degrading bacteria may make up 5–15% of the total cultivable bacteria in the soil and rhizosphere (Latour et al., 2013). Although small, it is a non-negligible and an important resource for developing biocontrol formulations. Screening for such enzymes has therefore become increasingly important.

The ability of Actinobacteria to produce the innumerable secondary metabolites, enzymes, and commercially important biomolecules has attracted researchers to explore this phylum for their role in quorum quenching activity. Endophytic actinomycetes and their AHL-lactonase enzymes have shown great potential in this regard (Chankhamhaengdecha et al., 2013). The authors made a first attempt toward screening for quorum quenching enzyme-producing actinomycetes from soil and plant tissues. With 51.5% of the tested strains possessing the quorum quenching activity, endophytic actinomycetes possessed the activity at higher frequency than the soil isolates at 36.9% demonstrating a great diversity and abundance of AHL-degrading actinomycetes. While one would think that quorum quenching is most useful for organisms that produce the signals enabling them to use them as a source of energy and nitrogen source (Flagan et al., 2003), organisms that do not produce the signals are also known to quench them, presumably to gain an advantage over communicating bacterial species in the same ecological niche (Wuster and Babu, 2008). For example, *Rhodococcus* and *Microbacterium* can degrade AHL signals

without having any known ability to produce them. In fact, there is no evidence of quorum sensing for the latter, not even bioinformatic. Hence, more of such environmental screening studies that target Actinobacteria are warranted.

Specific members of the phylum *Actinobacteria* have also shown considerable potential in agro-environment due to their quorum quenching activity. Several Actinobacteria have the ability to colonize plant surfaces and thereby exclude plant pathogens either by competition or through inhibition by antibiotic production (Selvakumar et al., 2014). Over the last decade, a total of six actinobacterial genera: *Arthrobacter* (Flagan et al., 2003), *Microbacterium* (Wang et al., 2012), *Mycobacterium* (Chen and Xie, 2011), *Nocardioides* (Yoon et al., 2006), *Rhodococcus* (Park et al., 2006; Latour et al., 2013), and *Streptomyces* (Chen and Xie, 2011; Ooka et al., 2013) have been reported for their quorum quenching activity. Members of these genera known to exist as plant symbionts or as endophytes residing within plant hosts without causing disease symptoms are reported to produce AHL-inactivating enzymes. In fact, *Rhodococcus* has an unusually high number of AHL-inactivating lactonases (Wuster and Babu, 2008), that may play a role in the intracellular metabolism of lactone compounds such as GBL (Uroz et al., 2005). Due to its high AHL-degrading activity, *R. erythropolis* strain R138 has been used as a biocontrol agent to prevent soft-rot in plants. Genetic evidence suggests that the lactone catabolic pathway in the strain may not be the only pathway for AHL-inactivation. In addition, it possesses multiple homologs of various catabolic enzymes, thus enhancing the species' metabolic versatility (Latour et al., 2013). Recently, two more strains of *R. erythropolis*, PR4 and MM30 of marine origin have been reported to enzymatically degrade *N*-oxododecanoyl-L-homoserine lactone (Romero et al., 2011). Similarly, the soil isolate *Nocardioides kongjuensis* A2–4^{T} is able to grow with N-hexanoyl-L-homoserine lactone as the sole carbon source suggesting that its quenching ability is worth exploration against plant pathogens (Yoon et al., 2006). Further, the AHL-degrading lactonase enzyme activity was also reported from the potato leaf-associated *Microbacterium testaceum* StLB037 (Wang et al., 2012). Recently, *Arthrobacter* species have been reported to inhibit quorum sensing in a cross phylum interaction (Igarashi et al., 2015). The strain PGVB1 produces arthroamide and turnagainolide that showed potent inhibition of *agr*-signaling pathway of quorum sensing in *Staphylococcus aureus* at 5–10 μM without showing cell toxicity. Similarly, a major metabolite piericidin A1 secreted by *Streptomyces* sp. TOHO-Y209 and TOHO-O348 demonstrated quorum sensing inhibiting activity against *C. violaceum* CV026 (Ooka et al., 2013). The piericidin class of metabolites are known inhibitors of NADH-ubiquinone oxidoreductase, with A1 specifically inhibiting both mitochondrial and bacterial NDAH- ubiquinone oxidoreductases. These studies suggest that Actinobacteria offer a unique system which, if exploited well, is likely to play a major role in controlling the spread of plant and human pathogens.

CONCLUSION

The enormous metabolic and phylogenetic diversity that exists in Actinobacteria offers a unique opportunity to explore its multifactorial abilities for biotechnological applications. Quorum sensing is one such property that is evidently under-explored in this phylum. Based on the limited information that is known, quorum sensing systems in Actinobacteria show considerable diversity in terms of the types of signals and the mechanisms it controls. However, there exists a taxa specific segregation within the phylum. For instance, GBL-mediated regulation is not only limited to *Streptomyces* but is also species specific. Interspecific signaling is therefore likely to expand the list of compounds and mechanisms involved in quorum sensing. The lack of good detection systems is a major limitation for further exploration of the communication system in Actinobacteria. Developing newer systems which can respond to a wider range of signals and that too at very low quantities are the need of the hour. Further exploration using these systems within and between multiple taxa is likely to reveal an even greater diversity of signals. Similarly, the quorum quenching ability of Actinobacteria exhibit a great potential, especially through their use as bio-control agents for plant pathogens and in controlling the spread of antibiotic-resistant organisms. However, systematic screening of specific ecosystems is required to fully exploit the quorum quenching potential. Using the knowledge gained from an in-depth understanding of the existing quorum sensing systems, Actinobacteria are likely to exhibit a wider array of properties that are likely to have significant implications for plant, animal and human health.

AUTHOR CONTRIBUTIONS

KJ and AP designed the review. SM and UP did the referencing, preliminary sequence analysis, and compilation of data. KJ and AP finalized the structure of the review, analyzed the sequence data, and wrote the review.

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Proteome profiling of heat, oxidative, and salt stress responses in *Thermococcus kodakarensis* KOD1

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The thermophilic species, *Thermococcus kodakarensis* KOD1, a model microorganism for studying hyperthermophiles, has adapted to optimal growth under conditions of high temperature and salinity. However, the environmental conditions for the strain are not always stable, and this strain might face different stresses. In the present study, we compared the proteome response of *T. kodakarensis* to heat, oxidative, and salt stresses using two-dimensional electrophoresis, and protein spots were identified through MALDI-TOF/MS. Fifty-nine, forty-two, and twenty-nine spots were induced under heat, oxidative, and salt stresses, respectively. Among the up-regulated proteins, four proteins (a hypothetical protein, pyridoxal biosynthesis lyase, peroxiredoxin, and protein disulphide oxidoreductase) were associated with all three stresses. Gene ontology analysis showed that these proteins were primarily involved metabolic and cellular processes. The KEGG pathway analysis suggested that the main metabolic pathways involving these enzymes were related to carbohydrate metabolism, secondary metabolite synthesis, and amino acid biosynthesis. These data might enhance our understanding of the functions and molecular mechanisms of thermophilic Archaea for survival and adaptation in extreme environments.

Keywords: proteome, stress responses, *Thermococcus*, archaea, metabolic pathway

Introduction

Thermococcus kodakarensis KOD1 is a hyperthermophilic anaerobic archaeon, isolated from a solfatara (102°C, pH 5.8) on the shore of Kodakara Island, Kagoshima, Japan (Morikawa et al., 1994). The environmental conditions are not always conducive to steady growth, as fluctuations in temperature regime, fluid flux, and carbon substrate supply create a spatial and temporal mosaic of microenvironments (Edgcomb et al., 2007). The different environmental conditions over time have facilitated the evolution of Archaea for adaptation to extreme environments, and indeed, these bacteria experience difficulties acclimating to less extreme conditions (Reed et al., 2013). *T. kodakarensis* KOD1 senses the environment and responds to changing environmental conditions (Izumi et al., 2001). Many proteins have been reported to play important roles in cellular protection against different stresses. For example, osmotically inducible protein C (OsmC) from *T. kodakarensis* plays a role in cellular defense against oxidative stress induced through exposure to hyperoxides or elevated

osmolarity (Park et al., 2008). *T. kodakarensis* also possesses four prefoldin genes, encoding two alpha subunits (pfdA and pfdC) and two beta subunits (pfdB and pfdD) of prefoldins on the genome. The PfdA/PfdB complex functions at all growth temperatures, while the PfdC/PfdD complex contributes to survival in high-temperature environments (Danno et al., 2008). Proteins involved in oxidative stress were well studied in *Pyrococcus*, which belong to the same order Thermococcales, along with *T. kodakarensis* KOD1. In *Pyrococcus horikoshii*, a significant increase of a 25 kDa alkyl hydroperoxide reductase (PH1217) was observed when the microorganism was cultivated under aerobic conditions (Kawakami et al., 2004). *P. furiosus* is surprisingly tolerant to oxygen, growing well in the presence of 8% (vol/vol) O_2. Superoxide reductase (SOR) and putative flavodiiron protein A play important roles in resisting O_2 (Thorgersen et al., 2012). Most cellular stress responses are highly conserved cellular defense mechanisms for protection against sudden environmental changes or frequent fluctuations in environmental factors (Feder and Hofmann, 1999). The cellular stress response has been associated with essential aspects of protein and DNA processing and stability in all three superkingdoms of life: Archaea, Bacteria, and Eukarya (Kültz, 2003). In Archaea, *T. kodakarensis* has emerged as a premier model system for studies of archaeal biochemistry, genetics, and hyperthermophily (Hileman and Santangelo, 2012). However, the current knowledge of the stress proteome of *T. kodakarensis*, i.e., the proteins expressed in response to cellular stress, remains fragmented.

Proteomics techniques are powerful tools for the identification of the quantitative changes in protein expression in response to stress exposure in cells, tissues or biological fluids. The first proteomics studies of thermophilic Archaea, involving the proteome of *Sulfolobus solfataricus* P2, were reported Chong and Wright (2005). Since then, the proteomics analysis of *Thermococcus* was conducted in 2009, which characterized the abundant expression of *Thermococcus onnurineus* NA1 proteins in enriched medium (Kwon et al., 2009). Recent developments in proteomics studies on extremophiles have provided unique information on the physiological characteristics required for adaptation to extreme conditions. For example, formate is used in gluconeogenesis and carbon monoxide is converted to carbon dioxide and assimilated into organic carbon in *T. onnurineus* NA1 (Yun et al., 2014).

In the present study, we simultaneously analyzed alterations in protein expression during heat, oxidative, and salt stresses based on two-dimensional (2-D) gel electrophoresis. We conducted proteomics analyses using matrix-assisted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF/MS) to identify the major proteins. The completed genome of *T. kodakarensis* KOD1 has facilitated the use of proteomics analyses under different stress conditions. The aim of the present study was to highlight the molecular adaptation mechanisms of *T. kodakarensis* KOD1 and reveal both common and distinct response pathways involved in the adaptation of this species to heat, salt, and oxidative stress.

Materials and Methods

Organism and Cell Culture

The *T. kodakarensis* strain KOD1 was obtained from the Japan Collection of Microorganisms (JCM). The cells were cultured in JCM medium 280[1].

Heat, Oxidative, and Salt Stress Procedure

Culture of *T. kodakarensis* KOD1were carried out in triplicate in 40 mL cultures in 50 mL serum bottles at 85°C anaerobically on a shaking incubator (150 rpm). For heat stress, the cells in the mid-log phase were shocked by exposure to 95°C and incubating for 4 h. For oxidative stress, the cells in the mid-log phase were cultured under aerobic conditions after adding oxygen (5 L/min) for 30 min. Each culture was maintained at 85°C for 4 h. For osmotic stress, *T. kodakarensis* KOD1 was grown until the mid-log phase and the cells were salt shocked after adding a final concentration of 1 M NaCl to the medium and incubating for 4 h. The cells treatment for 1 h was harvested through centrifugation at 12,000 rpm for 10 min at 4°C for two-dimensional gel electrophoresis (2-DE). Survival of the cells was estimated by the three-tube most probable number method per 30 min period after exposure to stress. Samples were diluted serially in growth medium, and cultures were incubated at 85°C.

2-DE

The cells were washed with 1X PBS (the salt stress cells including control were washed four times and others were washed twice), and the total proteins were solubilized in lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris, 100 mM DTT, and 0.5% carrier ampholyte) for 20 min. The soluble proteins were separated through centrifugation at 40,000 rpm for 1 h at 4°C. The soluble protein concentration was determined using a standard Bradford method (Bradford, 1976).

Isoelectric focusing (IEF) was conducted using the IPGphor/IsoDalt system (Bio-Rad, Hercules, CA, USA) at 20°C. IPG gel strips system (Bio-Rad., Hercules, CA, USA) were rehydrated in swelling solution (7 M urea, 2 M thiourea, 2% CHAPS, 100 mM DTT, 0.5% IPG buffer system (Bio-Rad, Hercules, CA, USA) and bromophenol blue containing 100 mg of protein for 12 h at 20°C, and subsequently, IEF was performed for 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, 1 h at 1000 V, 30 min at 8000 V, and 45000 Vh. The IPG strips were equilibrated for 15 min in Solution I (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 10 mg/mL DTT, and bromophenol blue), followed by 15 min in Solution II (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2% iodoacetamide, and bromophenol blue). For the second dimension, vertical slab gels were used. The 12% SDS gels were prepared, and an equilibrated IPG gel strip was laid on top of the gel filled with 0.5% agarose solution. Electrophoresis was performed at 5 mA/cm for 1 h at room temperature, followed by 10 mA/cm until the dye front reached the bottom of the gel. The proteins were detected through silver staining.

[1]http://www.jcm.riken.jp/cgi-bin/jcm/jcm_grmd?GRMD = 280&MD_NAME

Protein Visualization and Image Analysis

The stained gels were scanned and digitized using a Duoscan scanner (Agfa, Trenton, NJ, USA; Bio-Rad, Hercules, CA, USA). After background subtraction, normalization, and matching, the spot volumes in gels from each treated-cell sample were compared with the matched spot volumes in gels from control cells. Comparison of the test spot volumes with the corresponding standard spot volumes yielded a standardized abundance for each matched spot, and the values were averaged across triplicates for each experimental condition. Statistical analysis was performed to select the matching spots across all images, including spots displaying $a \geq 1.5$ average-fold increases in abundance between conditions and spots with $P < 0.05$. Spots differentially and markedly overexpressed were excised.

Protein Identification

The Voyager-DE^{TM} STR Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA) was used for MALDI-TOF/MS. The desired gel pieces were carefully excised, destained, and in-gel digested using trypsin. Briefly, the excised-gel pieces were washed with water for 2 × 15 min, followed by an additional wash with water/acetonitrile (1:1) for 2 × 15 min. After removing all liquid, acetonitrile was added to cover the gel pieces. Acetonitrile was removed after the gel pieces were shrunk. The gel pieces were rehydrated in 0.1 M ammonium bicarbonate for 5 min, and subsequently incubated for 15 min with an equal volume of acetonitrile. After removing all liquid, the gel pieces were dried in a vacuum centrifuge for 20 min. The gel pieces were swollen in 10 mM DTT/0.1 M ammonium bicarbonate and incubated for 45 min at 56°C, followed by cooling at RT. After removing the excess liquid, the same volume of freshly prepared 55 mM iodoacetamide in 0.1 M ammonium bicarbonate was added, followed by incubation in the dark for 30 min at room temperature. The iodoacetamide solution was removed, and the gel pieces were incubated in 30 mL of 0.1 M ammonium bicarbonate for 5 min, and subsequently further incubated for 15 min with an equal volume of acetonitrile. After an additional incubation with ammonium carbonate/acetonitrile, the gel pieces were dried in a vacuum centrifuge for 20 min, rehydrated in digestion buffer and placed on ice for 45 min. The buffer was replaced with 20 mL of digestion buffer with trypsin (12, 500 $\mu g\ mL^{-1}$). After overnight digestion at 37°C, a sufficient volume of 25 mM ammonium bicarbonate was added to cover the gel pieces and incubated for 15 min. The same volume of acetonitrile was added and incubated for 15 min, followed by the addition of 5% formic acid/acetonitrile (1:1) to the recovered supernatant and incubation for 30 min. After repeating this step, all the extracts were dried in a vacuum centrifuge for 1–2 h. The dried peptide was dissolved in 20 mL of 5% formic acid and sonicated for 5 min in a water bath sonicator. The peptide sample (2 mL) with standard calibrant (1 mL) was mixed with 2 mL of a 2:1:1 (v:v:v) matrix mixture containing matrix solution (20 mg a-cyano-4-hydroxycinnamic acid/1 mL acetone):nitrocellulose solution (20 mg nitrocellulose/1 mL acetone): 2-propanol. Two microliters of sample was loaded onto a MALDI plate, dried for 30 min at room temperature, rinsed with 5 mL of 5% formic acid, and washed with 5 mL of water. After drying at room temperature, the plate probe was inserted into a MALDI mass spectrometer. For protein identification, we performed searches in the NCBInr, Swiss-Prot/TrEMBL, and MSDB sequence databases using MS-Fit[2], Mascot[3], and ExPASy[4]. The complete experiment was repeated three times, including cell growth, proteome purification, 2-DE, and protein identification.

Agar Plate Bioassay

Polymerase chain reaction (PCR) using *T. kodakarensis* KOD1 genomic DNA as a template was performed to isolate *TK0108, TK0217, TK0537, and TK1085* using the following oligonucleotide primers listed in supplementary **Table 1**. The PCR products and the pET28a vector were digested by the restriction enzymes. The ligation products were transformed into *Escherichia coli* BL21 (DE3) cells by electroporation and confirmed by sequencing. *E. coli* cells containing the four recombinant plasmids were named as pET28a-TK0108, pET28a-TK0217, pET28a-TK0537, and pET28a-TK1085, respectively. The *E. coli* cells were cultured in 10 mL of LB broth containing 30 $\mu g\ mL^{-1}$ kanamycin at 37°C for 3 h. When the OD_{600} reached 0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce protein expression. After 4 h of culture with shaking, the OD_{600} were adjusted to 0.5 and the protein expression were checked by SDS-PAGE. Petri plate-based dilution bioassays were performed after the cells were treated at 50°C for 20 min or the cells were spotted onto LB plates with 5 mM H_2O_2 and 1 M NaCl, respectively. The images were taken after incubation at 37°C for 12 h. This assay was performed in triplicate for three times and the representative images were shown.

Data Analysis

Gene ontology (GO) enrichment was performed using BLAST2GO (Conesa and Gotz, 2008). The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to determine the position of the identified proteins in respective pathways (Kanehisa and Goto, 2000). Protein–protein interactions were predicted using STRING set at high confidence (Franceschini et al., 2013), and Cytoscape was used for network visualization (Shannon et al., 2003). The protein function was predicted by BLAST (Altschul et al., 1997), SMART (Roy et al., 2010), and I-TASSER (Letunic et al., 2015).

Result

Cell Growth, Proteome Analysis, and Protein Identification

Thermococcus kodakarensis KOD1 has been reported to strictly anaerobic. Temperature range of growth is 60–100°C, with an optimum of approximately 85°C. Range of NaCl concentration allowing growth is between 0.17 and 0.86 M, with an optimum of 0.52 M (Atomi et al., 2004). Further research showed that

[2]http://prospector.ucsf.edu

[3]http://www.matrixscience.com

[4]http://www.expasy.org

TABLE 1 | List of up-regulated proteins under heat stress in *Thermococcus kodakarensis* KOD1.

No	Protein name	Protein ID	SC(100%)[a]	Fold change	pI[b]	pI[c]	Mw[d]	Mw[e]
1	Thermosome alpha subunit	TK0678	48	3.2	4.84	4.8	59.12	59.2
2	ATP-dependent glucokinase	TK1110	19	2.1	5.52	5.6	50.70	50.0
3	Aspartyl – tRNA synthetase	TK0492	17	1.6	5.35	5.4	50.88	51.5
4	Hypothetical protein	TK0300	16	2.5	5.76	5.7	50.80	51.0
5	Ornithine carbamoyltransferase	TK0871	28	2.2	5.76	5.7	35.02	35.0
6	Probable transcription regulator	TK0471	43	2.9	6.01	6.5	30.81	31.0
7	RNA – binding protein	TK2097	15	2.1	6.02	5.5	18.03	18.0
8	Hypothetical protein	TK1561	26	2.6	5.32	5.4	21.77	23.0
9	6,7-dimethyl-8-ribityllumazine synthase	TK0429	25	2.8	5.70	5.7	15.69	16.0
10	Hypothetical protein	TK0108	61	1.6	4.99	5.0	22.39	23.0
11	Cobalamin adenosyltransferase	TK1045	29	3.1	6.19	6.3	19.26	19.0
12	2-dehydropantoate 2-reductase	TK1968	37	3.3	4.43	4.5	34.03	34.5
13	Hypothetical protein	TK1937	17	2.5	4.82	5.0	16.36	17.5
14	*N*-acetyltransferase	TK0232	41	3.2	5.76	5.8	31.78	32.0
15	Hypothetical protein MJ0668	TK0823	24	3.0	6.53	6.7	10.13	10.0
16	Predicted exonuclease	TK0458	18	2.5	6.15	6.4	20.05	20.0
17	ABC-type maltodextrin-binding periplasmic component	TK1771	15	3.2	4.56	4.5	49.44	51.5
18	Thermosome beta subunit	TK2303	36	3.1	4.86	4.8	59.13	60.2
19	Sugar-phosphate nucleotidyltransferase	TK0955	19	2.5	5.15	5.2	46.80	47.7
20	Acyl-CoA synthetase	TK0944	8	3.3	5.51	5.7	51.83	50.9
21	Hypothetical protein	TK0077	28	1.9	4.94	5.1	5.34	5.4
22	Zinc-dependent protease	TK0689	31	3.8	5.20	5.2	48.52	49.0
23	ATPase, ParA/MinD family	TK0701	42	3.7	4.81	4.9	31.93	30.9
24	Hypothetical protein	TK1972	15	1.6	4.89	4.9	39.80	40.0
25	Glycine cleavage system protein P	TK1379	38	2.3	5.51	5.5	55.96	56.2
26	Methionine synthase II	TK1447	12	2.6	5.90	5.9	35.25	35.0
27	Deoxyribose-phosphate aldolase	TK2104	27	2.3	5.18	5.2	24.49	26.0
28	Metallophosphoesterase	TK0547	19	2.6	5.22	5.3	24.12	23.0
29	Protein disulphide oxidoreductase	TK1085	39	3.8	4.72	4.8	25.28	25.6
30	Deblocking aminopeptidase	TK0781	31	1.9	5.46	5.5	38.27	38.5
31	Hypothetical protein	TK0163	46	1.8	5.60	5.6	28.74	29.7
32	Oxidoreductase	TK0845	22	3.0	5.36	5.4	31.57	31.8
33	Eukaryotic-type DNA primase	TK1790	17	2.8	6.24	6.2	40.27	40.0
34	Inorganic pyrophosphatase	TK1700	47	1.7	4.84	4.8	20.78	22.0
35	Acetyltransferase	TK1174	31	1.8	5.98	6.0	18.79	19.1
36	Hypothetical protein	TK1584	36	1.9	5.71	5.7	10.92	11.9
37	2-oxoisovalerate:ferredoxin oxidoreductase, alpha subunit	TK1980	26	2.1	4.97	5.0	44.37	44.5
38	Pyridoxine/pyridoxal 5-phosphate biosynthesis protein	TK0217	25	2.6	5.57	5.5	36.64	37.7
39	Thermophile-specific fructose-1,6-bisphosphatase	TK2164	60	2.7	5.36	5.3	41.63	41.8
40	Serine hydroxymethyltransferase	TK0528	41	2.0	5.80	5.2	48.20	47.3
41	Glutamate dehydrogenase	TK1431	34	1.8	5.88	5.5	47.03	47.9
42	Deblocking aminopeptidase	TK1177	54	1.8	5.39	5.3	38.17	38.0
43	ATPase involved in chromosome partitioning	TK2007	36	2.2	5.71	5.6	27.61	27.5
44	Hydrolase	TK2232	21	2.5	5.42	5.4	24.24	24.5
45	Peroxiredoxin	TK0537	48	3.5	5.02	4.9	24.63	24.0
46	Myo-inositol-1-phosphate synthase	TK2278	23	2.5	5.31	5.0	42.39	43.0
47	2-amino-3-oxobutylrate Co A ligase	TK2217	18	3.0	5.53	5.5	43.94	44.9
48	DNA polymerase sliding clamp	TK0535	32	2.5	4.49	4.4	28.22	28.0
49	Anthranilate synthase	TK0254	14	2.2	5.20	5.6	48.51	49.5
50	Cell division GTPase	TK1421	29	2.3	4.80	4.4	40.03	40.0
51	Hydrolase	TK0251	14	1.9	4.91	4.2	27.41	29.4
52	Chromosome partitioning protein ParB homologue	TK0378	24	2.4	5.85	5.0	35.97	38.0
53	Glyceraldehyde-3-phosphate dehydrogenase	TK0765	25	2.5	5.30	5.9	37.21	36.2
54	Distant homolog of phosphate transport system	TK1967	26	1.6	4.58	4.0	23.99	22.0
55	ABC-type phosphate transport system	TK1868	22	2.5	5.30	5.9	28.41	30.5
56	Serine-glyoxylate aminotransferase	TK1548	17	2.2	5.93	6.5	42.88	44.0
57	Hypothetical protein	TK1160	42	1.9	6.84	6.5	14.79	15.5
58	*N*-acetyltransferase	TK1054	15	2.4	6.64	6.0	20.77	20.0
59	Transcription regulator	TK0126	23	2.5	6.77	6.2	20.70	21.0

[a]*Sequence coverage,* [b]*Theoretical pI,* [c]*Experimental pI,* [d]*theoretical mass (kDa), and* [e]*experimental mass (kDa) of the identified proteins.*

T. kodakarensis KOD1 could grow after aerobic inoculation, at which the cells were initially under oxygen saturation at the cultivation temperature (Kobori et al., 2010). To study the effect of stresses on *T. kodakarensis* KOD1, the cells were exposed to 95°C, 1 M NaCl, or saturated oxygen condition for 4 h. The effect of the stresses on cells viability was assayed using the most probable number method. The results showed that there were no significant differences in the frequency of viable cells compared to control (Supplementary Figure S1). To better understand the molecular mechanism underlying the responses of *T. kodakarensis* KOD1 to heat, oxidative, and salt stresses, we conducted comparative proteomics assays to identify proteins differentially expressed in this strain based on 2-D gel electrophoresis using cells grown under the stresses for 1 h. The cytosolic proteins were subjected to 2-DE, and MALDI was used to identify the proteins involved in heat, oxidative, and salt responses. Proteins extracted under conditions without any stress were used as a control. The gels (Supplementary Figures S2–S4) were silver stained and subsequently analyzed using PDQuest 7.1. After optimization of the 2-DE gels and image processing, the proteins showing at least 1.5-fold (control reference gel) increased expression were further subjected to mass spectrometry. The experiments were repeated three times, and only the reproducible differences were considered.

Based on the 2-DE gels, we identified 83, 33, and 56 up-regulated proteins in response to heat, osmotic, and oxidative stresses, respectively. Among these proteins, 59, 42, and 29 up-regulated proteins were identified using MALDI-TOF/MS, and these results are summarized in **Tables 1–3** under heat, oxidative, and salt stresses, respectively. The pIs of the protein spots ranged from 4.0 to 6.5, and the molecular masses ranged from 5.4 to 92.6 kDa. A homology-based search using the available protein databases revealed that proteins of *T. kodakarensis* KOD1 origin as the best results in all cases. The molecular masses and pIs for each protein, estimated from the spot positions on the gels, were compared with those of the homologous proteins retrieved. In most cases, these values were comparable (**Tables 1–3**).

Among the up-regulated proteins under the three stresses, 27 proteins were up regulated under both heat and oxidative stresses, representing 46 and 53% of the total proteins under a single stress, and seven proteins were up regulated under both heat and salt stresses (**Figure 1**; Supplementary Table S2). Only six proteins were present in the catalog of up-regulated proteins in the presence of both oxidative and salt stresses. Moreover, four proteins (TK0108, TK0217, TK0537, and TK1085) were over-expressed under all three stresses. These results suggested that *T. kodakarensis* KOD1 utilized similar defense mechanisms to a certain extent against heat and oxidative stresses. On the other hand, 29, 30, and 20 proteins were up regulated specifically under heat, oxidative, and salt stress, respectively, (**Figure 1**; Supplementary Table S2). These results suggested that there were also distinct mechanisms for *T. kodakarensis* KOD1 to defense against different stresses. For example, TK0189 (OsmC) was overexpressed in response to osmotic stress, but not under heat and oxidative stress (Park et al., 2008).

Functional Assay of the Co-Over-Expressed Proteins under Stresses

To examine the function of the co-over-expressed proteins, the effects of the overexpression of TK0108, TK0217, TK0537, and TK1085 on the growth of *E. coli* under different environment stresses were analyzed. After induction by IPTG, the expression of the proteins was checked by SDS-PAGE (data not shown). Cultures of *E. coli* cells either expressing the four proteins or containing the pET28 vector were diluted and spread on different plates. **Figure 2A** showed that recombinant and control cells have similar growth on LB medium in overnight grown culture. The growth of the strain containing the pET28 vector was inhibited by high temperature treatment or by the addition of a high concentration of H_2O_2 and NaCl to the medium. Whereas, the *E. coli* expressing TK0108, TK0217, TK0537, and TK1085 displayed the higher tolerance to heat stress. In high oxidative and salinity supplemented medium, the recombinant cells also increased the number of colonies as compared to control cells.

As an additional way to examine the possible function of identified proteins, we used the STRING tool to prepare an interaction map (**Figure 2B**). As might be expected, TK0537 and TK1085 have the high connectivity (score > 0.80) with proteins involved in oxygen detoxifying. The molecular chaperones displayed connectivity with TK0217. Interestingly, TK0108 showed high connectivity (score > 0.75) with proteins in DNA repair and transcription. These results indicate that the four proteins may contribute to the stress tolerance in different pattern.

Functional Categorization Analysis

We conducted a GO analysis to characterize protein function. The proteins up-regulated during the three stresses were categorized according to molecular functions and biological processes based on GO classification, using BLAST2GO. GO categories were assigned to all proteins according to molecular functions and biological processes.

The classification of heat stress proteins based on biological processes generated ten different groups (**Figure 3A**). More than 80% of the total proteins were classified into three categories: metabolic processes (40%), cellular processes (26%), and single-organism processes (20%). The classification of oxidative stress proteins based on biological processes generated eight different groups, and more than 80% of the total proteins were classified into three categories: metabolic processes (38%), cellular processes (26%), and single-organism processes (22%; **Figure 3A**). For salt stress proteins, six different groups were generated, and the ratios in metabolic processes, cellular processes, and single-organism processes were 37, 27, and 19%, respectively, (**Figure 3A**).

The classification according to molecular function showed six different groups of proteins up-regulated in response to heat (**Figure 3B**), and 94% of these proteins belonged to either (1) catalytic activity (54%) or binding activity (40%). Other categories included transporter activity, enzyme regulator activity, electron carrier activity, and antioxidant activity. Whereas the classification of proteins under oxidative stress yielded five different groups, with 90% of the proteins belonging

TABLE 2 | List of up-regulated proteins under oxidative stress in *T. kodakarensis* KOD1.

No	Protein name	Protein ID	SC(100%)[a]	Fold change	pI[b]	pI[c]	Mw[d]	Mw[e]
1	ABC-type dipeptide transport system	TK1804	15	1.9	4.64	4.8	92.13	92.6
2	DNA/RNA repair helicase	TK0928	12	2.1	4.33	4.5	53.15	54.0
3	Thermophile-specific fructose-1,6-bisphosphatase fructose-1,6-bisphosphatase	TK2164	15	2.6	5.36	5.4	41.63	43.5
4	Archaeal ATPase	TK1465	21	3.0	6.36	6.4	53.84	54.2
5	Zinc-dependent protease	TK0699	10	2.9	5.49	5.9	53.56	54.8
6	Thioredoxin reductase	TK2100	15	3.2	5.85	5.9	35.97	37.0
7	Ferredoxin oxidoreductase	TK1980	18	2.7	4.97	5.0	44.40	45.4
8	Glutamate dehydrogenase	TK1431	21	2.6	5.88	5.5	46.90	47.9
9	Glyceraldehyde-3-phosphate dehydrogenase	TK0765	26	3.4	5.96	6.4	37.21	37.8
10	Peptide methionine sulphoxide reductase	TK0819	21	2.2	5.04	5.6	39.09	38.3
11	Cell division ATPase	TK1421	28	1.8	4.80	5.3	40.03	41.6
12	2-deoxyribose 5-phosphate aldolase	TK2104	25	1.9	5.18	5.6	24.49	25.8
13	ATPase	TK0701	10	2.0	4.81	4.9	31.93	31.0
14	Transcription regulator	TK1962	21	2.4	5.67	5.9	22.02	23.0
15	Hypothetical protein	TK0083	41	1.8	4.23	4.6	11.67	12.0
16	Hypothetical protein	TK0361	14	2.1	4.82	4.9	16.40	16.7
17	Molydopterin converting factor	TK2118	36	2.4	4.77	4.9	9.15	9.8
18	Thermosome alpha subunit	TK0678	48	3.5	4.84	4.3	59.12	59.9
19	ABC-type maltodextrin transport system	TK1771	15	2.5	4.56	4.4	49.44	48.5
20	Thermosome beta subunit	TK2303	36	3.3	4.86	4.3	59.13	60.4
21	Sugar-phosphate nucleotidyltransferase	TK0219	19	2.1	5.15	5.2	46.80	45.8
22	Acyl-CoA synthetase	TK0944	8	2.1	5.51	5.0	51.83	50.6
23	Hypothetical protein	TK1792	28	1.6	4.83	4.9	40.20	42.2
24	Zinc-dependent protease	TK0689	31	2.3	5.20	5.3	48.52	49.7
25	Hypothetical protein	TK0443	15	1.9	5.4	5.4	40.97	41.5
26	Glycine cleavage system protein	TK1379	38	2.8	5.51	5.2	55.96	56.6
27	Methionine synthase II	TK1447	12	2.1	5.90	5.5	35.25	36.2
28	Metallophosphoesterase	TK0547	19	2.1	5.22	5.6	24.12	24.8
29	Protein disulphide oxidoreductase	TK1085	39	4.1	4.72	4.6	25.28	25.7
30	Deblocking aminopeptidase	TK0781	31	1.8	5.46	5.9	38.27	38.6
31	Hypothetical protein	TK2125	46	1.9	5.82	5.9	28.73	29.2
32	Oxidoreductase	TK0845	22	3.2	5.36	5.5	31.57	32.4
33	Eukaryotic-type DNA primase	TK1791	17	2.4	6.24	6.5	40.27	40.9
34	Inorganic pyrophosphatase	TK1700	47	2.1	4.84	4.2	20.78	21.3
35	Hypothetical protein	TK0108	56	2.0	4.99	5.5	22.39	23.4
36	Acetyltransferase	TK1174	31	2.8	5.98	6.4	18.79	18.0
37	Pyridoxine/pyridoxal 5-phosphate biosynthesis protein protein, SOR/SNZ family biosynthesis	TK0217	25	3.1	5.57	5.5	36.64	37.6
38	Serine hydroxymethyltransferase	TK0528	41	2.5	5.80	5.0	48.20	47.6
39	Ornithine carbamoyltransferase	TK0871	28	2.4	5.76	5.0	35.02	36.0
40	ATPase involved in chromosome partitioning	TK2007	36	2.7	5.71	5.1	27.61	26.6
41	Hydrolase	TK2232	21	2.8	5.42	5.0	24.24	25.2
42	Peroxiredoxin	TK0537	48	4.8	5.02	4.8	24.63	26.5

[a]*Sequence coverage,* [b]*Theoretical pI,* [c]*Experimental pI,* [d]*theoretical mass (kDa), and* [e]*experimental mass (kDa) of the identified proteins.*

to either catalytic activity (53%) or binding activity (37%; **Figure 3B**). The salt stress proteins were classified into seven different groups, with 49% of the proteins belonging to catalytic activity and 32% of the proteins belonging to binding activity (**Figure 3B**). The different proteins with catalytic activity were highly represented, suggesting that these proteins might function in metabolic pathways that deserve further attention.

Metabolic Pathway Analysis

The results of the GO analysis showed that these stresses influenced a variety of cellular processes, particularly metabolic processes (**Figure 4**). The up-regulated proteins were further analyzed using the KEGG to explore potential metabolic pathway functions. Among these proteins, 30 proteins were associated with specific KEGG pathways. These proteins were involved in

TABLE 3 | List of up-regulated proteins under salt stress in *T. kodakarensis* KOD1.

No.	Protein name	Protein ID	SC(100%)[a]	Fold change	pI[b]	pI[c]	Mw[d]	Mw[e]
1	Thioredoxin reductase	TK2100	24	3.1	5.85	6.0	39.44	38.4
2	Xaa-Pro aminopeptidase	TK0967	27	2.1	5.07	5.5	39.20	39.2
3	Phosphoribosyl transferase	TK0853	21	2.8	5.40	5.8	36.24	38.2
4	Deblocking aminopeptidase	TK1177	23	2.2	5.39	5.8	38.17	39.2
5	2-dehydro-3-deoxyphosphoheptonate aldolase	TK0268	25	2.8	5.28	5.5	33.43	35.4
6	Peptide methionine sulphoxide reductase	TK0819	28	2.1	5.29	5.1	29.25	28.2
7	Archaeal glucosamine-6-phosphate deaminase	TK1755	23	1.8	5.41	5.6	36.72	36.7
8	2-dehydropantoate 2-reductase	TK1968	37	2.5	4.43	4.6	34.03	34.9
9	Pyridoxine/pyridoxal 5-phosphate protein	TK0217	25	2.8	5.57	5.1	36.64	37.6
10	Hypothetical protein, conserve, DUF75	TK1919	27	1.6	5.58	5.1	26.19	28.2
11	DNA polymerase sliding clamp	TK0535	32	2.2	4.49	4.9	28.22	29.2
12	Inositol-1-monophosphatase	TK0787	27	2.9	5.27	5.9	27.97	26.0
13	Metal-dependent phosphohydrolase	TK1944	25	2.4	5.76	5.0	30.00	30.8
14	Prephenate dehydrogenase	TK0259	38	2.6	5.29	5.9	29.25	31.3
15	Ferredoxin: NADP oxidoreductase	TK1685	28	3.0	5.76	5.0	32.50	33.5
16	Protein disulphide oxidoreductase	TK1085	39	3.2	4.72	4.0	25.28	24.3
17	Hypothetical protein	TK0108	20	1.7	4.99	5.0	22.39	24.4
18	Metal-dependent phosphohydrolase	TK0014	45	1.9	5.15	5.9	21.24	20.2
19	Peroxiredoxin	TK0537	48	4.5	5.02	5.8	24.63	22.6
20	Acid phosphatase	TK1137	30	2.4	5.90	5.0	28.25	29.3
21	Hypothetical protein	TK1561	52	2.0	5.32	4.8	21.77	23.8
22	Osmotically inducible protein C (OsmC)	TK0189	34	3.8	5.85	5.1	15.34	13.3
23	Transcription regulator	TK0834	28	2.5	6.67	6.0	22.22	23.2
24	Peptidyl-prolyl cis-trans isomerase	TK1850	39	2.8	4.32	5.0	17.54	18.5
25	Hydrogenase maturation protease	TK2004	30	2.1	4.73	4.0	17.03	18.0
26	Predicted nucleic acid-binding protein	TK0066	43	2.3	4.80	4.4	16.88	15.9
27	Hypothetical protein	TK1409	44	1.6	4.74	4.0	9.59	10.0
28	Hypothetical protein, conserve	TK0783	41	2.1	4.87	5.3	11.84	12.8
29	LSU ribosomal protein L7AE	TK1311	40	2.7	5.20	5.9	13.69	14.7

[a]*Sequence coverage,* [b]*Theoretical pI,* [c]*Experimental pI,* [d]*theoretical mass (kDa), and* [e]*experimental mass (kDa) of the identified proteins.*

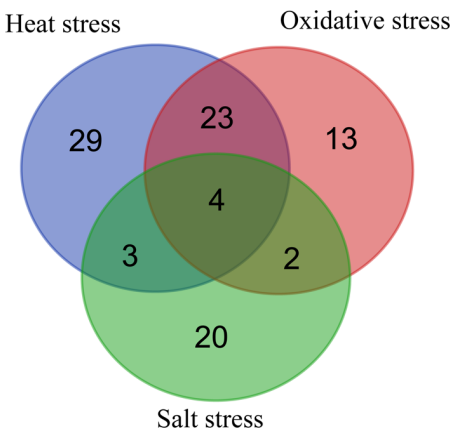

FIGURE 1 | Venn diagram representing the distribution of up-regulated proteins during heat, oxidative, and salt stresses.

pentose phosphate pathway, glycolysis, amino acids metabolism, the urea cycle, secondary metabolite synthesis, transporter, and electron transfer chain. Two enzymes in gluconeogenic pathway (TK2164 and TK0765) were up regulated under both heat and oxidative stresses. TK1771 involved in carbohydrate uptake was also increased under both heat and oxidative stresses. TK0955 and TK1110 in mannose metabolism were only up regulated under heat stress. TK0254, TK0259, TK0268, TK1379, TK1431, TK1447, and TK2217 that were up-regulated by different stresses may participate in amino acids synthesis. Among them, TK1379, TK1431, and TK1447 were increased under both heat and oxidative stresses. TK0254 and TK2217 were up regulated by only heat stress while TK0268 and TK0259 were increased under salt stress. TK0787 and TK0217 involved in compatible solute synthesis were abundant under salt stress. Interestingly, TK0217 were also up regulated by heat stress. Further function of these enzymes were discussed in the following section.

Discussion

All living organisms must adapt to changing environmental conditions to survive. The success of *Thermococcus* largely reflects an ability to survive under extreme conditions. However, these strains are constantly exposed to different stresses. In

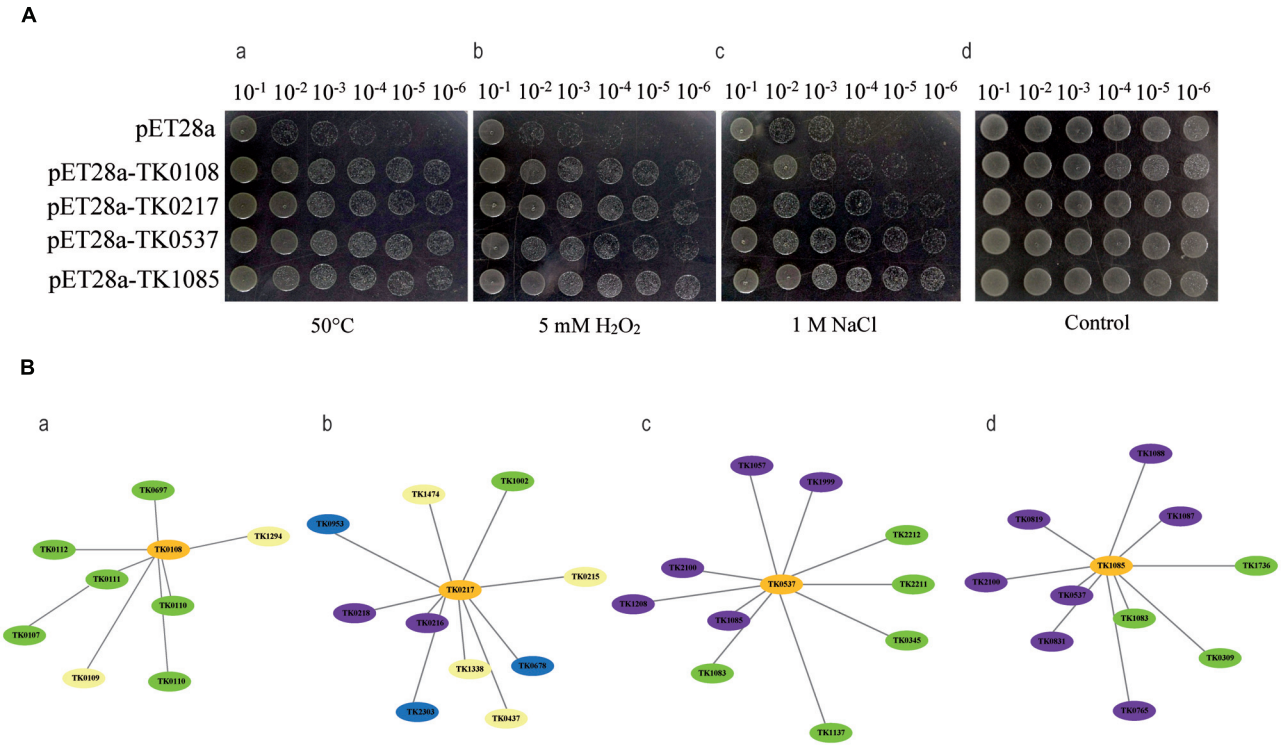

FIGURE 2 | Functional analysis of the co-over-expressed proteins by Agar plate bioassay and protein-protein interaction. (A) TK0108, TK0217, TK0537, and TK1085 confer stress tolerance to *Escherichia coli*. (a) Growth tolerance to heat stress. *E. coli* cells expressing the four proteins (OD600 = 0.5) were preheated at 50°C for 20 min and then incubated at 37°C for 12 h. Various dilutions (10^{-1} to 10^{-6}) were spotted on LB plates. (b,c) Survival of different strains in response to H_2O_2 and NaCl exposure. IPTG was added to the cultures of *E. coli* cells to induce the expression of recombinant proteins. The cultures were adjusted to OD600 = 0.5, 10 μL from 10^{-1} to 10^{-6} dilutions were spotted onto LB plates with 5 mM H_2O_2 (b) and 1 M NaCl (c) and then incubated at 37°C for 12 h. (d) The same amount of *E. coli* cells either expressing the four proteins or containing the pET28 vector grown at 37°C for 12 h were used as a control. **(B)** Predicted protein-protein interaction network, using STRING v9.1, to examine co-expressed proteins (a), the hypothetical protein (TK0108); (b), pyridoxal biosynthesis lyase PdxS (TK0217); (c), peroxiredoxin (TK0537); and (d), protein disulphide oxidoreductase (TK1085)) in heat, oxidative, and salt-stressed *Thermococcus kodakarensis* KOD1 cells. The graph was constructed in the STRING tools using standard parameters. The proteins that may be involved in oxygen detoxifying are shown in violet and the proteins that may regulate DNA repair and transcription are shown in green. The chaperones are shown in blue. Protein functions were predicted using the software listed in 'Materials and Methods.'

the present study, we conducted a proteomics analysis on *T. kodakarensis* KOD1 to globally identify differences in protein expression under heat, oxidative, and salt stresses. Some proteins, such as thermosome, OsmC, and peroxiredoxin, were over-expressed under the examined stresses. The proteomics data further revealed that many interesting proteins were up regulated and some proteins were co-expressed under different stresses. GO and KEGG pathway analyses indicated that sugar, amino acids, and compatible solutes metabolic pathways were involved. The proteins in transmembrane transport and electron transfer chain were also increased.

Cellular stress is induced through the abrupt disruption of the local cell environment. Cells primarily react to various stresses through a number of specific and well conserved adaptive intracellular signaling pathways to alleviate damage and maintain or re-establish homeostasis, and this process has been collectively referred to as the as cellular stress response (Simmons et al., 2009; Jiang et al., 2011). When different stresses are causally and functionally related, certain degrees of overlap, defined as 'crosstalk,' between the respective defense programs are expected (Logemann and Hahlbrock, 2002). Under the three stresses examined, we observed the over-expression of four proteins, including a hypothetical protein (TK0108), pyridoxal biosynthesis lyase PdxS (TK0217), peroxiredoxin (TK0537), and protein disulphide oxidoreductase (TK1085) in *Thermococcus* (**Figure 2**). The function of TK0108 remains unknown; however, this protein might bind manganese-dependent transcription regulators (TK0107), HAD superfamily hydrolases (TK0110), RNA-binding proteins (TK0111), and elongation factors (TK0112) based on predictions of protein–protein interactions. Based on the protein interaction prediction, we assumes that TK0108 might regulate transcription activity through binding these enzymes under stress conditions. For the other three proteins, a recent study has shown that peroxiredoxin (TK0537) belongs to a 1-Cys Prx6 subfamily. This enzyme exhibits oligomeric forms with reduced peroxide reductase activity as well as decameric and dodecameric forms that can act as molecular chaperones by protecting

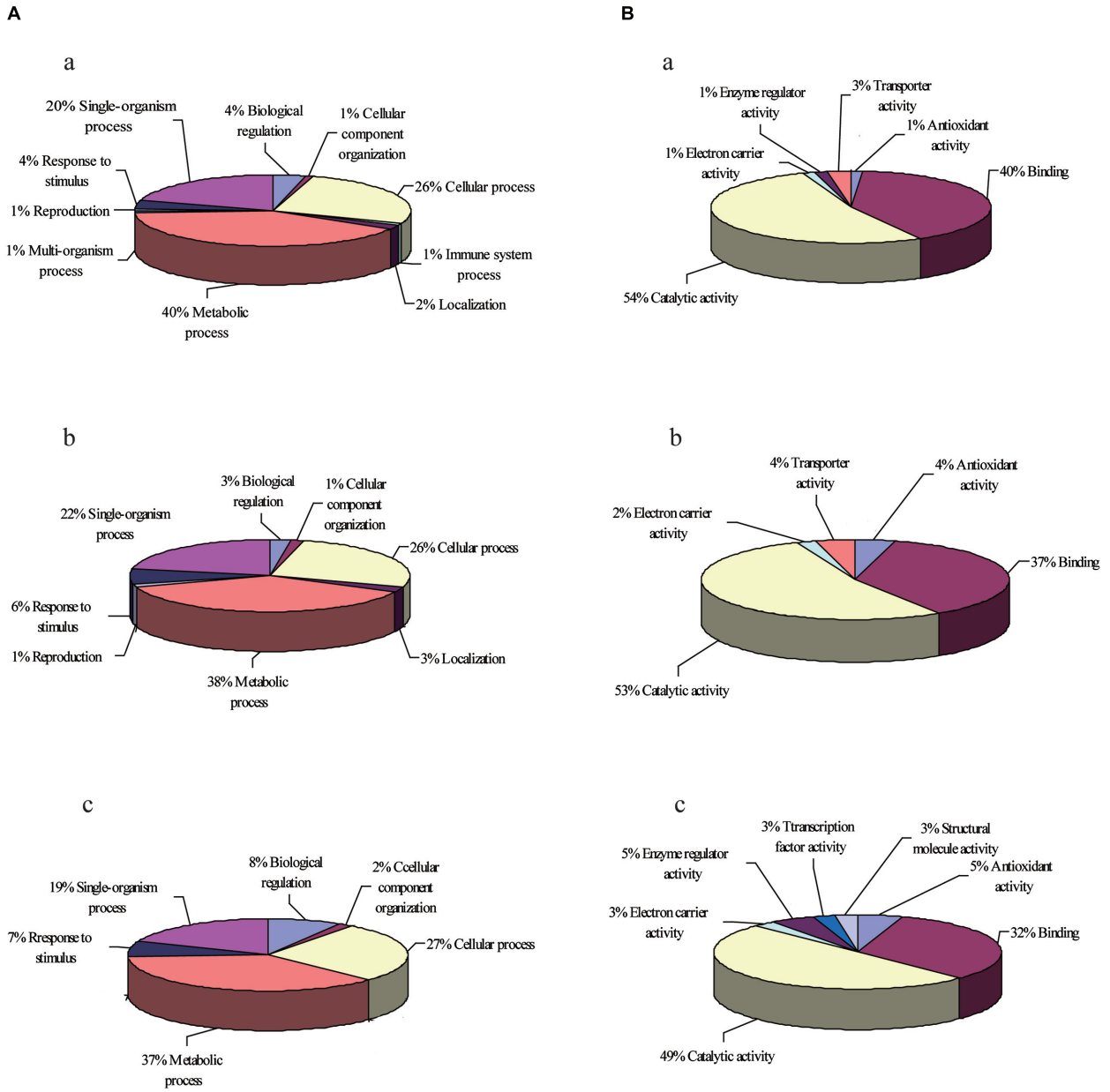

FIGURE 3 | GO enrichment analysis of up-regulated proteins. The GO terms in two biological processes **(A)** and molecular functions **(B)** under heat stress (a), oxidative stress (b), and salt stress (c) were analyzed.

both proteins and DNA from heat and oxidative stresses (Lee et al., 2015). Furthermore, peroxiredoxin (TK0537) and protein disulphide oxidoreductase (TK1085) are important enzymes for the regulation of reactive oxygen species (ROS) production and redox balance across human, yeast, and bacterium. Based on predictions of protein–protein interactions, TK0537 and TK1085 interact with one another and with thioredoxin reductase, glutaredoxin-related protein, and ferritin-like protein. TK0217, the pyridoxal biosynthesis lyase PdxS, and TK0126 are essential for the biosynthesis of pyridoxal 5′-phosphate, the active form of vitamin B6 (Matsuura et al., 2012). Vitamin B6 has long been considered as an enzymatic cofactor. However, it was recently shown that this vitamin is also a potent antioxidant that effectively quenches ROS and is highly important for cellular well-being (Mooney et al., 2009). Increased ROS generation is a common response in cells exposed to stresses; thus, it has been suggested that redox regulation might represent a critical second messenger system upstream of the cell stress signaling network (Kültz, 2005; Jiang et al., 2011), suggesting that these three enzymes are critical factors for cellular stress responses to different stresses.

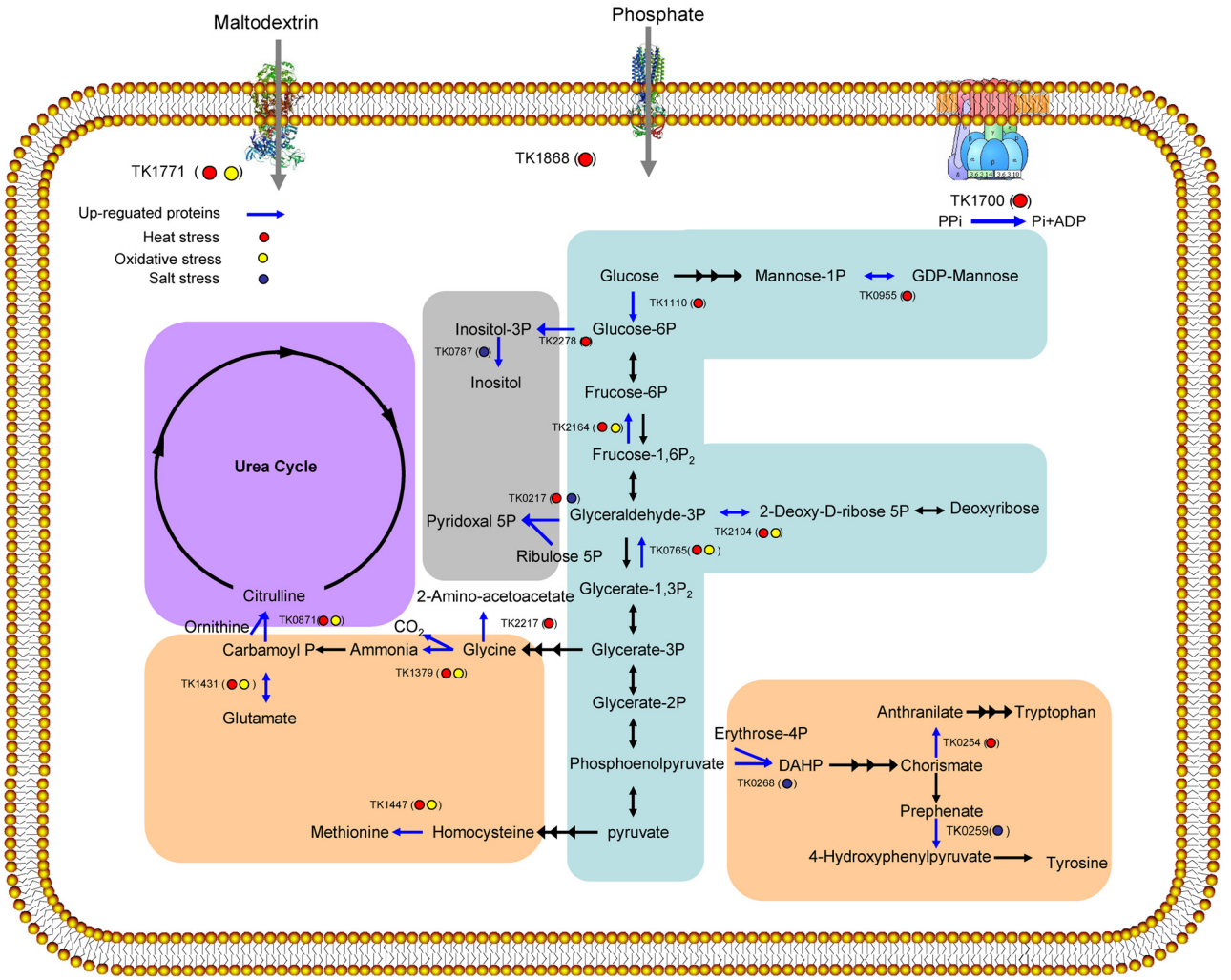

FIGURE 4 | Overview of the up-regulated proteins (bright blue line) involved in cell metabolism under heat stress (red cycle), oxidative stress (yellow cycle), and salt stress (dark blue cycle). Pathways involving carbohydrate metabolism (blue), amino acid biosynthesis (orange), the urea cycle (purple), and secondary metabolite biosynthesis (gray) were induced.

Six enzymes (TK0765, TK0955, TK1110, TK1771, TK2104, and TK2164), involved in carbohydrate metabolism, were abundant in *T. kodakarensis* KOD1 under the examined stresses (**Figure 4**). In eukaryotes, it has been proposed that enhanced saccharides uptake and glycolysis protect cells from oxidative stress (Kondoh et al., 2007). TK1771, the maltodextrin-binding periplasmic component of the ABC-type maltodextrin transport system, is in the same operon with TK1774. Recently, we have shown that this TK1774 can produce maltotriose (Guan et al., 2013; Sun et al., 2015). This facts suggests that TK1771 might mediates the uptake of maltotriose. Furthermore, the members of *Thermococcus* are characterized by the presence of unique, modified variants of classical glycolytic pathways, such as the Embden–Meyerhof–Parnas (EMP) pathway (Brasen et al., 2014). ADP-dependent glucokinase (TK1110), which catalyzes the first step in the EMP pathway to phosphorylate glucose to glucose 6-phosphate, was abundantly expressed under heat and oxidative stress conditions. Increasing of glycolytic flux contributes to NADH production, which can be converted to NADPH by NADH kinase. Additionally, NADPH can be used by cells to prevent against stress (Jia et al., 2010). Interestingly, two gluconeogenic enzymes, fructose-1,6-bisphosphatase (TK2164) and phosphorylating GAP dehydrogenase (TK0765), were also abundantly expressed, potentially redirecting carbon flux away from the EMP pathway. The observed increase in the levels of the gluconeogenic enzymes could signify a boost in the synthesis of glucose-6-phosphate and also favor flux through the ribulose monophosphate pathway, the substitution for the missing pentose phosphate pathway in *T. kodakarensis* KOD1 to produce NADPH (Orita et al., 2006). Carbon flux could also be redirected through deoxyribose-phosphate aldolase (TK2104) to deoxyribose, the precursor of DNA, suggesting that even under severe stress conditions, equilibrium is maintained with respect to intracellular sugar levels and glycolysis intermediates.

A few amino acid biosynthesis proteins, such as glutamate dehydrogenase (TK1431), were significantly expressed during heat and oxidative stresses (**Figure 4**). TK1431 plays a central role in metabolism, as this enzyme is one of the most abundant proteins in *Thermococcales* cells, exceeding 10% of the total cytoplasmic protein in *T. kodakarensis* KOD1 (Altschul et al., 1997). In addition to activity toward Glu, the activity of TK1431 toward Gln, Ala, Val, and Cys has also been detected. Furthermore, TK1431 is responsible for NADH generation in *T. kodakarensis* KOD1 (Yokooji et al., 2013). Ornithine carbamoyltransferase (TK0871), which was up-regulated under heat and oxidative stresses, might catalyze the conversion of ornithine and carbamoyl phosphate into citrulline in a *de novo* pathway for arginine synthesis or the detoxifying urea cycle (Legrain et al., 2001). Two additional enzymes (TK0259 and TK0268), involved in tyrosine biosynthesis, were up-regulated under salt stress. While TK0254 catalyzing tryptophan biosynthesis from chorismate and TK2217 catalyzing glycine synthesis from glycerate-3P were abundant under heat stress (**Figure 4**). The up-regulation of these enzymes ensures the supply of amino acids for protein biosynthesis and protection against stress. In addition, amino acids might also play an important role in stress resistance through osmotic adjustment, osmolytes accumulation and ROS detoxification.

In the previous study, responses of *Thermococcus* and *Pyrococcus* to stresses have been reported. In both *T. kodakarensis* and *P. furiosus*, di-*myo*-inositol phosphate will be accumulated under heat and osmotic stresses (Borges et al., 2010; Esteves et al., 2014). In our study, we found that Inositol-1-monophosphatase (TK0787) and *myo*-inositol-1-phosphate synthase (TK2278) playing pivotal roles in the biosynthesis of di-*myo*-inositol phosphate are increased under heat and osmotic stresses, respectively. In the case of oxidative stress, both *Thermococcus* and *Pyrococcus* can tolerate high concentration of oxygen (Marteinsson et al., 1997; Kobori et al., 2010; Thorgersen et al., 2012). An NAD(P)H oxidase (TK1481) participates in the oxygen sensitivity the expression of the enzyme is constitutive in *T. kodakarensis* (Kobori et al., 2010). This result is consistent with our research as we do not find the over-expression of the protein in any stress. In *Pyrococcus*, the expression of SOR and related enzymes which protect aerobes from the toxic effects of oxygen, is also constitutive (Jenney et al., 1999). In the current proteomics result, SOR is not in the list of over-expressed proteins of *T. kodakarensis*. Interestingly, an alkyl hydroperoxide reductase (PH1217) in *P. horikoshii*, whose transcription and translation increased by the addition of exogenous oxygen, showed 91% identity to TK0537. Together with molecular chaperone function of the enzyme (Lee et al., 2015), all of the evidences indicates that TK0537 plays several roles in response to stress.

In the present study, we used 2-D gel electrophoresis and MALDI-TOF/MS in a proteomics approach to obtain insight into the intricate mechanisms of *T. kodakarensis* KOD1 for survival under heat, oxidative, and salt stresses. Herein, we identified 92 differentially expressed proteins belonging to major processes, including carbohydrate and amino acid biosynthesis, protein folding, and cell redox homeostasis. Most of the proteomics studies under stress have been performed in bacteria and eukaryotes. In the present study, we conducted a proteomics analysis involving Archaea to improve our current understanding of the unique mechanisms in Archaea and explore the evolutionary relationships of stress responses among Archaea, Bacteria, and Eukarya.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00605

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