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

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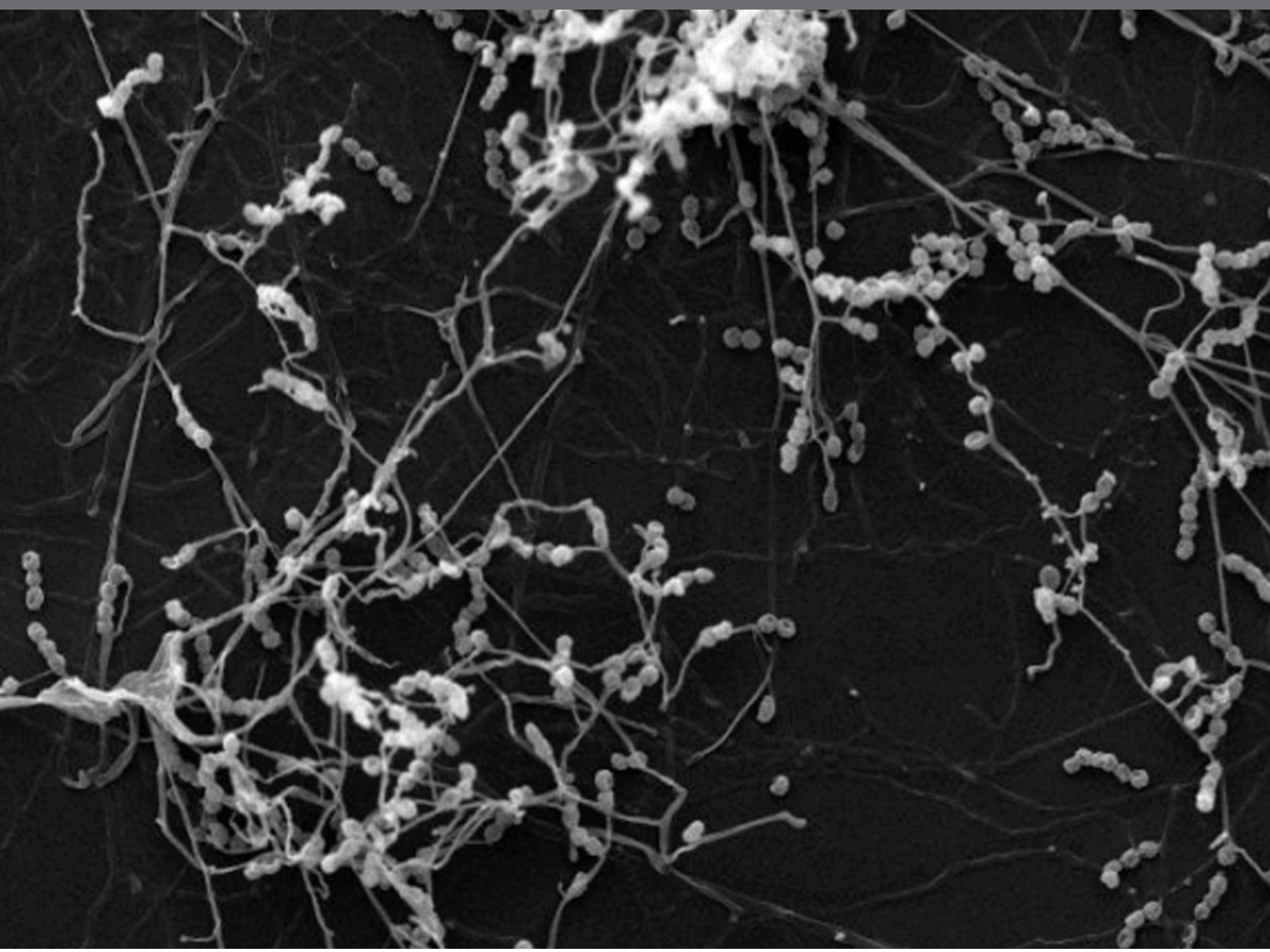

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ACTINOBACTERIA IN SPECIAL AND EXTREME HABITATS: DIVERSITY, FUNCTION ROLES AND ENVIRONMENTAL ADAPTATIONS, SECOND EDITION

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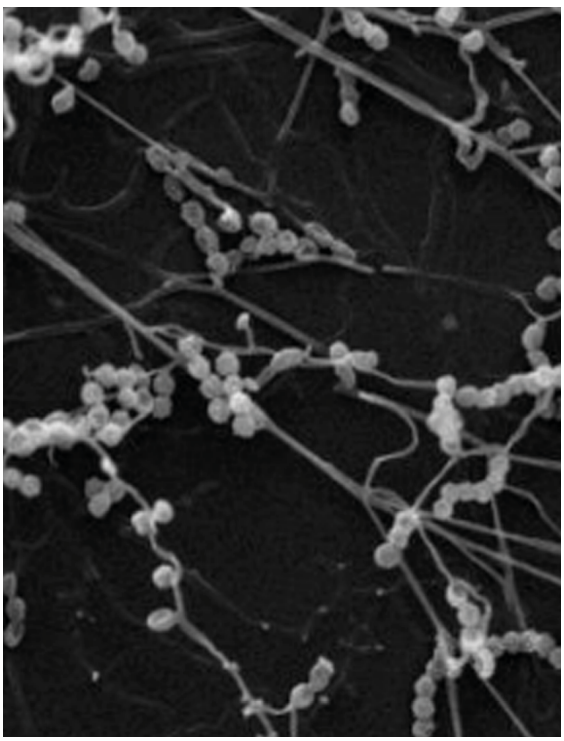

Scanning electron micrograph of strain *Thermocatellispora tengchongensis* YIM77521 grown on ISP 3 medium for 7 days at 45 °C.
Image by Wen-Jun Li.

Actinobacteria are well-known producers of a vast array of secondary metabolites. Compared with actinobacteria from temperate habitats, the community structure, diversity, biological activities and mechanisms of environmental adaptation of those actinobacteria in special and extreme environments are relatively unstudied and unclear, and their functions and utilization are even less reported. These actinobacteria are potential new sources of novel natural products and functions for exploitation in medicine, agriculture, and industry. Recent advances in cultivation, DNA sequencing technologies and -omics methods have greatly contributed to the

rapid advancement of our understanding of microbial diversity, taxonomy, function and they interactions with environment. Following the success of the Research Topic "Actinobacteria in special and extreme habitats: diversity, functional roles and environmental adaptations" organized in 2015, we are happy to launch a second edition. This Research Topic second edition, comprising reviews and original articles, highlights recent discoveries on rare actinobacterial diversity, phylogenomics, biological compounds, ecological function and environmental adaptations of actinobacteria in special and extreme habitats; and broadens our knowledge of actinobacterial diversity and their ecophysiological function.

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

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Editorial on the Research Topic

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

Actinobacteria produce structurally diverse bioactive natural products, such as enzymes, antibiotics, antitumor and immune regulatory agents. *Actinobacteria* are not only the main producers of microbial-derived drugs, they also play an important role as symbionts in plant-associated microbial communities (Barka et al., 2015). At the same time, members of the phylum *Actinobacteria* were found to be widely distributed in different ecological environments, including diverse special and extreme habitats of aquatic and terrestrial ecosystem (Qin et al., 2011; Dhakal et al., 2017; Goodfellow et al., 2018). Compared with actinobacteria from temperate habitats, the community structure, diversity, biological activities, and mechanisms of environmental adaptation of those actinobacteria in special and extreme environments are relatively unstudied and unclear, and their functions and utilization are even less reported. These actinobacteria are potential new sources of novel natural products and functions for exploitation in medicine, agriculture, and industry.

It's exciting that there are more and more reports in this field recently. These discoveries make us consider some intriguing and new questions, such as, are actinobacteria ubiquitous in the special and extreme environment on Earth, and where are the limits of their survival? At the same time, the discovery of more and more pure cultures and new taxa of actinobacteria from extreme environments has raised new questions for the taxonomy of the phylum *Actinobacteria*. How can we establish a more accurate taxonomic system to reflect the natural evolutionary relationship of *Actinobacteria*? Moreover, how can we recognize the specific ecological functions of these ecologically adapted actinobacteria and their potential unique environmental adaptation mechanisms? Following the success of the Research Topic, "Actinobacteria in special and extreme habitats: diversity, functional roles, and environmental adaptations" (Qin et al., 2016), organized in 2015, we are happy to launch a second edition. More than 100 authors, from 14 different

countries, contributed a total of 16 articles in this new edition, including one review paper and 15 original research articles, covering a variety of topics related to actinobacteria in special and extreme habitats. These articles addressed issues related to the cultivation methods of rare actinobacteria, metagenomic analyses of diversity, phylogenomic taxonomy, genome mining, bioactive compounds, and their habitat adaptation mechanism using omics approaches. We are grateful to all authors who have submitted their manuscripts to the second edition of this Research Topic.

The special and extreme environments are likely to contain abundant rare actinobacteria and novel species. However, the acquisition of pure culture is a prerequisite for the further study of their classification and function. Caves spread all over the world, being dark, humid, and nutrient-limited. The cultivation of these cave microorganisms has proven to be challenging (Ghosh et al., 2017). An original article by Fang et al. explores the effects of heat pretreatment, pH, and calcium salts on isolation of rare actinobacteria from Karstic Caves in Yunnan, China. A total of 204 isolates were cultured, and the authors obtained a high number of 29 different rare actinobacterial genera. Actinobacteria from caves have been found to produce a variety of secondary metabolites. However, studies of microbial ecology in caves are still very limited. Recently, members of actinobacteria were reported to be possibly involved in the moonmilk genesis (Bindschedler et al., 2014). Interestingly, the article by Maciejewska et al. provides novel evidences that some filamentous *Streptomyces* could be key protagonists in the genesis of moonmilk through a wide spectrum of biomineralization processes. These studies enlarged our knowledge on cave actinobacterial diversity and their special ecological functions. Desert is the most extreme non-polar biome on Earth. Recent metagenomic analyses of hyper-arid and extreme hyper-arid desert soils revealed a remarkable degree of actinobacterial "dark matter" (Idris et al., 2017). The diversity of actinobacterial taxa in the Badain Jaran (BJD) and Tengger Deserts (TGD) of China were assessed using combined cultivation-dependent and high-throughput sequencing techniques (Sun et al.). These authors found that the phylum *Actinobacteria* was the predominant, comprising 35.0 and 29.4% of the communities in the two desert sands, respectively. Taxonomic classification of 1,162 actinobacterial strains revealed a high diversity of 73 genera, including 37 new taxa, and 10.36 % of the tested isolates showed antimicrobial activities (Sun et al.). However, their ecological significance in deserts deserved further exploration.

Marine actinobacteria have attracted more and more attention because of their special physiological characteristics and capacity of producing various natural compounds with diverse bioactivities (Schinke et al., 2017). However, marine actinobacteria producing anti-complement agents are still poorly explored. Xu et al. analyzed the genome of a marine *Streptomyces* sp. DUT11, which showed a strong anti-complement activity, and isolated the active compounds tunicamycins I, V, and VII. Another marine actinobacterium, *Glycomyces sediminimaris* UTMC 2460, which showed anti-microfouling activity, was analyzed for its active compounds. These authors concluded that diketopiperazines produced by this strain could be used as environmentally safe anti-fouling agents to prevent the fouling process in marine habitats (Heidarian et al.). The article by Sun et al. reveals the marine adaptation mechanism of a sponge-derived actinobacterium, *Kocuria flava* S43, by comparative genomics analysis. These authors found that gene acquisition was probably a primary mechanism of environmental adaptation in *K. flava* S43 (Sun et al.). These studies indicated that marine actinobacteria are rich sources of diverse biological compounds.

In this Research Topic, we collected five papers related to endophytic actinobacteria, which is also a research hotspot in recent years. Habitat-adapted, symbiotic, indigenous endophytic actinobacteria from special and extreme habitats probably contain novel taxa and compounds, and enhance their host tolerance of harsh environments (Mesa et al., 2017; Qin et al., 2018). The article by Singh and Dubey reviews the taxonomic and chemical diversity of endophytic actinobacteria in arid, mangrove, non-mangrove saline and aquatic ecosystems and discusses their potential biotechnological applications. Similarly, Jiang et al. explores the diversity and antibacterial activities of endophytic actinobacteria from five different mangrove plants in Guangxi Zhuang Autonomous Region, China; they found 28 actinobacterial genera and four potential new species. The two articles by Bibi et al. and Wei et al. report on the endophytic actinobacteria and their biological secondary metabolites from the halophyte *Salsola imbricate* and Chinese tea plants; their results confirm again that endophytic actinobacteria might be an undeveloped bioresource library for active compounds. Lasudee et al. report the actinobacteria associated with arbuscular mycorrhizal spores of *Funneliformis mosseae*, and explore their potential plant growth promotion effects in agriculture; results showed that the isolates could produce indole-3-acetic acid (IAA) and siderophores, solubilize phosphate, and promote rice plant growth.

Genome sequencing and the phylogenomic strategy have been explored for the research of taxonomy and prokaryotic systematics. For instance, the class *Acidimicrobiia* is comprised of few cultivable species at present, containing only the order *Acidimicrobiales*, two families *Acidimicrobiaceae* and *Iamiaceae* with few genera (Ludwig et al., 2012). Hu et al. analyzed 20 sequenced members of this class and identified 15 conserved signature indels (CSIs) in widely distributed proteins and 26 conserved signature proteins (CSPs); the phylogenomic analysis revealed another three major lineages in addition to the two recognized families. Furthermore, Sangal et al. revisit the taxonomic status of the biomedically and industrially important genus *Amycolatopsis*, using a phylogenomic approach. According to the genome sequences analysis and the core genome phylogeny, genus *Amycolatopsis* was subdivided into four major clades and several singletons (Sangal et al.). These results indicate that whole genome sequencing analysis can provide more accurate taxonomic status for prokaryotes.

The developments of omics methods have provided a robust support for our understanding of the actinobacterial adaptation mechanisms to the special and extreme habitats. Cornell et al. obtained 76 plasmid-containing isolates of actinomycetes from the Great Salt Plains of Oklahoma. Eleven isolates were chosen for genome sequencing, and the results revealed the

presence of series genes involved in antibiotic production, antibiotic, and heavy metal resistance, osmoregulation, and stress response, which likely facilitate their survival in the extreme halophilic environment (Cornell et al.). By transcriptome analysis, physiological, and molecular experiments, Han et al. found that accumulation of ectoine played a vital role for the salt stress tolerance of the halotolerant *Nocardiopsis gilva* YIM 90087^T. The article by Yin et al. report that a hybrid strategy was used to utilize carbon sources at different temperatures by an aerobic, and cellulose degrading thermophilic actinomycete, *Thermoactinospora rubra* YIM 77501^T, by using combined genomic and transcriptomics methods.

In summary, this Research Topic second edition presents recent discoveries on diversity, function roles, and environmental adaptations of actinobacteria in special and extreme habitats; and broadens our knowledge of actinobacterial diversity and their ecophysiological function. We are delighted to present this Research Topic in Frontiers in Microbiology. We hope that readers of the Journal will not only enjoy this Research Topic but also will find it a useful reference. Future research still looks forward to the innovation and application of new technologies, such as the application of single cell microfluidic technique to obtain new pure cultures. At the same time, the cooperation of different disciplines, and international cooperation of scientists from different countries should be strengthened. We also believe that in the future, more "dark matter" from actinobacteria in special and extreme environments will be discovered and utilized for the benefit of human beings.

AUTHOR CONTRIBUTIONS

W-JL and SQ organized this topic. SQ wrote the editorial article. H-PK, WH, and IA are co-editors of the topic and discussed the writing.

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ORIGINAL RESEARCH
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Insights on the Effects of Heat Pretreatment, pH, and Calcium Salts on Isolation of Rare *Actinobacteria* from Karstic Caves

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The phylum *Actinobacteria* is one of the most ubiquitously present bacterial lineages on Earth. In the present study, we try to explore the diversity of cultivable rare *Actinobacteria* in Sigangli Cave, Yunnan, China by utilizing a combination of different sample pretreatments and under different culture conditions. Pretreating the samples under different conditions of heat, setting the isolation condition at different pHs, and supplementation of media with different calcium salts were found to be effective for isolation of diverse rare *Actinobacteria*. During our study, a total of 204 isolates affiliated to 30 genera of phylum *Actinobacteria* were cultured. Besides the dominant *Streptomyces*, rare *Actinobacteria* of the genera *Actinocorallia*, *Actinomadura*, *Agromyces*, *Alloactinosynnema*, *Amycolatopsis*, *Beutenbergia*, *Cellulosimicrobium*, *Gordonia*, *Isoptericola*, *Jiangella*, *Knoellia*, *Kocuria*, *Krasilnikoviella*, *Kribbella*, *Microbacterium*, *Micromonospora*, *Mumia*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Nocardiopsis*, *Nonomuraea*, *Oerskovia*, *Pseudokineococcus*, *Pseudonocardia*, *Rhodococcus*, *Saccharothrix*, *Streptosporangium,* and *Tsukamurella* were isolated from these cave samples.

Keywords: Sigangli Cave, rare *Actinobacteria*, heat pretreatment, pH, calcium salts

INTRODUCTION

Caves provide a quasi-extreme environment for living organisms owing to relatively low organic nutrient input and lack of light (Pedersen, 2000). Some 'sojourners' such as crickets, spiders, olms and bats are, however, adapted to these unassumingly harsh environments through modifications in their body morphology and other physiological changes (Culver and Pipan, 2009). Unlike these organisms, the microscopic counterparts are abundantly present within the cave environment (Wu et al., 2015; Tomczyk-Żak and Zielenkiewicz, 2016), and they are involved in the dissolution and precipitation of karst minerals (Castanier et al., 2000). Despite many studies on the potential function and diversity of these microbes in the oligotrophic environments, our knowledge on cave microbial diversity and related bioactivities are still limited (Engel et al., 2004).

Earlier studies on caves indicated that bacteria and archaea constitute the majority of the microbial diversity (Barton and Jurado, 2007). Pyrosequencing analyses had determined phyla

Proteobacteria, *Acidobacteria,* and *Actinobacteria* to be among the dominant taxa on cave environments (Pasić et al., 2010; Wu et al., 2015; Tomczyk-Żak and Zielenkiewicz, 2016). Among the major bacterial lineages, the phylum *Actinobacteria* are of special interest due to their versatile metabolic activities (Genilloud, 2014; Remenár et al., 2014). They are found ubiquitously in nature. Besides their role in biodegradation and production of ecologically important bioactive metabolites, they are also involved in biomineralization (Gillieson, 1996; Dhami et al., 2013). Based on the biotechnological significance of the phylum *Actinobacteria*, the basic aim of this work is to study the diversity of cultivable rare *Actinobacteria* in a karst cave located in Yunnan, China.

Though bacterial richness and diversity within specific environmental samples and their possible physiological role in nature can be established with NextGen sequencing techniques and other bioinformatics tools (Green and Keller, 2006), physiologies can only be verified with pure cultures (Leadbetter, 2003). It is, however, estimated that 99% of the existing microbes have not been cultivated yet (Whitman et al., 1998). It is therefore necessary to utilize various enrichment techniques or media to bring these uncultivated cells into cultures. Some of these techniques of culturing previously uncultivable soil bacteria have been reviewed by Pham and Kim (2012). In their review, major emphases are given upon the modification of growth conditions and use of new culturing methods. In the current, we try to explore the option of using a combination of enrichment techniques including heat-pretreatments of the samples, adjusting the isolation media into a pH gradient and supplementing the media with different calcium salts at different concentration. These techniques have already been established as an effective measure for isolation of diverse rare *Actinobacteria* (Alferova and Terekhova, 1988; Hayakawa, 2008; Lauber et al., 2009), but have not been exploited to determine the cultivable actinobacterial diversity in caves.

MATERIALS AND METHODS

Site Description and Samples Collection

The Sigangli Caves, located in Cangyuan County, Yunnan Province, China, are part of a series of karst caves of the Yunnan–Guizhou Plateau formed from the dissolution of limestone and other calcareous rocks. The plateau, covering an area of over 1.3×10^5 sq. km, was formed during tectonic shifts of Eurasian plate and is the center of karst area in East Asia (**Figure 1**).

Samples for isolation of *Actinobacteria* were collected from different part of the caves (23°32′ N, 99°33′ E). The samples (**Table 1**) include the hard rock forms (sedimentary rocks and cave coral, referred to here as Type 1 sample) and the weathered rock forms (saprolites, sand, debri and arene, referred to here as Type 2 sample). The samples were collected using sterile scalpels or spades and were transferred immediately in falcon tubes or Ziploc bags. These samples were then stored under a low temperature environment until processing for isolation.

Isolation and Preservation of *Actinobacteria*

A set of pretreatments and inoculation procedures adopted for the isolation of culturable *Actinobacteria* is listed in **Table 2**. Samples (2 g) were suspended in 20 ml sterile distilled water and kept in a rotary shaker (180 rpm, 28°C) for 1 h. The suspensions were serially diluted and aliquots of 100 μl of the diluted suspension was plated on freshly prepared agar media (in triplicates). The following isolation media were used: Humic acid-vitamin medium (HV) (Hayakawa and Nonomura, 1987); International *Streptomyces* Project (ISP) 5 medium (Shirling and Gottlieb, 1966); Cellulose-casamino acid (CC) medium (Yuan et al., 2017); Trehalose-proline agar (HP) (Li et al., 2014); Starch-Casien (SC) medium (Küster and Williams, 1964); B-4 medium (Boquet et al., 1973), and Water agar containing 11 g of agar per liter of water. Each of these media was supplemented with nystatin (50 mg L^{-1}) and nalidixic acid (20 mg L^{-1}) to prevent the growth of fungi and fastidious bacteria. Following incubation for 2 weeks at 28°C, all the colonies developed on the isolation media were counted. Depending on the isolation conditions and the media used, total colony forming units (CFUs) from each treatment were determined. Apart from the spore-forming strains, viabilility of the other vegetative cells were determined by subculturing on YIM 38 medium (Zhao et al., 2010). Heatmaps representing the distribution of these CFUs across the different physiological parameters were generated with R software (R Core Team, Vienna, Austria). The heatmaps are generated by applying 'heatmap.2' function of 'gplots' package. Further, morphologically distinct colonies were selected and purified on YIM 38 medium. The purified cultures were preserved as lyophilized cultures in skim milk and as glycerol suspensions (20%, v/v) at −80°C.

DNA Isolation, PCR Amplification, and Sequencing

Genomic DNA was extracted using TIANGEN^{TM} Genomic DNA purification kit according to manufacturer's instructions. The DNA preparations were used as template for PCR amplification using the universal primers 27F: 5′-CA GAGTTTGATCCTGGCT-3′ and 1492F: 5′-AGGAGGTGA TCCAGCCGCA-3′. PCR reactions were conducted using iCycler Thermal Cycler (Bio-Rad, USA Laboratories, Inc.) by applying the following conditions: initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. Amplified PCR products were verified on 0.8% agarose gel with 2 Kb DNA ladder (Fermentas) as a molecular size reference and sent for sequencing (Sangon Biotech, Shanghai, China).

16S rRNA gene sequences of the isolates were compared with the published 16S rRNA gene sequence database in EzBioCloud server (Yoon et al., 2017) on the basis of pairwise alignment. The strains were identified based on the sequence similarity to their closest homolog. Strains showing identity to bacterial phyla, other than *Actinobacteria*, were not reflected in the current study. Relative abundance of the *Actinobacteria* based on the number of

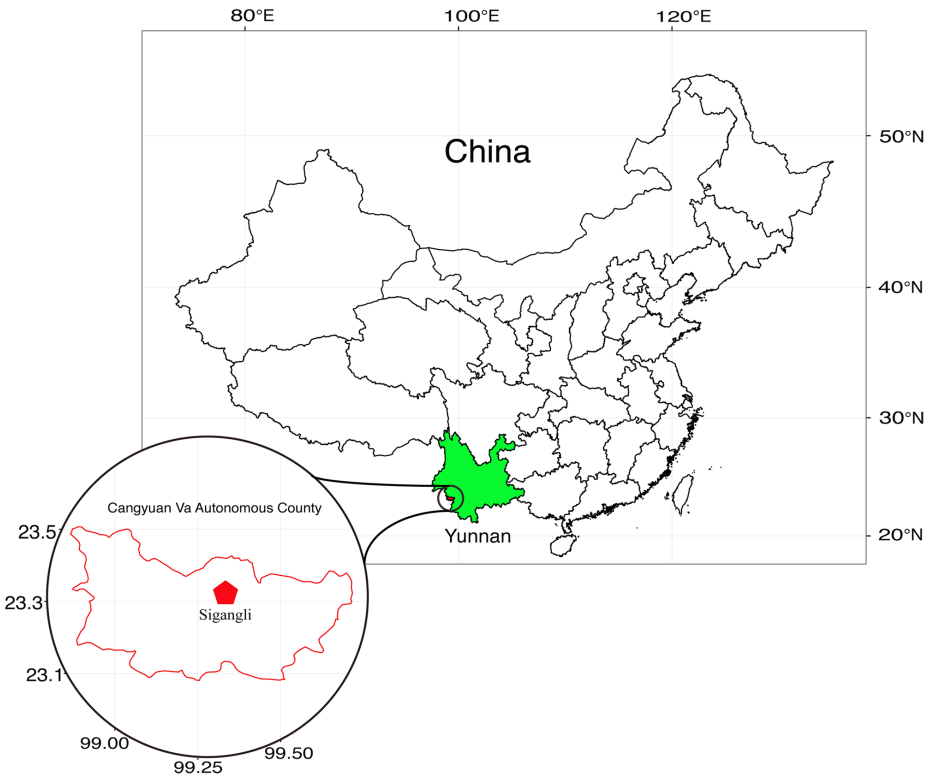

FIGURE 1 | Geographical location of Sigangli Caves, Yunnan Province, China.

strains from each genera were plotted into a scatter-plot using Microsoft Excel 2013.

Nucleotide Accession Numbers

The partial 16S rRNA gene sequences of all the cultivated actinobacterial strains isolated during the study were deposited in GenBank with the following accession numbers: KX274728–KX274786, MF431270–MF431414 (Supplementary Table S1).

RESULTS

Effects of Temperature, pH, and Calcium Salts on Isolation of *Actinobacteria*

Figure 2 represents the effects of the different enrichment methods on the isolation of *Actinobacteria*. When heat pretreatments was used as the enrichment techniques (**Figure 2A**), more CFUs was determined in samples incubated at 40°C for 2 days prior to isolation (Treatment c) than in samples incubated at higher temperatures (Treatments d, e, and f). It is, however, interesting to note that samples kept at room temperature (Treatment b) yield lower CFUs than the one incubated at 40°C. Among the five media during this process, HV agar seems to be a better isolation medium (**Figure 2A**). When pH was used as the isolation criteria, it was determined that more CFUs were obtained in media adjusted to neutral pH, fewer in alkaline pH and least in circumneutral pH (**Figure 2B**). If the sample pH is taken into consideration, there is a gradual decrease in the number of CFUs with increase in sample pH, irrespective of the sample types. Lastly, considering the use of calcium salts in isolation, it was determined that $CaCO_3$ yielded more *Actinobacteria* than $(CH_3COO)_2Ca$ and $CaCl_2$ (**Figure 2C**). However, it was not just the salt that is important, the concentration of each salt in the selection media also played an important role. In our case, it was determined that higher CFUs was determined when salt concentration was proportionately at lower concentration (i.e., 0.1 and 0.01%, w/v) than in its absence or at high concentration.

Relative Abundance of Rare *Actinobacteria*

Of these total colonies observed, morphologically distinct colonies were further selected, subcultured, and preserved. These include 87 isolates from Type 1 samples and 117 from the Type 2 samples. Sequence analysis of 16S rRNA gene indicated that the strains from the Type 1 samples were distributed to 20 genera in 14 families of the phylum *Actinobacteria*, while the strains isolated from Type 2 samples were distributed to 21 genera and 16 families. The relative abundance of the strains is represented in **Figure 3**, and the 16S rRNA gene sequence profile listed in Supplementary Table S1. Besides the most abundant genus *Streptomyces*, the rare actinobacterial genera *Nocardia* and *Rhodococcus* were relatively abundant in both the sample types

TABLE 1 | Description of the samples used for isolation.

Samples	Sample form	pH of samples	Sampling date	Sampling site	Coordinates
STRS01	Dark saprolite	7.8	30-03-2013	Sigangli	E 99.334′
SST6	Sedimentary rock	7.3			N 23.325′
SS16	Sandy soil	8.3			
SS19	Debris	8.0			
CS7	Arene	8.5	01-04-2013		
CST1	Cave coral	7.1			
YS5	Saprolite	7.5			
CS4	Stony and sandy soil	7.6	02-04-2013		
BS1	Dark saprolite	8.1	03-04-2013		

TABLE 2 | Effects of physiological parameters on isolation of *Actinobacteria*.

S. no.	Physiological conditions for isolation	Pretreatment conditions	Samples for the study	Isolation media used
1	Sample pretreatment methods	(a) Fresh samples w/o pretreatment (b) Air dried in room temperature for 2 weeks (c) Samples kept in oven at 40°C for 2 days (d) Samples kept in oven at 65°C for 4 h (e) Samples heated in oven at 110°C for 1 h (f) Pretreatment e followed by c	SST6 (Type 1), SS19 (Type 2)	HV, ISP5, CC, HP, SC
2	Effect of pH	Isolation media adjusted to pH 6, 7, 8, and 9	SS16 (pH 8.3), SS19 (pH 8.0), YS5 (pH 7.5), CS7 (pH 8.5), CS4 (pH 7.6), BS1 (pH 8.1)	CC, HP
3	(a) Preference of calcium salts (b) Concentration of the salts	(a) Isolation media supplemented with one of the three calcium salts $CaCO_3$/$CaCl_2$/$(CH_3COO)_2Ca$ (b) Concentration of each calcium salts adjusted to 0, 0.01, 0.1, and 1% (w/v)	CST1 (Type 1), STRS01 (Type 2)	B-4, HP, Water agar

(18 and 7 strains respectively in Type 1 samples, and 17 and 7 strains in Type 2 samples). While the genus *Micromonospora* was relatively more abundant in Type 2 samples (19 strains, as compared to 2 in Type 1 samples), the genus *Mycobacterium* was more in hard rock (7) than in the weathered rock samples (3). Other rare genera that were common to both the sample types include *Jiangella*, *Kribbella*, *Nocardioides*, *Nocardiopsis*, *Nonomuraea,* and *Streptosporangium*. Apart from these common genera, few rare actinobacterial genera were restricted to only one particular sample type. The genera *Amycolatopsis*, *Beutenbergia*, *Cellulosimicrobium*, *Gordonia*, *Isoptericola*, *Microbacterium*, *Mumia*, *Oerskovia,* and *Pseudokineococcus* were isolated only from Type 1 samples while the rare genera *Actinocorallia*, *Actinomadura*, *Agromyces, Alloactinosynnema, Knoellia, Kocuria, Krasilnikoviella*, *Pseudonocardia*, *Saccharothrix,* and *Tsukamurella* were isolated from Type 2 samples.

DISCUSSION

Karstic caves are characterized by low stable temperature (10–15°C), relatively high humidity (90–100%) and total darkness or low level of light, and are often mystical and inaccessible for study. Above that, caves usually constitute a oligotrophic ecosystem with total organic carbon of less than 2 mg/L (Tomczyk-Żak and Zielenkiewicz, 2016). Despite the oligotrophic condition, the average number of microorganisms in this ecosystem have been estimated to be in the range of 10^6 cells/g of rock (Barton and Jurado, 2007). Despite the estimated large number of bacterial cells in cave rocks, they could isolate around 400 bacteria belonging to phyla *Proteobacteria*, *Firmicutes,* and *Actinobacteria* from a sample of Lechuguilla Cave, New Mexico. Of these strains, nearly 40 strains are assumed to be previously uncultivated species indicating that the diversity of microbes within the caves is impressive (Barton and Jurado, 2007). In another study in Kartchner Caverns, Arizona, 90 unique isolates belonging to *Proteobacteria*, *Firmicutes,* and *Actinobacteria* were isolated, but these bacteria have 16S rRNA gene sequence similarity profiles to known bacteria (Ikner et al., 2007).

Among these group of bacteria, class *Actinobacteria*, because of its different versatile morphology, were detected and isolated from even the most extreme of environments (Mohammadipanah and Wink, 2016). The main mechanism for the survival of these actinobacteria in these environments is through the formation of different types of spores. Most actinobacterial spores are developed either endogenously (e.g., *Dactylosporangium*, *Thermoactinomyces*) or exogenously

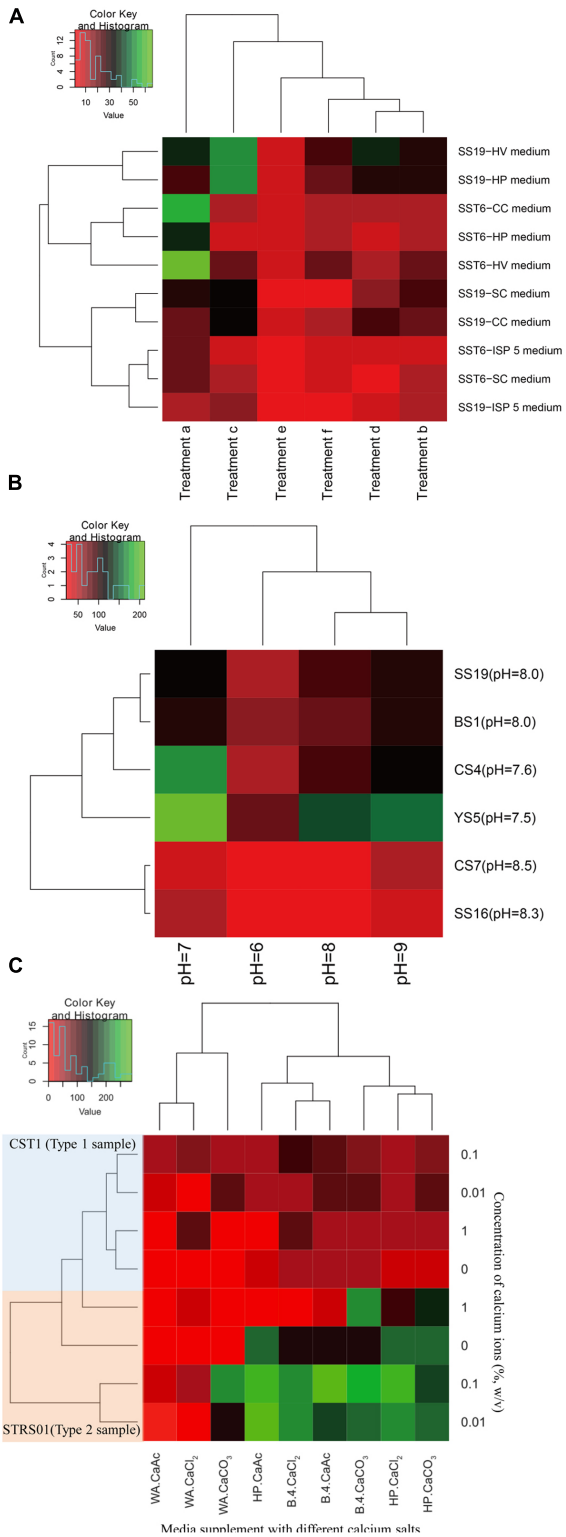

FIGURE 2 | Heatmaps indicating the number of CFUs obtained after enrichments of samples collected from a karstic cave in Sigangli, China. The labels **(A–C)** represent the effects of temperature, pH, and calcium salts on the isolation of rare *Actinobacteria*, respectively. The heatmaps are generated in R software by applying 'heatmap.2' function in 'gplots' package.

(e.g., *Streptomyces*) in response to environmental stress (Kalakoutskii and Agre, 1976). While in karst environments, the presence of minerals, particularly different forms of calcium rocks, trigger sporulation in many *Actinobacteria* (Kalakoutskii and Agre, 1976). These spores usually remain in dormant state with minimum respiration, but could be made to germinate in defined media by providing an energy source (Salas et al., 1983). In most case of germination, a mild stimuli is required either in the form of heating or supplying germinants that stimulate the disruption of spore cortex (Warth and Strominger, 1972; Ensign, 1978). In our effort to select and isolate diverse *Actinobacteria*, we considered three sample enrichments methods involving chemical and physical treatments as discussed below.

Among the physical enrichments method for actinobacterial isolation, air drying, dry heating, moist incubation, and desiccation have been found to be an effective for selection of spore-forming rare *Actinobacteria* (Hayakawa, 2008). Air-drying of soil at 120°C for an hour is usually preferred for isolation of genera *Dactylosporangium*, *Microbispora* and *Streptosporangium*, while limiting the growth of streptomycetes (Jiang et al., 2016). Similarly, air-drying at 100°C for 15 min have been used effectively for isolation of *Actinomadura* (Jiang et al., 2016). Air-drying the sample at an ambient temperature for a week preferentially select *Herbidospora* among other bacteria (Jiang et al., 2016). Genus *Micromonospora* were selectively isolated by pretreatment of samples at 55–65°C for 30 min (Jiang et al., 2016). An effective method proposed for isolation of rare *Actinobacteria* involved air drying at 80°C for 2 h (Goodfellow, 2010). Preferential selection of several rare *Actinobacteria* on heat treatments might be related to the spore-forming capability of several groups of *Actinobacteria* (Jiang et al., 2016). In the current study, we considered to pretreat our samples by air-drying at room temperature, 40, 65, and 110°C for different time intervals for effective isolation of diverse rare actinobacteria (**Table 2**). An advantages of air-drying is that many *Actinobacteria* produces spores, and that dry spores have low respiration rate and can survive for longer period of time. During this period of low level of endogenous respiration, the spores did not germinate, but can be germinated readily when a defined medium with organic energy source is provided (Ensign, 1978). During our study, four cells in the heatmap representation indicated CFUs' count in the range of 50 or above (**Figure 2A**). These highest OTUs were represented in samples treated at low ambient temperatures. The levels of CFUs was found to decrease with increased pretreatment's conditions. Least CFUs were obtained in the samples pretreated at 110°C for 1 h. However, if the pretreatment at 110°C for 1 h is accompanied by incubation at 40°C for 2 days, the numbers of sporulating cells that survived the heat treatment increased as indicated in the **Figure 2A**. This finding could be related to the study of Lapteva et al. (1976) whereby the authors suggested that the germination inhibitors produced in the spores due to heating were neutralized during subsequent incubation at lower temperature. If a comparative analyses were made between the number of CFUs obtained and the strains cultured during our study, positive correlation could be established between the number of spore-forming *Actinobacteria*

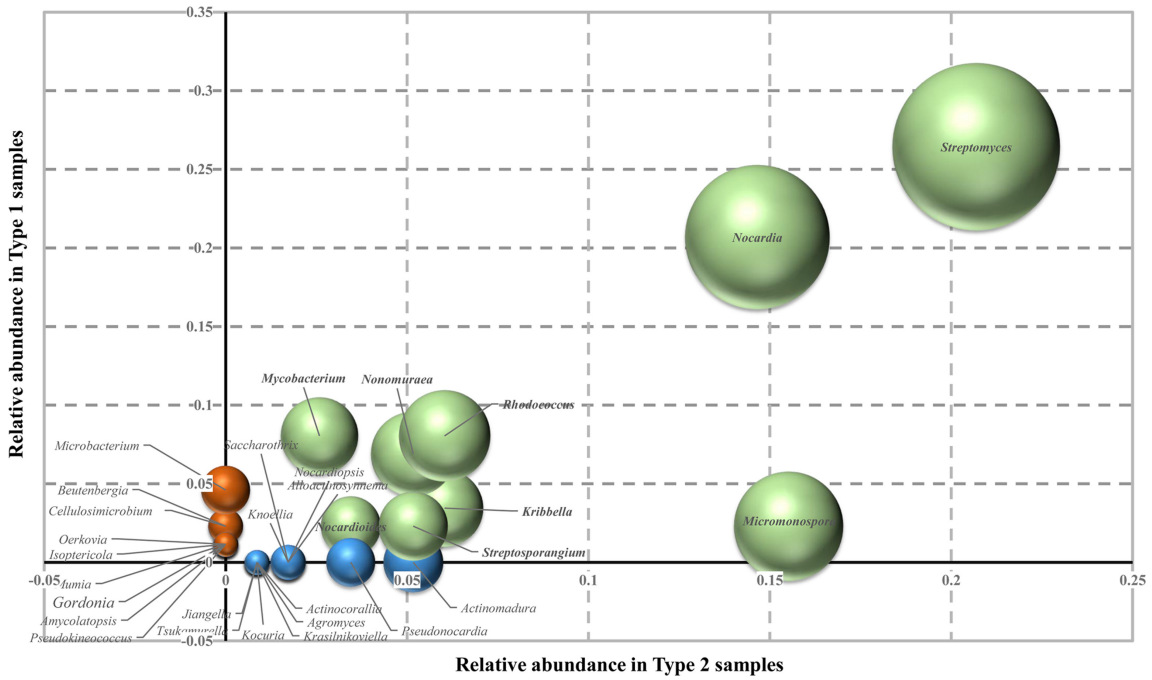

FIGURE 3 | Relative abundance of rare *Actinobacteria* on the two types of cave samples used on this study. Values on the axes represented the relative abundance of each genus on the different samples. Green spheres indicate actinobacterial genera present in both the sample types; red, genera isolated from Type 1 samples only; and blue, genera found only in Type 2 samples. Sizes of the sphere quantify the relative number of strains of each genera. The scatter-plot is generated using Microsoft Excel 2013.

obtained and the temperature used for pretreatment. While no strict thermophilic *Actinobacteria* such as members of the genera *Thermomonospora*, *Dactylosporangium,* etc. were isolated during our study, a fair number of rare *Actinobacteria* with aleurispores (*Micromonospora*), arthrospores (*Actinomadura*), sporangiospores (*Streptosporangium*), or other spore-bearing structures (*Amycolatopsis* etc.) were obtained. However, in addition to these *Actinobacteria*, endospore-forming non-*Actinobacteria* such as *Bacillus* strains were also obtained during the isolation process. The co-occurrence of these bacteria are, however, found to be very less as confirmed by our preliminary sequencing analysis of the 16S rRNA gene (data not shown).

Actinobacteria are capable of growing under selective conditions of pH or salinity (Mohammadipanah and Wink, 2016). It is because pH of soil strongly influence the biomass, activity and composition of the microbial community, and therefore pH in the isolation media provide a selective pressure for the growth of bacteria (Bååth, 1996; Matthies et al., 1997; Rousk et al., 2009). Unlike fungi which grow preferentially in acidic and moist condition, most *Actinobacteria* showed optimum growth on slightly alkaline condition (Kontro et al., 2005; Lewin et al., 2016). The isolation of strictly acidophilic *Actinobacteria* like the genus *Streptacidiphilus* (Kim et al., 2003; Cho et al., 2008; Golinska et al., 2016) from diverse ecosystems have provided a platform for isolation of *Actinobacteria* under acidic environments, in addition to the normally preferred slightly alkaline condition. Considering the wide range of pH on which *Actinobacteria* can dwell with, we considered to compare the CFU's count under a gradient of pH range of isolation, despite all the sample pHs being in the range of 7.5 to 8.5 (**Table 2**). During the current study, more actinobacterial CFUs were detected in neutral pH (**Figure 2B**). This may be because of the easy maintanence of cell's cytoplasmic pH at close to neutrality (Kontro et al., 2005). While the actinobacterial CFUs did not dwindle much in alkaline pH, it had the least count in acidic isolation media. This finding could also be related with the findings of Rousk et al. (2010) whereby the relative abundance of *Actinobacteria* was not affected by soil pH, but rather depended on the isolation condition (Lauber et al., 2009).

Actinobacteria often colonize the rock walls of caves. In a study on biogeochemical role of *Actinobacteria* in Altamira Cave (Spain), *Actinobacteria*-coated spots on the cave walls was found to uptake carbon dioxide gas which is available in abundance in cave (Cuezva et al., 2012). This uptake gas is used by the bacteria to dissolved rock and subsequently generate crystals of calcium carbonate (Cañaveras et al., 2001). While its role in biomineralization is plausible, calcium ions do play specific role in various spore-forming microorganism as well. While measuring the metal ion content in five *Streptomyces* strains (Salas et al., 1983), the level of calcium was found to be higher in dormant spore than in the vegetative cells. Calcium is mostly found as a complex with dipicolinate and this complex could be acting as secondary stabilizing agent for the spore against environmental stresses (Moir and Smith, 1990). However in the

presence of suitable germinants, the spore release the calcium-dipicolinate complex from the core to initiate the process of spore germination (Moir, 2003). One process through which the complex acts is by initiating cortex degradation through structural modification of the peptidoglycan (de Vries, 2004). The use of calcium carbonate in pretreatment for selective isolation of *Actinobacteria* (El-Nakeeb and Lechevalier, 1963; Alferova and Terekhova, 1988) may be related with the spore formation in *Actinobacteria*. On the other hand, calcium chloride, when added to isolation media, was found to stimulate the growth of a rare heterotrophic *Actinobacteria*, *Sporichthya* (Suzuki et al., 1999). We, therefore, considered to compare the effect of supplementation of three calcium salts in the isolation media including $CaCO_3$ and $CaCl_2$. During our study, all three calcium salts facilitated the isolation of *Actinobacteria* (**Figure 2C**). This finding is also in congruence with the finding of Chen et al. (2016) whereby the actinobacterial community structures showed significant correlations with calcium. Warth and Strominger (1972) have determined that germination of bacterial spore required a optimum concentration of approximately 10 mM calcium ions. This observation is similar with our findings where a lower concentration of calcium ions (0.1% or ∼10 mM) provide more CFUs than higher (1%, w/v or ∼0.1 M) or in the absence of calcium salts (**Figure 2C**). Among the two types of samples, more CFUs were observed in Type 2 than in Type 1 samples. The reason could be implicated on the lower cell concentration on the surface of hard rock (Barton and Jurado, 2007).

In all the above cases, isolation media play the key role for providing the favorable condition for isolation and growth of rare *Actinobacteria*. It is therefore important to use isolation media that preferentially isolate different group of rare *Actinobacteria* and select/design set of media with different components to maximize our chance for isolation of unique and other rare *Actinobacteria* (Tiwari and Gupta, 2013), lest *Actinobacteria* will be at competitive disadvantages on the solid media against the fastidious bacteria and fungi that usually occupy a larger living space. In the current study, we had selected seven isolation media that have been found effective in isolation of *Actinobacteria*. Among them, HV agar with/without chemical supplements had been used efficiently by Hayakawa's group for isolation of many rare *Actinobacteria* including strains of genera *Actinokineospora*, *Actinomadura*, *Actinoplanes*, *Actinosynnema*, *Catenuloplanes*, *Cryptosporidium*, *Dactylosporangium*, *Geodermatophilus*, *Herbidospora*, *Kineosporia*, *Microbispora*, *Micromonospora*, *Microtetraspora*, *Nonomuraea*, *Spirilliplanes*, *Sporichthya*, *Streptosporangium,* and *Virgosporangium* (Hayakawa, 2008). It may be the wide applicability of this media in isolation of different group of rare *Actinobacteria*, that we are able to found more CFUs in this medium (**Figure 2A**). While SC and ISP media were introduced for the isolation of mycelial-producing *Actinobacteria* particularly genus *Streptomyces* (Küster and Williams, 1964; Shirling and Gottlieb, 1966), B-4 media was established to be good for isolation of *Actinobacteria* precipitating calcium carbonate crystals (Boquet et al., 1973). The large amount of *Steptomyces* among our isolates could be correlated with the use of ISP and SC during our isolation (Supplementary Table S1). The media CC and HP were especially designed in our laboratory to isolate rare *Actinobacteria* that could utilize complex energy sources (Li et al., 2014; Yuan et al., 2017). Among these two media, HP was more efficient than CC in giving larger CFUs count (**Figures 2A,C**). The reason behind the larger CFUs in HP could not be ascertained from the current study, however, it is possible that degradation of cellulose required complex enzyme-system and that many *Actinobacteria* were not able to use cellulose as their energy sources. On the other hand, water agar, which is found to stimulate growth of spore-forming microorganisms, was not effective during our study for the growth of rare *Actinobacteria* (**Figure 2C**). Inhibition of certain rare actinobacterial strains by the preferential treatments, however, cannot be completely ruled out.

In the karstic caves, the primary production usually depends on chemoautotrophic bacteria (Sarbu et al., 1996). Recent studies have, however, revealed that considerable input of organic matter could support the growth of heterotrophic bacteria including *Actinobacteria* (Arroyo et al., 1997; Groth and Saiz-Jimenez, 1999). These findings instigated the study on diversity of *Actinobacteria* in several caves located around the world such as Niu Cave (Zhou et al., 2007), Pajsarjeva jama (Pasić et al., 2010), Wind Cave (Chelius and Moore, 2004), Kartchner Caverns (Ikner et al., 2007), Altamira Cave (Cuezva et al., 2009), and Altamira and Tito Bustillo Caves (Schabereiter-Gurtner et al., 2002). Studies of Shabarova and Pernthaler (2010) have resulted in the isolation of *Actinobacteria* belonging to the genera *Arthrobacter*, *Blastococcus*, *Curtobacterium*, *Kribella*, *Micrococcus*, *Nocardia*, *Promicromonspora*, *Pseudonocardia*, *Rhodococcus,* and *Streptomyces*. Unlike the above study, significant diversity of rare *Actinobacteria* were observed in the present study. These *Actinobacteria* were affiliated to genera *Actinocorallia*, *Actinomadura*, *Agromyces*, *Alloactinosynnema*, *Amycolatopsis*, *Beutenbergia*, *Cellulosimicrobium*, *Gordonia*, *Isoptericola*, *Jiangella*, *Knoellia*, *Kocuria*, *Krasilnikoviella*, *Kribbella*, *Microbacterium*, *Micromonospora*, *Mumia, Mycobacterium, Nocardia*, *Nocardioides*, *Nocardiopsis*, *Nonomuraea*, *Oerskovia*, *Pseudokineococcus*, *Pseudonocardia*, *Rhodococcus*, *Saccharothrix*, *Streptosporangium,* and *Tsukamurella*. The presence of genera *Micromonospora*, *Nocardia,* and *Rhodococcus* as the dominant rare *Actinobacteria* in our study was consistent with other related studies (Arroyo et al., 1997; Zhou et al., 2007; Valme et al., 2010).

Despite the isolation of varied actinobacterial groups after applying a set of pretreatments and modification of isolation media, our study suffers from few limitations. Firstly, the physicochemical parameters of the sampling site and the co-existence of different minerals/metals were not measured during the study. Lack of these data prevent us from indirect establishment of the interrelationship between the occurrence of different actinobacterial groups and their physiological roles in cave. Secondly, the study was limited to isolation of culturable *Actinobacteria*. As such, we could not equally verify if the applied methods were effective to deselect non-*Actinobacteria*. It is also equally possible that certain culturable rare *Actinobacteria* particularly non-spore formers were deselected due to the stressors provided in our pretreatments. Lastly, the isolation

methods have not been replicated in other karst environments or any other habitats. A study on the total microbial composition using NextGen sequencing could provide an idea of the effectiveness of the isolation method. However, it can certainly be stated that the methods provided above proved effective for the isolation of many rare *Actinobacteria*, comprising of both spore-formers (e.g., *Actinocorallia*, *Alloactinosynnema*, *Jiangella*, *Oerskovia* etc.) and non-spore formers (such as *Agromyces*, *Beutenbergia*, *Cellulosimicrobium*, *Gordonia*, *Isoptericola*, *Kocuria*, *Tsukamurella*).

AUTHOR CONTRIBUTIONS

W-JL, B-ZF, and JC designed research and project outline. JC, M-XH, MX, and NS performed isolation, deposition, and identification. B-ZF, J-YJ, and D-QW contructed the heatmap and other related bioinformatic plots. B-ZF, NS, MX, and W-JL drafted 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.2017.01535/full#supplementary-material

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Assessment of the Potential Role of *Streptomyces* in Cave Moonmilk Formation

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Moonmilk is a karstic speleothem mainly composed of fine calcium carbonate crystals ($CaCO_3$) with different textures ranging from pasty to hard, in which the contribution of biotic rock-building processes is presumed to involve indigenous microorganisms. The real microbial input in the genesis of moonmilk is difficult to assess leading to controversial hypotheses explaining the origins and the mechanisms (biotic vs. abiotic) involved. In this work, we undertook a comprehensive approach in order to assess the potential role of filamentous bacteria, particularly a collection of moonmilk-originating *Streptomyces*, in the genesis of this speleothem. Scanning electron microscopy (SEM) confirmed that indigenous filamentous bacteria could indeed participate in moonmilk development by serving as nucleation sites for $CaCO_3$ deposition. The metabolic activities involved in $CaCO_3$ transformation were furthermore assessed *in vitro* among the collection of moonmilk *Streptomyces*, which revealed that peptides/amino acids ammonification, and to a lesser extend ureolysis, could be privileged metabolic pathways participating in carbonate precipitation by increasing the pH of the bacterial environment. Additionally, *in silico* search for the genes involved in biomineralization processes including ureolysis, dissimilatory nitrate reduction to ammonia, active calcium ion transport, and reversible hydration of CO_2 allowed to identify genetic predispositions for carbonate precipitation in *Streptomyces*. Finally, their biomineralization abilities were confirmed by environmental SEM, which allowed to visualize the formation of abundant mineral deposits under laboratory conditions. Overall, our study provides novel evidences that filamentous Actinobacteria could be key protagonists in the genesis of moonmilk through a wide spectrum of biomineralization processes.

Keywords: biomineralization, moonmilk genesis, geomicrobiology, carbonatogenesis, cave microbiology

INTRODUCTION

The hypogean environment, although highly deprived of nutrients, sustains a diverse microbial life. This subterranean microbiome plays an important ecological role in caves, with secondary effects on mineralogy, including host rock dissolution or mineral precipitation, leading to the formation of various secondary mineral deposits termed speleothems (Barton and Northup, 2007; Jones, 2010; Cuezva et al., 2012). A biogenic origin has been hypothesized for a number of speleothems, including coralloids (Banks et al., 2010), pool fingers (Melim et al., 2001), ferromanganese deposits (Northup et al., 2003; Spilde et al., 2005), helictites (Tisato et al., 2015), and moonmilk (Canaveras et al., 2006). Unlike typical mineral deposits, moonmilk is present as a soft and pasty precipitate on cave surfaces and within pools (Richter et al., 2008; Cacchio et al., 2014). The origin of moonmilk has been controversial for many years due to the complex mineralogy, the atypical crystalline morphology, and also the size of its crystals (Verrecchia and Verrecchia, 1994; Canaveras et al., 1999, 2006; Bindschedler et al., 2010, 2014). Notably, moonmilk deposits are characterized by several crystal habits including nano-fibers, and micro-meter sized needle-fiber crystals in a form of monocrystalline rods and polycrystalline chains (Canaveras et al., 1999, 2006; Bindschedler et al., 2010, 2014). While initially postulated as a speleothem of abiotic origin (Harmon et al., 1983; Borsato et al., 2000), recent studies attributed the genesis of moonmilk to indigenous microbial population (Canaveras et al., 2006; Cailleau et al., 2009; Baskar et al., 2011; Braissant et al., 2012).

Microbial carbonate precipitation (MCP) is a broad spectrum phenomenon either mediated by autotrophic pathways, such as photosynthesis and methanogenesis that lead to depletion of local CO_2, or heterotrophic pathways that alter local conditions leading to $CaCO_3$ precipitation (Castanier et al., 2000; Barton and Northup, 2007; Banks et al., 2010). Caves are devoid of sunlight, ruling out photosynthesis, while methanogenesis has been documented rarely in these systems. Heterotrophic processes may therefore play an important role (Banks et al., 2010). Active calcite precipitation by heterotrophs in calcium-rich environments has been hypothesized to be the consequence of a detoxification process, wherein the acidification of the local environment induced by passive influx of Ca requires growing cells that would actively export the excess of this metal to maintain cellular calcium homeostasis (Banks et al., 2010). Such metal detoxification strategies have also been linked to the formation of the unusual speleothems known as helictites (Tisato et al., 2015). Heterotrophic growth can also increase the environmental pH, which can in turn increase the saturation index of $CaCO_3$ and drive precipitation. Calcite precipitation through nitrogen metabolism is thought to operates by different metabolic pathways, including ureolysis, ammonification through amino acid and peptide catabolism, and dissimilatory nitrate reduction to ammonia (DNRA), all of which increase the local pH (**Figure 1**; Castanier et al., 2000).

Moonmilk was reported to host wide spectrum of microbiota, including Archaea, Bacteria, and Fungi (Rooney et al., 2010; Portillo and Gonzalez, 2011; Engel et al., 2013; Reitschuler et al., 2014, 2015, 2016; Axenov-Gibanov et al., 2016; Maciejewska et al., 2016). Among this microbiome, fungi, and filamentous microorganisms, particularly members of Actinobacteria phylum were reported to be possibly involved in the moonmilk genesis (Canaveras et al., 2006; Bindschedler et al., 2010, 2014). Bindschedler et al. (2010, 2014) suggested that the presence of nano-fibers within the crystalline structure of moonmilk was associated with biomineralized fungal hyphae. The authors suggest that organized networks of nano-fibers, often observed in moonmilk, could represent fibrous fungal cell wall polymers, such as chitin and β-(1→3)glucans (Bindschedler et al., 2010). On the other hand, the observation of unstructured aggregates of unconsolidated microcrystalline fibers with calcified Actinobacteria-like filaments led Canaveras et al. (2006) to propose a model of moonmilk formation wherein Actinobacteria promoted calcium carbonate precipitation by creating locally favorable conditions, with the bacterial cell walls serving as nucleation zones (Canaveras et al., 2006). The presence of metabolically active microorganisms in moonmilk was demonstrated using isothermal microcalorimetry (Braissant et al., 2012), although the progressive accumulation of $CaCO_3$ (and presumably entombment) ultimately leads to a decrease of the microbial activity (Canaveras et al., 2006; Sanchez-Moral et al., 2012). As a result, microorganisms would play a significant role in the initiation of moonmilk formation, which ultimately would be overtaken by abiotic processes leading to the growth of the deposit, which can reach up to 1 m in thickness (Sanchez-Moral et al., 2012).

In all of these studies, there has been no clear distinction as to whether the increase or decrease in local pH is ultimately leading to the precipitation of moonmilk by the dominant actinobacterial species observed. In this work we use a combination of microscopy, cultivation and genomic approaches to provide an *in vitro* and *in silico* assessment of the actinobacterial metabolic activities that could promote $CaCO_3$ precipitation. Our data suggest that the *Streptomyces* species would play an important role in nitrogen metabolism, which could locally raise pH and contribute to moonmilk formation.

MATERIALS AND METHODS

Moonmilk Sampling and *Streptomyces* Strains Used in This Study

Samples for microscopy and cultivation were taken from moonmilk deposits originating from three sampling points (collection points COL1, COL3, COL4, Supplementary Figure 1 from Maciejewska et al., 2016) in the upper Viséan limestone cave "Grotte des Collemboles" (Springtails' Cave), Comblain-au-Pont, Belgium (more detailed cave description in Supplementary Figure 1).

Moonmilk samples for strains isolation were brought to the laboratory on ice and stored at 4°C prior to *lyophilization*. Extensive attempts at the cultivation of Actinobacteria led to the isolation of 31 phylogenetically distinct *Streptomyces* strains representing phylogenetically distinct phylotypes (as previously described in Maciejewska et al., 2016 and Supplementary

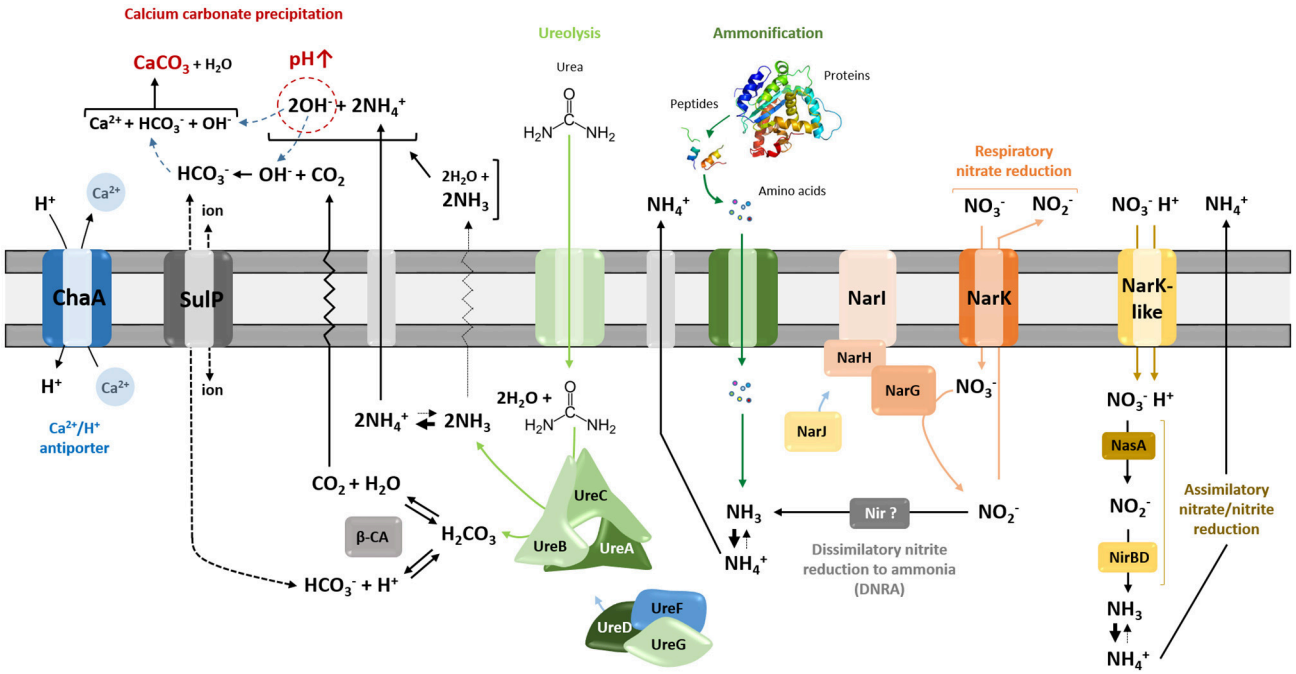

FIGURE 1 | Heterotrophic pathways associated with carbonatogenesis presumed to occur in *Streptomyces* species. Microbially mediated carbonate precipitation might be either linked to active transport of calcium ions across cellular membrane through $Ca^{2+}/2H^{+}$ antiporter system—ChaA, or to nitrogen cycle-related pathways—ureolysis, ammonification, and dissimilatory reduction of nitrate to ammonium/ammonia (DNRA). All those pathways lead to alkalinization of the bacterial environment through the generation of ammonium, shifting the equilibrium toward carbonate/bicarbonate ions, which, upon the presence of calcium, precipitate as calcium carbonate ($CaCO_3$). ChaA, apart from providing calcium ions for potential precipitation, locally increase pH through simultaneous incorporation of protons. The urease activity seems to be linked with cytoplasmic carbonic anhydrases (β-CA), which catalyze dehydration of carbonic acid produced during ureolysis into carbon dioxide that can constitute an additional source of bicarbonate ions for precipitation. The export or import of bicarbonate ions could be potentially mediated via the sulfate transporter family protein in cluster with CA (SulP-type permease). Assimilation of organic nitrogen from amino acids releases extracellular ammonia through deamination, once the metabolic demand for nitrogen is fulfilled. The inorganic nitrogen source (NO_3^-), which is imported in *Streptomyces* by Nark-type transporters, can participate into biomineralization exclusively through dissimilatory reduction to ammonia mediated by respiratory nitrate reductases (Nar) together with potential nitrite reductases (Nir), which would be functional under dissimilatory conditions. Reduction of nitrite to ammonia in streptomycetes was found to operate through assimilatory pathway catalyzed by NasA and NirBD reductases.

Figure 2). Moonmilk samples for scanning electron microscopy (SEM) were preserved in two separate fixative solutions, 2.5% glutaraldehyde in 0.1 M Na-acetate buffer (pH 7.4), and 100% ethanol, and stored at 4°C until analysis. To exclude the effect of fixatives on the crystalline structure of moonmilk, the lyophilized samples were also observed under the microscope as controls (data not shown).

Environmental Scanning Electron Microscopy (ESEM) and Elemental X-Ray Energy Dispersive Microanalysis (EDS)

Glutaraldehyde-fixed moonmilk samples were post-fixed in 1% osmium tetroxide (OsO_4) in distilled water, rinsed, and dehydrated through a graded ethanol series (30–100%). Glutaraldehyde-fixed samples and ethanol-preserved samples were then processed by critical point drying, prior to mounting on the glass slides covered by double-side carbon tape together with lyophilized samples. Parts of each sample were mounted to expose the outer surface, a vertical section or fracture made with a scalpel blade. Dry samples were subsequently sputter coated with platinum on Balzers sputtering Unit SCD 030 (Balzers, Lichtenstein).

The production of mineral deposits by isolates MM24 and MM99 following growth in two different culture conditions—calcite precipitation agar (CPA) and modified B-4 medium (more details in $CaCO_3$ precipitation section) was detected from the living bacterial colonies after being air-dried. Morphological observations were performed by light microscopy (reflected, transmitted, and polarized light) using an Olympus Provis AX-70 microscope fitted with a Visicam 5.0 videocamera for image capture. SEM observations were performed in an environmental scanning electron microscope FEI XL30 ESEM-FEG (Eindhoven, The Netherlands). Platinum-coated samples were observed under high vacuum (HV) conditions with the ET-secondary electron (SE) detector at 10 mm working distance and 15 kV accelerating voltage. Air-dried cultures, were observed under low vacuum (LV) conditions (0.4 Torr) with the large field gaseous secondary electron (GSE) detector and the backscattered electron (BSE) detector at 10 mm working distance and 10 and 20 kV accelerating voltage, respectively. As a result of applying two types of detection—GSE and BSE, the contrast of the images

due to the surface morphology or due to the atomic number of the elements and the density of minerals could be obtained. Elemental X-ray microanalysis and mapping was carried out using a Bruker silicon drift energy dispersive detector (SDD Quantax 129 eV, Billerica, MA, USA) at 10–20 kV accelerating voltage with the Esprit 1.9 software. Semi-quantitative analyses of the elemental composition were done using the standard-less ZAF method with automatic background subtraction.

Genomic Analysis of Moonmilk-Derived Isolates

The genes of interest (see **Figure 1**) including those coding for (i) the ureolytic system (*ure*), (ii) the $Ca^{2+}/2H^{+}$ antiporter system (*chaA*), (iii) the nitrate/nitrite reductases (*nar/nas/nir*) with the corresponding transporters (*narK*), and (iv) the carbonic anhydrase (CA) together with sulfate transporter in cluster with CA (*sulP*; Felce and Saier, 2005), were retrieved from the genomes of moonmilk *Streptomyces* sequenced at the Luxembourg Institute of Science and Technology, as previously described (Maciejewska et al., 2016). These genes were first identified within the chromosome of the model *Streptomyces* species—*Streptomyces coelicolor* (Bentley et al., 2002). The designations of the selected genes are listed in Supplementary Table 1. Subsequently, genes sequences encoding the corresponding proteins were identified within the genomes of additional 54 reference *Streptomyces* strains (Supplementary Table 2) for which completely assembled genomes are available in NCBI FTP server (data retrieved on January 8th, 2016).

A total of 407,461 protein sequences were organized in clusters of orthologous groups (COGs) using Proteinortho v 5.12 (Lechner et al., 2010) with the PoFF extension to further discriminate similar sequences based on synteny. Created COGs were used as models to screen moonmilk *Streptomyces* genomes. For every *S. coelicolor* gene, the collection of protein sequences in the corresponding COG was used to build a hidden Markov model (HMM) profile (Eddy, 1998). As an example, the gamma sub-unit of the urease metallo-protein of *S. coelicolor* (UreA, SCO1236) clusters in a COG with 52 sequences from other *Streptomyces* (Supplementary Table 1). This COG is used to construct a HMM profile representing the UreA protein that is used to search a database of translated predicted coding sequences of the moonmilk *Streptomyces*. Partial coding sequences resulting from the fragmented nature of the moonmilk *Streptomyces* genomes were also considered in the screening. The moonmilk *Streptomyces* coding sequences were predicted with Prodigal v2.6.2 (Hyatt et al., 2010). HMM profile building and HMM search were carried out using the HMMER3 software package (v3.1b2, http://hmmer.org/). The accession numbers of genes recovered from moonmilk *Streptomyces* are compiled in the Supplementary Table 3.

Metabolic Assays

Ammonification

The ability of isolates to decompose organic nitrogen into ammonia was tested on nutrient agar containing: peptone, 5 g/l; beef extract, 3 g/l; NaCl, 5 g/l; phenol red, 0.012 g/l; agar, 15 g/l; pH 7.0 (Food and Agriculture Organization of the United Nations, 1983). Each representative moonmilk *Streptomyces* isolate was spot-inoculated on an individual Petri dish and incubated for 7 days at 28°C. *Citrobacter freundii* ATCC 43864 was used as a positive control strain, while uninoculated medium was used as a negative control. The development of a pink color, indicating a pH increase due to the formation of ammonia following peptides/amino acids degradation was monitored every day during the incubation.

Ureolysis

Rapid screening of urease activity was performed on Christensen's Urea Agar Base (UAB) solid media as described previously (Hammad et al., 2013). The UAB medium was prepared as follows: urea, 20.0 g/l; NaCl, 5.0 g/l; peptone, 1.0 g/l; glucose, 1.0 g/l; KH_2PO_4, 2.0 g/l; phenol red, 0.012 g/l, and agar, 15.0 g/l; pH 6.5. All components of the media were autoclaved except urea which was filter-sterilized and added after autoclaving. The UAB medium without urea was used as a negative control. Both types of media were inoculated with representative of each phylotype together with urease negative control strain (*Escherichia coli* ATCC 25922) and urease positive control strain (*Klebsiella pneumoniae* ATCC 13883) and incubated at 28°C. Plates were examined continually to record development of the pink color indicating a pH increase as a result of urease enzyme activity, leading to generation of ammonia through urea degradation, according to the following reaction: $(NH_2)_2CO + H_2O \rightarrow 2NH_3 + CO_2$.

Nitrate and Nitrite Reduction

The assessment of nitrate and nitrite reduction was performed as described by Li et al. (2016), with small modifications. Briefly, the phylotypes were inoculated in nitrate or nitrite agar slants (potassium nitrate/sodium nitrite, 1 g/l; peptone, 5 g/l; beef extract, 3 g/l; agar, 12 g/l; pH 7.0) and nitrate/nitrite broth tubes (potassium nitrate/sodium nitrite, 1 g/l; peptone, 5 g/l; beef extract, 3 g/l; pH 7.0) equipped with the Durham tubes. *Escherichia coli* ATCC 25922 was used as a control strain for exclusive reduction of nitrate to nitrite, while *Pseudomonas aeruginosa* ATCC 27853 as a complete reducer of nitrate into the nitrogen gas, collected in the Durham tube. Uninoculated media were used as an additional control. Inoculated test tubes were incubated at 28°C for 9 days in static conditions, to reduce amount of dissolved oxygen. After incubation time few drops of sulfanilic acid and alpha-naphthylamine were added to each test tube, which together react with nitrite generating red/pink color. Reduction of nitrate (NO_3^-) is indicated by appearance of red color in nitrate broth, while red color disappearance in nitrite broth indicates nitrite (NO_2^-) reduction.

Oxidative Glucose Breakdown

The glucose oxidative test was carried out according to Hugh and Leifson (1953). Briefly, each representative of the phylotypes was spot-inoculated on Hugh and Leifson's Oxidative-Fermentative (OF) basal medium, prepared as follows: peptone, 2.0 g/l; NaCl, 5.0 g/l; bromothymol blue, 0.03 g/l; K_2HPO_4, 0.3 g/l; agar, 3.0 g/l; pH 7.1. Filter-sterilized glucose was added after autoclaving to a final concentration of 1%. The OF medium not supplemented

with glucose was used as a negative control, and oxidative *Pseudomonas aeruginosa* ATCC 27853 strain as a positive control. The inoculated plates were incubated at 28°C during 7 days and monitored continually to observe development of yellow color indicating on the acid production due to glucose metabolism.

$CaCO_3$ Precipitation

The screening for isolates able to precipitate calcium carbonate through ureolysis was performed using calcite precipitation agar (CPA) as previously described (Stocks-Fischer et al., 1999). The CPA medium was prepared as follows: (g/l); nutrient broth, 3.0 g/l; urea, 20.0 g/l; $CaCl_2{*}2H_2O$, 28.5 g/l; $NaHCO_3$, 2.12 g/l; NH_4Cl, 10.0 g/l; agar, 15.0 g/l. All components were autoclaved apart from urea, which was added filter-sterilized. The same medium, but without urea, was used as an additional control. Alternatively, precipitation of $CaCO_3$ was tested on modified B-4 medium (pH 7.0) composed of: yeast extract, 4 g/l; calcium acetate, 2.5 g/l; agar, 15 g/l. The plates inoculated with MM24 and MM99, following incubation at 28°C, were examined under ESEM after 2 months of incubation for CPA media, and 1 month of incubation for B-4.

$CaCO_3$ Solubilization

Isolates were tested for their ability to solubilize calcium carbonate on two different solid media including (i) minimal medium (MM; Kieser et al., 2000) containing $CaCO_3$ (2 g/l), supplemented or not with glucose (5 g/l), and (ii) 1:100 diluted nutrient agar (Portillo et al., 2009), containing $CaCO_3$ (2.5 g/l), supplemented or not with glucose (2 g/l). The spot-inoculated plates were incubated for 4 weeks at 28°C and the capability of calcium carbonate solubilization was confirmed by observation of the clear halo around a colony.

RESULTS AND DISCUSSION

Microscopic Evaluation of Indigenous Moonmilk Filamentous Bacteria as Nucleation Sites for Carbonate Precipitation

Filamentous bacteria, particularly Actinobacteria, have been proposed to participate in the genesis of moonmilk deposits by serving as nucleation sites for carbonate deposition (Canaveras et al., 2006). Regardless the fixation procedure (glutaraldehyde, ethanol, and freeze-drying), classical SEM observations revealed the same crystal morphologies that have also been described in the literature (data not shown; Canaveras et al., 1999, 2006; Bindschedler et al., 2010, 2014). The surface of moonmilk samples revealed the presence of dense, unstructured meshes of micrometer-size filaments known as needle-fiber calcite (**Figure 2a**), which, based on EDS analysis, were shown to be mainly composed of calcium, carbon, and oxygen (Supplementary Figure 3A). The structure of moonmilk deposits was characterized by the presence of abundant, randomly oriented, monocrystalline rods, and polycrystalline fibers composed of stacked rhombohedra (**Figure 2a**). Those crystals showed variable dimensions, ranging from 0.5 to 1 μm width and 30–100 μm length for monocrystals, and 2–20 μm width, 10–100 μm length for polycrystals, as previously reported (Canaveras et al., 1999). Interestingly, we observed within the moonmilk microscopic composition, abundant networks of filaments with nano-sized width (50–150 nm), which either formed compacted pellets (**Figure 2b**) or mats (**Figure 2c**). The organized networks of nano-fibers, similar to the one observed in **Figure 2c**, were previously reported from moonmilk and associated with fungal wall polymers (Bindschedler et al. 2010; Bindschedler et al., 2014). However, randomly-oriented, compacted pellets of nano-fibers as presented on **Figure 2b**, were comparable to filamentous Actinobacteria, but characterized by a much smaller cell size which could be a result of the oligotrophic nature of the cave environment. It has been demonstrated that the cell size is largely dependent on the nutritional status of the environment, with resource-poor ecosystems stimulating dwarfism (Young, 2006; Portillo et al., 2013). In this work, the observed size of the putative bacterial nano-filaments, ranging from 0.05 to 0.15 μm, could also be a consequence of the oligotrophic nature of the moonmilk niche as cell elongation/filamentation has been shown to be the result of nutritional stress in some bacteria (Steinberger et al., 2002), including Actinobacteria (Pine and Boone, 1967; Wills and Chan, 1978; Deutch and Perera, 1992). However, even in nutrient-rich soils the majority of bacteria can display a diameter <0.2 μm (Hahn, 2004). In addition, the imprints of nano-sized microorganisms were already reported from other geological formations, such as sedimentary rocks (Folk, 1993; Folk and Chafetz, 2000). Finally, recent findings of ultra-small marine Actinobacteria with an average diameter of about 0.3 μm, provides an additional evidence for the existence of nano-bacteria in oligotrophic ecosystems (Ghai et al., 2013). Unlike crystal fibers, the tiny filaments observed in glutaraldehyde- and ethanol-fixed samples, were not completely straight and regular in their shape but rather displayed plasticity and were often curved, either without preferential orientation (**Figure 2b**) or in the same direction (**Figures 2d,e**). The tiny, curved filaments were not observed—probably not preserved—in freeze-dried samples that suffered of ice crystal growth (data not shown). One-directional growth is not a typical behavior of growing actinobacterial filaments that are commonly branching in diverse directions in order to form a complex mycelial network. This rigid and unidirectional growth might suggest that they represent calcified filaments potentially still actively growing at their tip, which remains curved (**Figure 2d**). Elemental analysis performed with EDS revealed that the observed nano-size filaments contained higher content of carbon and oxygen in comparison to the surrounding crystals, suggesting a possible biological origin (Supplementary Figure 3B). However, these EDS analyses should be viewed with great prudence as the difference in the elemental compositions between filaments and crystals could simply be a consequence of the structure and not the nature (organic vs. mineral) of the analyzed areas. Additionally, along (**Figure 2f**) or on the tip (**Figure 2g**) of some filaments, a possible initiation of calcium carbonate deposition was observed. Besides nano-sized filaments, reticulated filaments were sporadically observed within moonmilk crystals (**Figure 2h**). Those particular filamentous

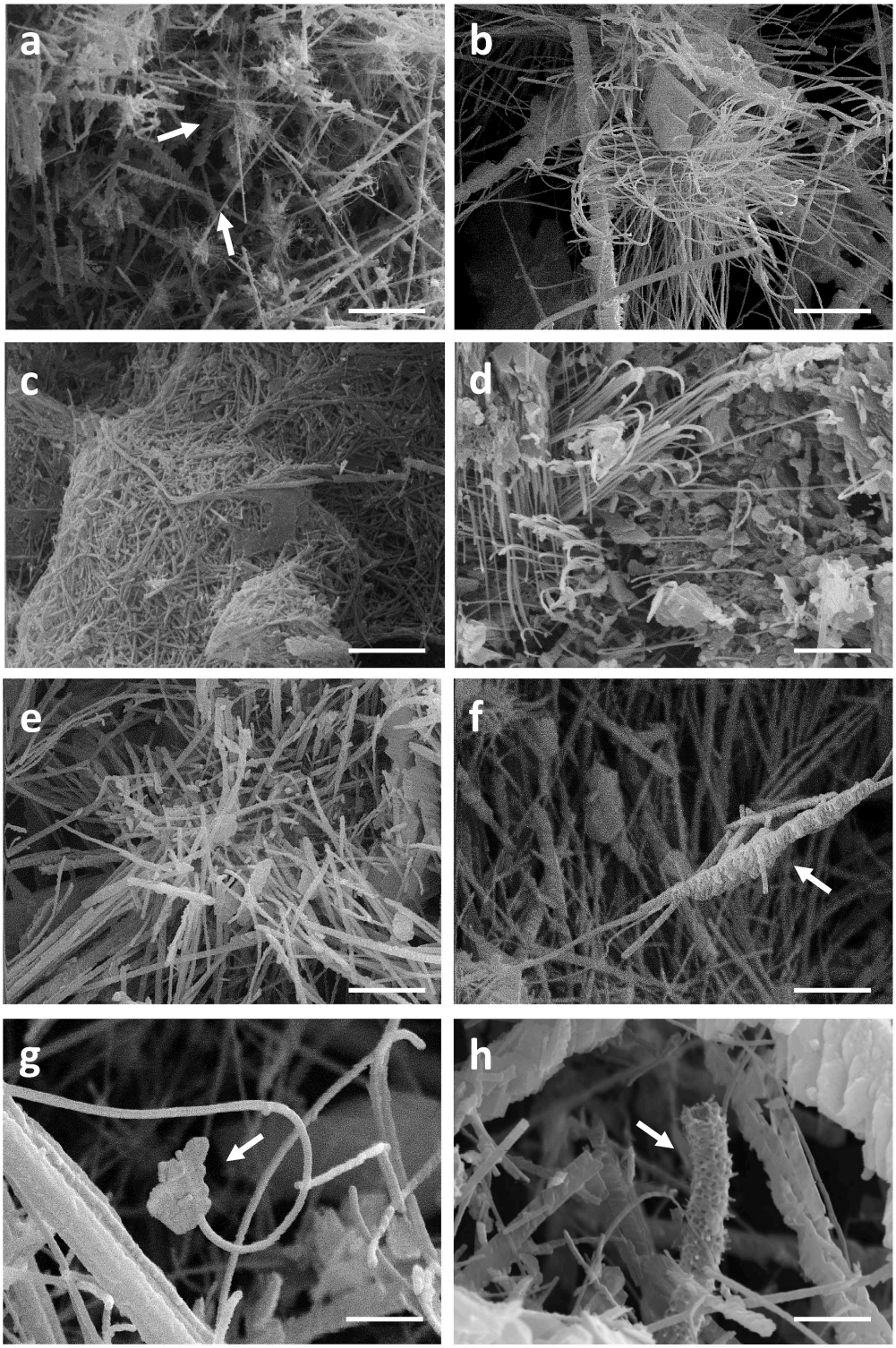

FIGURE 2 | SEM-images of nano-size filaments found in the crystal structure of moonmilk deposits from the cave "Grotte des Collemboles" (Comblain-au-Pont, Belgium). Among typical moonmilk monocrystalline rods and polycrystalline fibers indicated by white arrows **(a)**, dense meshes of tiny filaments were observed, which were compacted into the stacked pellets **(b)** or dense biofilms **(c)**, mostly randomly orientated, but occasionally one-way directed **(d,e)**. Along **(f)** or on the tip **(g)** of some of those filaments, calcium carbonate deposition was identified, with some of the filaments presenting reticulated morphology **(h)** as indicated by arrows.

forms, with the size of about 0.5 μm width and up to 75 μm length, are often found in subsurface environment, including limestone or lava caves, and possess higher carbon content than the one typically observed for calcite minerals, suggesting their biogenic origin. However, the associated microorganisms are not yet identified (Melim et al., 2008, 2015; Northup et al., 2011; Miller et al., 2012). Overall, these observations tend to confirm the hypothesis of filamentous microorganisms (bacteria and fungi) serving as a nucleation sites for moonmilk mineral deposition (Canaveras et al., 2006).

Potential Role of Cultivable Moonmilk-Derived *Streptomyces* in Carbonate Precipitation

A nucleation site itself is not sufficient to promote $CaCO_3$ precipitation, as dead bacterial cells lose the ability to precipitate minerals (Banks et al., 2010). The dominant role of bacteria in calcification is attributed to metabolic activities which increase the pH of the environment (above pH 8) and therefore favor a shift of the CO_2 – HCO_3^- – CO_3^{2-} equilibrium toward carbonate ions which precipitate with Ca^{2+} ions. We screened 31 representative *Streptomyces* strains isolated from moonmilk (see Supplementary Figure 2 retrieved from Maciejewska et al., 2016) for their metabolic activities that could lead to a raise in pH, including ureolysis, peptides/amino acids ammonification, and dissimilatory nitrate/nitrite reduction to ammonia (**Figure 1**). Although these assays were performed under laboratory conditions, a qualitative assessment of these processes allowed ranking the tested phylotype representatives according to their metabolic performance, and therefore their potential to drive biomineralization through an increase in the extracellular pH. The results are shown in **Figure 3**, along with a compilation of activities and phylogenetic relationships (**Figure 4**). The results of each metabolic activity assay for all the tested strains are presented in Supplementary Figure 4.

Ureolysis

Among the 31 isolates tested, 15 showed an ability to increase the pH by hydrolysis of urea (**Figure 4**). MM99 and MM122 were the strongest ureolytic strains, with comparable metabolic performance to the positive control strain—*Klebsiella pneumoniae* ATCC 13883 (**Figure 3A** and Supplementary Figure 4A). The majority of urease-positive moonmilk isolates displayed weak and moderate activities, which were observed either within 3 days of incubation (five strains) or after extended time (more than 1 week; six strains; **Figure 3A** and Supplementary Figure 4A).

In order to know if a relation could be established between the assessed *in vitro* activity and the genetic predispositions for ureolysis, we examined the genomes of the phylotype representatives for the presence of urease genes. We used HMM profiles constructed from Clusters of Orthologous Groups (COGs) of proteins of the urease structural subunits [UreA/UreB/Ure(AB)/UreC], as well as the accessory proteins (UreF/UreG/UreD). The corresponding genes originate from functional clusters of three types i.e., *ureABCFGD. ure(AB)C*, and *ure(AB)CFGD,* which are all present in the urease-positive species. The large majority of moonmilk strains (90%) was found to encode all the urease genes, in some cases present in several copies (**Table 1**), suggesting that even the strains displaying urease-negative phenotypes are capable of urease activity. This suggests that in urease-negative strains urease activity is not expressed under the conditions tested, or that they harbor mutations that prevent expression or activity. Nonetheless, urea transport appears to be functional in all urease-negative isolates as urea exerted a toxic effect in the growth media of these strains (an effect that could be reversed by growth in the same medium lacking urea; data not shown).

Urease activity has been recently linked to an activity of the zinc-containing enzyme—carbonic anhydrase (CA; Achal and Pan, 2011). While urease maintains an alkaline environment by generating ammonia, carbonic anhydrase would provide carbon dioxide for biomineralization through dehydration of carbonic acid (H_2CO_3) also produced during ureolysis (**Figure 1**). It was recently demonstrated that the activities of CA and urease were correlated along the bacterial growth and corresponded to maximum calcite production (Banks et al., 2010; Achal and Pan, 2011), while inhibition of carbonic anhydrase activity decreased the rate of calcification, with calcite precipitation occurring more efficiently with the synergistic action of both enzymes—CA and urease (Dhami et al., 2014).

The presence of this highly efficient enzyme in cave settings was previously confirmed through metagenomic study of a speleothem in Tjuv-Ante's Cave (Sweden; Mendoza et al., 2016), and highly acidic cave biofilms, known as "snottites" (Jones et al., 2011). However, it has never been directly associated to a specific taxonomic group through genome-based approach. The only link was suggested by Cuezva et al. (2012), who proposed CA to be responsible for CO_2 sequestration by gray spot colonization found on the walls of Altamira cave, which were dominated by Actinobacteria.

Evaluation of the genomes of moonmilk phylotypes for the presence of β-CA (Smith and Ferry, 2000), together with the sulfate transporter family protein in cluster with CA (SulP-type permease) revealed that all the investigated strains encode at least one copy of carbonic anhydrase [100% strains encoded β-CA (1) and 87% β-CA (2)] and 90% of them possessing sulfate transporter (**Table 1**). High copy number of this intracellular zinc metalloenzyme, reaching up to seven copies in MM17, revealed their ubiquitous distribution among karstic bacteria, and suggests their applications in multiple and essential cellular processes beyond their presumed role in carbonate precipitation. Interestingly, a high representation of sulfate permeases of SulP family was also characteristic for the studied population. These broad specificity inorganic anion transporters were suggested to assist bicarbonate (HCO_3^-) transport (Felce and Saier, 2005), which was experimentally confirmed in marine cyanobacteria (Price et al., 2004). While in cyanobacteria SulP transporter mediates HCO_3^-/Na^{2+} symport, the substrate specificity in the case of *Streptomyces* is highly speculative. Nevertheless, fusion of this protein with carbonic anhydrases indeed strongly suggests its participation in HCO_3^- transport. However, whether it is an importer or exporter remains an open question. It

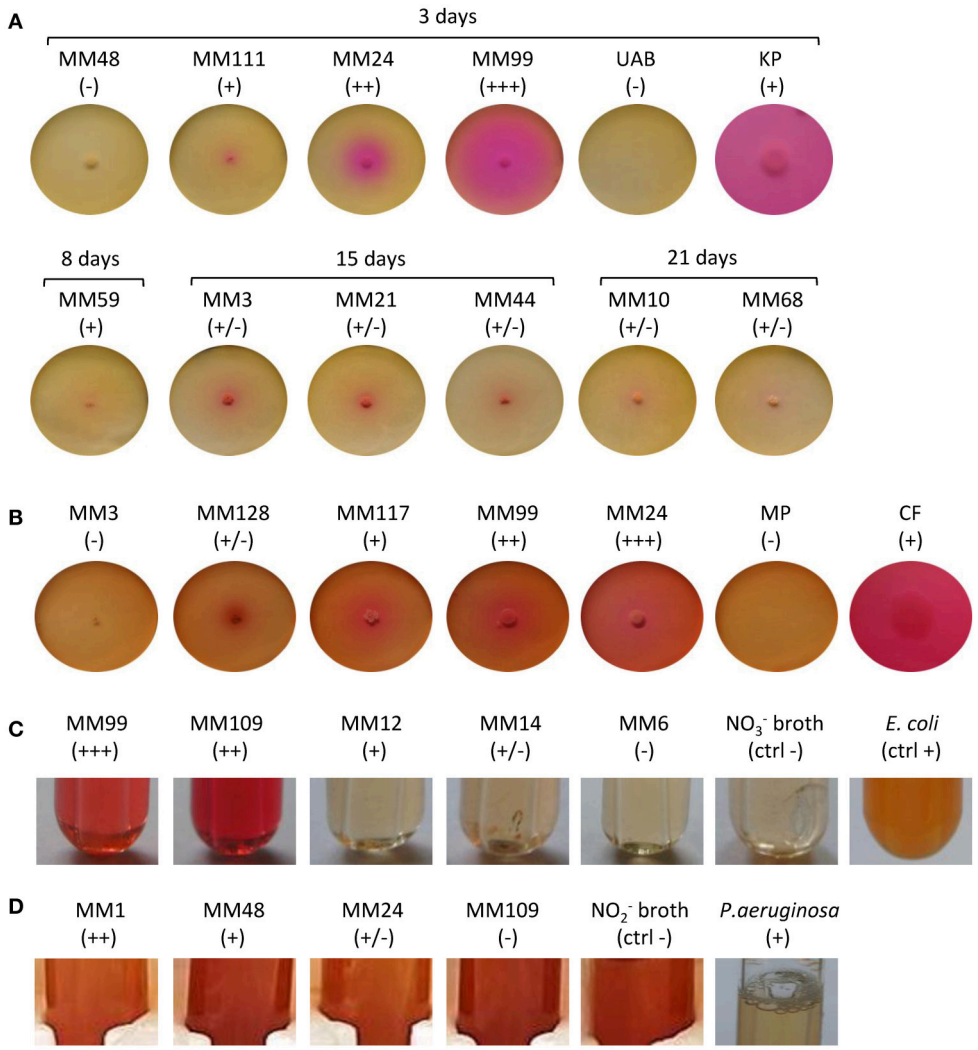

FIGURE 3 | Precipitation-related metabolic activities of the moonmilk *Streptomyces*. **(A)** Ureolysis. **(B)** Ammonification. **(C)** Nitrate reduction **(D)** Nitrite reduction. *Klebsiella pneumoniae* ATCC 13883 (KP), *Citrobacter freundii* ATCC 43864 (CF), *Escherichia coli* ATCC 25922 (EC), and *Pseudomonas aeruginosa* ATCC 27853 (PA) were used as positive controls for ureolysis, ammonification, nitrate, and nitrite reduction tests, respectively. The observed activities are visualized for representative strains demonstrating different metabolic performance for each activity tested, designated through the symbols: (–) lack, (+/–) weak, (+) moderate, (++) good, and (+ + +) strong.

could be possible that HCO_3^- uptake through SulP increases the intracellular pool of this inorganic carbon species, which potentially might be efficiently transformed into carbon dioxide by carbonic anhydrases, and be exported outside the cell, unless required for cellular metabolism. Alternatively, SulP-dependent export of intracellularly formed HCO_3^- through CA-mediated hydration of carbon dioxide, would be assisted with import of other ions (**Figure 1**). Altogether, these findings reveal abundant distribution of genes involved in the inorganic ionic transport and metabolism, which might be related to the biomineralization phenomenon.

Ammonification

Moonmilk *Streptomyces* were also evaluated for their ability to raise the pH through peptide/amino acid mineralization. Ninety-four percent of tested strains efficiently decomposed nitrogenous compounds into ammonia (**Figures 1**, **3B**, **4** and Supplementary Figure 4B). The majority displayed a strong metabolic phenotype (**Figure 5**), which is unsurprising given that *Streptomyces* are well-known ammonifying bacteria in soils, where they actively participate in the decomposition of organic matter (Prakash et al., 2012). In the isolated cave environment, with limited organic matter input from the surface, the source of such macromolecules is unclear, although peptides and amino acids might be entering the cave through water that has percolated through the soil, making such molecules more readily available than urea (Northup and Lavoie, 2001). Amino acid/peptide ammonification was found to be more widespread among the moonmilk isolates than ureolysis, supporting this hypothesis.

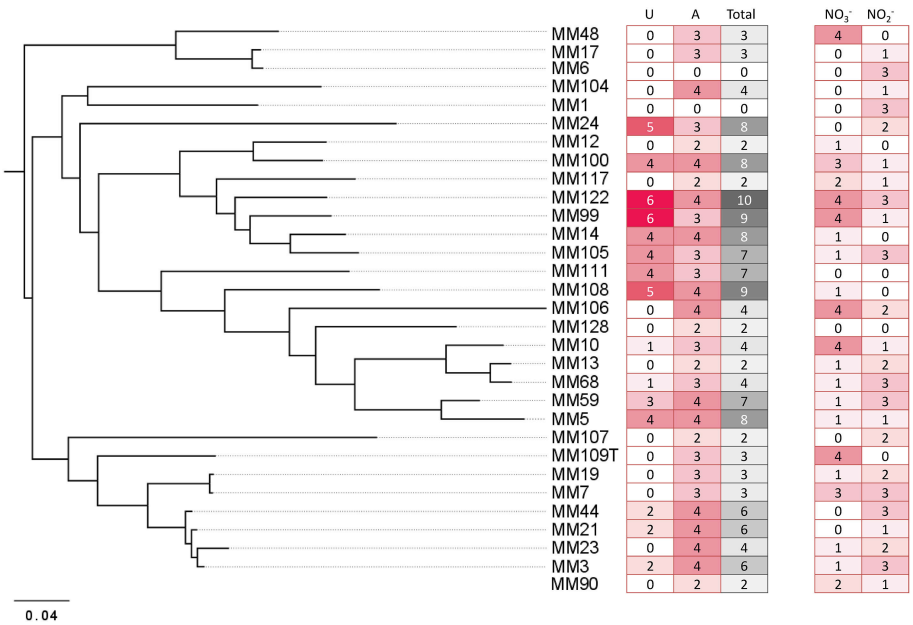

FIGURE 4 | The global pattern of carbonate precipitation-associated metabolic activities of moonmilk *Streptomyces*. The heatmap plot representing metabolic performance of moonmilk *Streptomyces* strains indicated by color scale, correlated with their MLSA-based phylogenetic classification (Maciejewska et al., 2016). The total value represents the sum of metabolic performance observed for ureolysis and amino acids ammonification, ranking the isolates according to their metabolic predispositions for calcium carbonate precipitation for the tested activities. The metabolic performance of nitrate/nitrite reduction test was not included into the final ranking. U, ureolysis; A, peptide/amino acid ammonification; T, total sum of activities for ureolysis and amino acid ammonification; NO_3^-, nitrate reduction; NO_2^-, nitrite reduction.

Dissimilatory Nitrate Reduction to Ammonium

Dissimilatory nitrate reduction to ammonium (DNRA) is another nitrogen cycle-related process considered to be involved in calcification (Castanier et al., 2000; **Figure 1**). This pathway operates in oxygen-limited environments, which could be encountered in the inner layers of the moonmilk deposits. Moreover, moonmilk develops alongside dripping water and its often pasty structure can become fluid, based on its water content, which can drastically reduce oxygen availability. Although *Streptomyces* are obligate aerobes, they are genetically capable of survival under oxygen-limited conditions, encoding genes related to anaerobic respiration, the so-called "anaerobic paradox" (Borodina et al., 2005). Indeed, the model species *Streptomyces coelicolor* was reported to anaerobically respire nitrate (Fischer et al., 2010, 2014). Though nitrite, the product of this process, was not reduced to ammonia, but detoxified through extrusion via NO_3^-/NO_2^- antiporter system (Fischer et al., 2010, 2012). In *S. coelicolor* the NirBD reductase was demonstrated to participate in nitrogen assimilation; however a *nirBD* null-mutant grown in the presence of nitrite and excess ammonium was still able to reduce nitrite suggesting the activity of an alternative and yet unknown enzyme (Fischer et al., 2012). We therefore questioned whether moonmilk isolates would be able to mediate DNRA and reduce nitrate and nitrite under oxygen-limited conditions. For this purpose, we incubated each strain in static (without agitation) liquid culture conditions to limit oxygen availability. Overall, 68 and 77% of strains revealed a capacity to reduce either nitrate (NO_3^-) or nitrite (NO_2^-), respectively, with 52% able to reduce both nitrate and nitrite (**Figures 3C,D**, **4**, **5**). No N_2 gas production was observed, excluding denitrification, which has been only rarely reported for *Streptomyces* (Albrecht et al., 1997; Shoun et al., 1998; Kumon et al., 2002). The lack of N_2 generation would suggest a complete reduction of nitrate/nitrite to ammonia via dissimilatory nitrate reduction. Ammonia produced by this pathway, unless not incorporated by other bacteria or oxidized to other nitrogenous compounds, might alkalinize the extracellular environment and thus stimulate $CaCO_3$ precipitation (**Figure 1**). In order to evaluate the presence of a DNRA pathway in moonmilk isolates, we screened their genomes for the presence of genes coding for respiratory nitrate reductases (*narGHJI*) and their associated NarK-type nitrate/nitrite transporter—NarK2 (**Figure 1**). While 40% of moonmilk strains encoded NarK2 transporter, 30% of them possessed the respiratory nitrate reductases genes (*narGHJI*), with isolates MM7, MM10, MM48, MM106, MM109, MM111, encoding a complete *nar* cluster (**Table 1**). Most of those strains were found to be among the strongest nitrate reducers under oxygen-limited conditions (**Figure 4**). The fact that only a minority of the moonmilk *Streptomyces* possessed the genetic material to perform the first step of DNRA, while a majority (68%) was able to reduce nitrate, suggests that another nitrate reduction pathway was operating under the condition tested. This alternative pathway is most likely the NO_3^- and NO_2^- assimilatory process that uses nitrate and nitrite as nutrient

TABLE 1 | *In silico* prediction of individual genes putatively involved in the moonmilk biomineralization process retrieved from moonmilk-originating *Streptomyces* strains, including genes related to ureolysis (*ure*), nitrate/nitrite transport (*narK*) and reduction (*nar/nas/nir*), carbon dioxide (CO_2) hydration (carbonic anhydrase—CA), and active transport of calcium ions through $Ca^{2+}/2H^+$ antiporter system (*chaA*).

	Ureolysis							NO_3^-/NO_2^- transport		Respiratory NO_3^- reduction					Assimilatory NO_3^- reduction	NO_2^- reduction			CO_2 hydration			Ca^{2+} transport
strain	*ureA*	*ureB*	*ureAB*	*ureC*	*ureF*	*ureG*	*ureD*	*narK2*	*narK*	*narG*	*narH*	*narJ*	*narJ2*	*narI*	*nasA*	*nirB1*	*nirB2*	*nirD*	β-CA (1)	β-CA (2)	*sulP*	*chaA*
MM1	1	1	2	3	1	1	2	–	+	–	–	–	–	–	1	1	1	1	2	1	1	–
MM3	1	1	2	3	2	2	3	–	+	–	–	–	–	–	1	1	1	1	2	1	1	–
MM5	1	1	2	3	2	2	2	–	+	–	–	–	–	–	1	1	1	1	3	3	1	1
MM6	1	1	1	3	1	1	1	–	+	–	–	–	–	–	1	1	2	2	4	–	1	1
MM7	2	2	2	3	2	2	2	+	+	1	1	–	1	1	1	1	1	1	1	1	1	–
MM10	1	1	1	2	2	2	2	+	+	3	2	1	1	2	1	1	1	1	2	1	1	–
MM12	1	1	2	3	3	3	3	–	+	–	–	–	–	–	1	1	1	1	2	2	–	1
MM13	1	1	1	2	2	2	2	+	+	1	–	–	–	1	1	1	1	1	2	1	1	1
MM14	1	1	2	3	2	2	2	–	+	–	1	–	1	1	1	1	1	1	1	2	1	–
MM17	1	1	3	3	2	2	2	–	+	1	–	–	–	–	1	1	1	1	6	1	1	1
MM19	1	1	1	2	1	1	1	–	+	–	–	–	–	–	1	1	1	1	1	1	1	–
MM21	1	1	2	3	2	2	2	–	+	–	–	–	–	–	1	1	1	1	2	1	1	–
MM23	1	1	2	3	2	2	2	–	+	–	–	–	–	–	1	1	1	1	1	1	1	–
MM24	2	2	2	5	2	2	4	+	+	–	–	–	–	–	1	1	1	1	2	2	1	1
MM44	1	1	2	3	2	2	2	–	+	–	–	–	–	–	1	1	1	1	2	1	1	–
MM48	2	2	2	4	2	2	2	–	+	1	1	–	1	1	2	1	2	1	5	1	1	1
MM59	1	1	–	1	1	1	1	–	+	–	–	–	–	–	1	1	1	1	3	2	1	1
MM68	1	1	1	2	3	2	2	–	+	–	–	–	–	–	1	1	1	1	2	2	1	1*
MM99	3	3	1	4	3	3	3	+	–	–	–	–	–	–	1	–	–	–	1	1	1	–
MM100	1	1	2	4	2	2	2	+	+	–	–	–	–	–	2	1	1	1	2	1	–	–
MM104	1	1	1	1	1	1	1	–	+	–	–	–	–	–	–	1	1	1	2	3	1	1
MM105	1	1	3	4	1	1	1	+	+	–	–	–	–	–	1	1	1	1	1	–	1	–
MM106	2	2	–	2	2	2	2	+	+	2	2	–	2	2	1	1	1	1	1	–	1	1
MM107	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	2	1	1
MM108	1	1	3	4	1	1	1	+	+	–	–	–	–	–	2	1	1	1	3	1	1	1
MM109	1	1	2	3	2	2	2	+	+	2	1	–	1	2	1	1	1	1	2	–	1	–
MM111	2	2	2	4	2	2	2	–	+	1	1	–	1	1	1	1	1	1	1	1	1	1
MM117	1	1	1	2	1	1	1	+	+	–	–	–	–	–	2	1	1	1	1	1	1	–
MM122	2	2	2	4	3	3	3	+	+	–	–	–	–	–	1	1	1	1	1	3	–	–
MM128	1	1	1	2	1	1	1	–	+	–	–	–	–	–	1	1	1	1	2	2	1	1

*CA_sulf, sulfate transporter family protein in cluster with carbonic anhydrase; *pseudogene.*

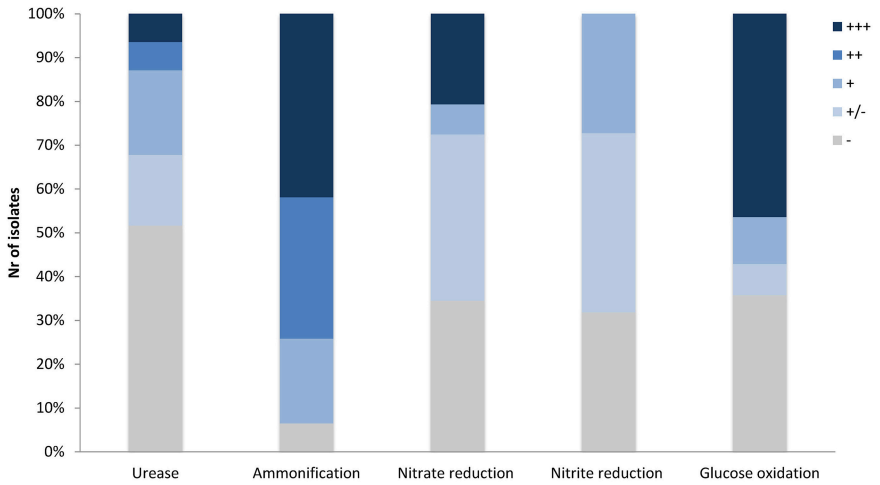

FIGURE 5 | Percentage of moonmilk *Streptomyces* displaying carbonatogenesis related activities *in vitro* within different metabolic categories. (–) lack, (+/–) weak, (+) moderate, (++) good, and (+ + +) strong.

sources through assimilatory *nasA* (nitrate reductase) and *nirBD* (nitrite reductase) genes, with the assistance of additional Nark-type NO_3^- transporter (Tiffert et al., 2008; Amin et al., 2012; Fischer et al., 2012). Assimilatory reduction of nitrate and nitrite to ammonium is highly plausible as 93% of moonmilk strains possess *nasA*, *nirBD*, and *narK* NO_3^- importer genes (**Table 1**). When monitored on solid medium and thus without oxygen limitation, 68 and 77% of the tested strains were able to reduce nitrate and nitrite, respectively (Supplementary Figures 4C,D), which confirmed the high potential of moonmilk *Streptomyces* to use nitrate and nitrite as a nitrogen source, as previously reported for terrestrial *Streptomyces* (Pullan et al., 2011; Fischer et al., 2012). Altogether, although moonmilk *Streptomyces* possess metabolic ability to reduce nitrate and nitrite, without production of gas, pointing on ammonia as a final product, we cannot at this point conclude whether this process represents assimilatory or dissimilatory pathway and whether DNRA is fully functional.

Active Calcium Transport

In addition to processes passively influencing carbonate precipitation, bacteria can also actively impact this phenomenon, through an active transport of calcium ions. Banks et al. (2010) suggested that the calcium-toxicity driven removal of this ion outside the bacterial cell is a factor driving calcification phenotype. Therefore, we have also retrieved through an *in silico* search ChaA, the $Ca^{2+}/2H^+$ antiporter system suggested to be involved in $CaCO_3$ deposition (Hammes and Verstraete, 2002; Banks et al., 2010; **Figure 1**). The presence of the *chaA* gene was confirmed for 50% of moonmilk cultivable phylotypes (**Table 1**), extending in those strains the calcium-detoxification system to their spectrum of biomineralization-related processes.

Production of $CaCO_3$ Deposits by Moonmilk *Streptomyces*

In order to confirm whether the moonmilk cultivable *Streptomyces* could indeed produce mineral deposits, we selected two phylotype representatives to be first investigated by polarized light microscopy then by ESEM in low vacuum mode for the presence of calcium carbonate precipitates. The selection of strains was based on their predispositions for $CaCO_3$ precipitation as judged by the sum of metabolic performance observed for ureolysis and peptide/amino acid ammonification—the two most significant activities observed for moonmilk *Streptomyces* (**Figure 4**). Strains MM24 and MM99, amongst one of the best isolates based on the metabolic ranking (**Figure 4**), were simultaneously cultivated on urea-containing CPA medium for 2 months, as well as for 1 month on the modified B-4 medium commonly used for $CaCO_3$ precipitation assays. Combined microscopic observations of bacterial colonies surfaces with BSE and GSE detectors revealed highly abundant calcite deposits produced by both strains in both media tested (**Figures 6**, **7**). Ureolysis-mediated mineral precipitation was confirmed by the lack of any calcite in urea-deficient CPA medium for both isolates (data not shown). All the produced mineral deposits appeared bright under polarized light (data not shown). The morphology of the calcite polymorphs was comparable between the isolates, however differed between the two culture conditions. On BSE-images, bacterial colonies grown in CPA medium showed a rocky surface with discoidal- or oval-shaped structures of variable diameter that were almost completely encrusting microbial colonies (**Figures 6a,c**). The mineral nature of the deposits was confirmed through their high (white) contrast on BSE-images compared to the surrounding dark organic matter of the colonies (**Figures 6a,c**). In addition, their $CaCO_3$ mineral composition was confirmed by the elemental X-ray analyses (Supplementary Figure 5) and elemental mapping (**Figures 6b,d**). Calcium, carbon and oxygen were present roughly in stoichiometric proportion of $CaCO_3$ in the spectra (Supplementary Figure 5), and the distribution of those elements was clearly visualized on the mapping (**Figures 6b,d**). The presence of calcium was associated with $CaCO_3$ minerals, while higher proportion of carbon

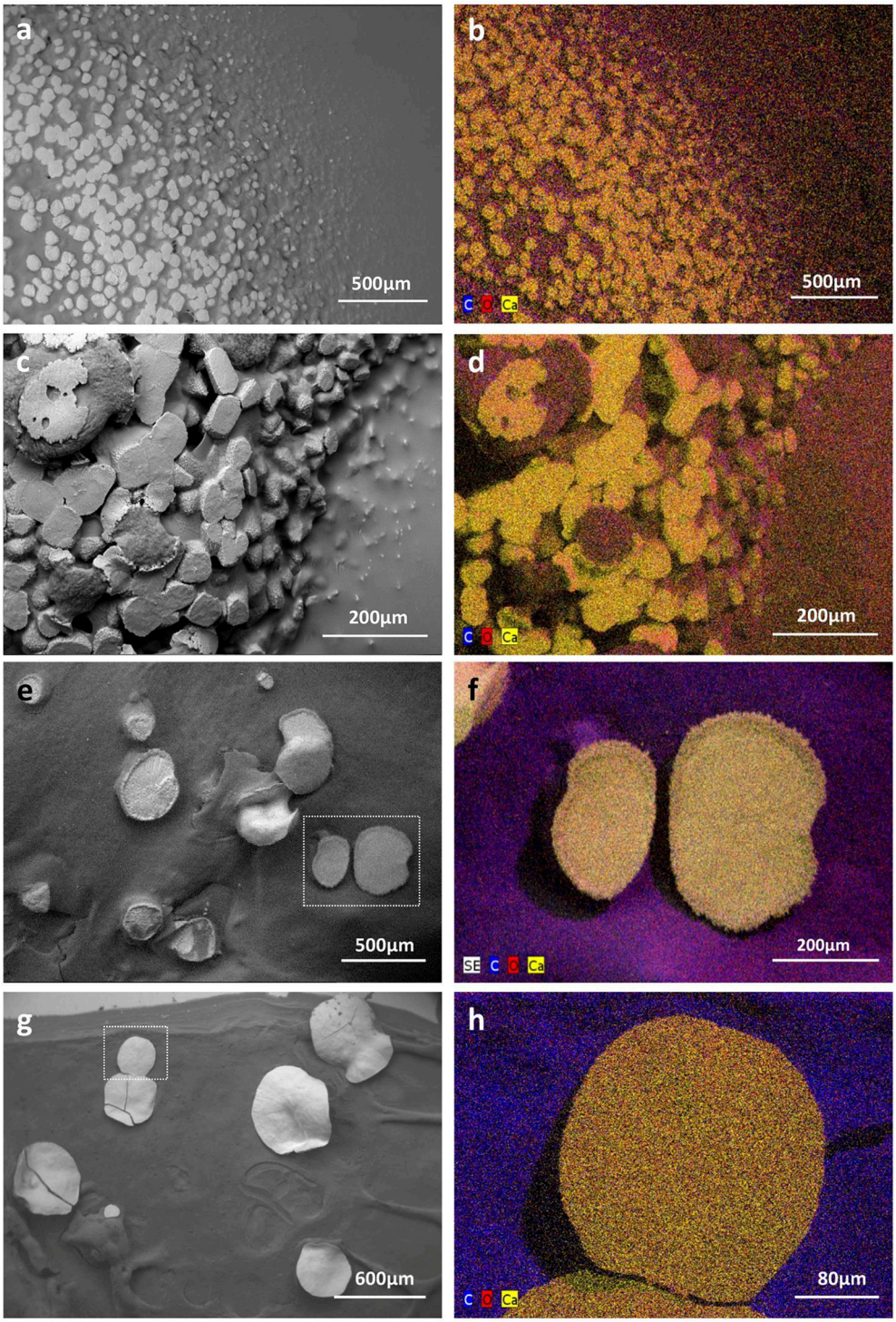

FIGURE 6 | Low vacuum-SEM-BSE images **(a,c,e,g)** and X-ray elemental mappings **(b,d,f,h)** of $CaCO_3$ deposits produced by MM24 **(a,b,e,f)** and MM99 **(c,d,g,h)** isolates. Abundant mineral deposits produced by isolates MM99 **(a)** and MM24 **(c)** in CPA medium, nearly encrusting the whole microbial colony, were morphologically different from less abundant, but much bigger, mineral polymorphs produced in modified B-4 agar by MM99 **(e)** and MM24 **(g)**. The observed precipitates were found to be $CaCO_3$ minerals as revealed by elemental spectra (Supplementary Figure 5) and mappings in both media and for both isolates [MM99 **(b)** and MM24 **(d)** in CPA, MM99 **(f)**, and MM24 **(h)** in modified B-4]. The mappings for MM99 and MM24 in B-4 are marked with the white squares. The mappings are combined images in which detected dominant elements, namely carbon (C), oxygen (O), and calcium (Ca) are assigned to a defined color.

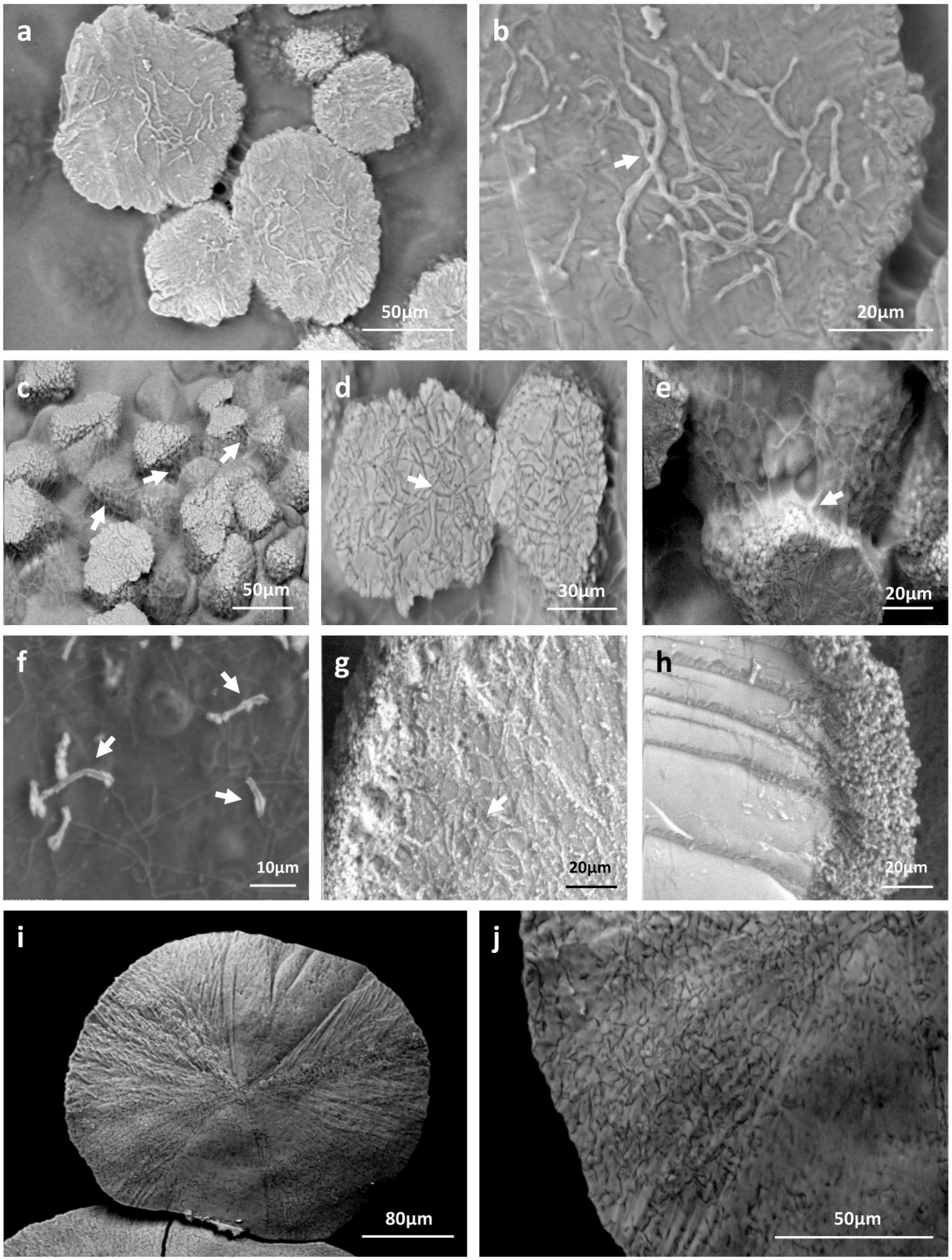

FIGURE 7 | Biogenic signatures within mineral deposits produced by moonmilk-originating *Streptomyces* (LV-SEM-BSE images). Along with the different morphologies of produced mineral deposits specific to the culture conditions used, numerous bacterial imprints were observed. *Streptomyces* filaments were completely incorporated into the produced minerals as observed for MM99 **(a,b)**, MM24 **(c–e)** in CPA medium, as well as for MM99 **(g)** and MM24 **(i,j)** in modified B-4 agar. Their mineralization also appeared as wrinkles each containing a bacterial filament suggesting their progressive encrustation within the mineral (**b,e,g,** arrows). Alternatively, non-mineralized microbial filaments were seen interconnecting adjacent mineral deposits, as detected for MM24 in CPA (**c,e,** arrows), or possible compacted aggregates of filaments were observed at the edges of the mineral produced by MM99 in B-4 **(h)**. Initial steps of mineral deposition were detected along bacterial filaments in modified B-4 culture of isolate MM99 (**f,** arrows).

was associated with organic colony biomass (**Figures 6b,d**). Interestingly, just on the mineral surface, dense webs of nano-sized filaments were observed (**Figures 7a–e**). On high resolution BSE-images, they appeared either as dark curved filaments at the mineral surface or in the middle of mineralized wrinkles suggesting that bacteria produced minerals in which they got progressively entombed (**Figures 7a–d**). The filaments free of mineral were also clearly seen interconnecting together and connecting adjacent mineral deposits (**Figure 7e**). While on the CPA medium both strains prolifically produced relatively small-sized calcite polymorphs (up to 100 μm; **Figures 6a,c**), on the B-4 medium the observed precipitates although being more scarce, were much larger (up to 400 μm; **Figures 6e,g**). The inorganic nature of the larger $CaCO_3$ deposits (**Figures 6e,g**) were confirmed by X-ray elemental spectra (Supplementary Figure 5) and elemental mapping (**Figures 6f,h**), which clearly distinguished $CaCO_3$ minerals from the surrounding bacterial biomass rich in carbon and oxygen. On BSE-image (**Figure 7f**) and under polarized light (data not shown), tiny $CaCO_3$ deposits were also detected along randomly distributed bacterial filaments. Additionally, unlike in CPA medium where the mineral surface was rather irregular and unstructured, mineral produced by both strains in B-4 showed morphologically distinct areas, either with a smooth, radiating texture (**Figure 7h**), with visible filamentous imprints (**Figure 7j**) or wrinkles (**Figure 7g**), that presumably corresponded to bacterial nano-sized filaments.

Potential Role of Cultivable Moonmilk-Derived *Streptomyces* in Carbonate Dissolution

In addition to constructive processes, bacteria are also believed to induce cave bedrock dissolution. As oppose to precipitation, a dissolution phenomenon is related to the acidification of the bacterial microenvironment, most likely as a result of organic acid production, which are the by-products of microbial carbon metabolism. The presence of detectable levels of organic acids in cave environment was previously demonstrated by *in situ* analysis via ATR-FTIR spectroscopy (Bullen et al., 2008). Released organic acids are able to bind cations such as Ca^{2+} and liberate carbonates, which can be subsequently re-precipitated to form cave secondary deposits or be used by bacteria. Therefore, we have tested the 31 phylotypes for their ability to decrease the pH of the medium through oxidative degradation of glucose based on the standardized oxidative/fermentative test (Hugh and Leifson, 1953). Over 68% of isolates were found to induce media acidification by this pathway, which was observed as yellow color to transparent halo development around the inoculum (**Figures 8A**, **9**). Among them, 52% of the *Streptomyces* strains exhibited either good or strong oxidative glucose respiration abilities, strongly reducing the extracellular pH (**Figures 5**, **9** and Supplementary Figure 6).

We have further tested solubilization abilities related to carbon metabolism by cultivating the phylotypes in calcium carbonate containing media—either in minimal medium or in diluted

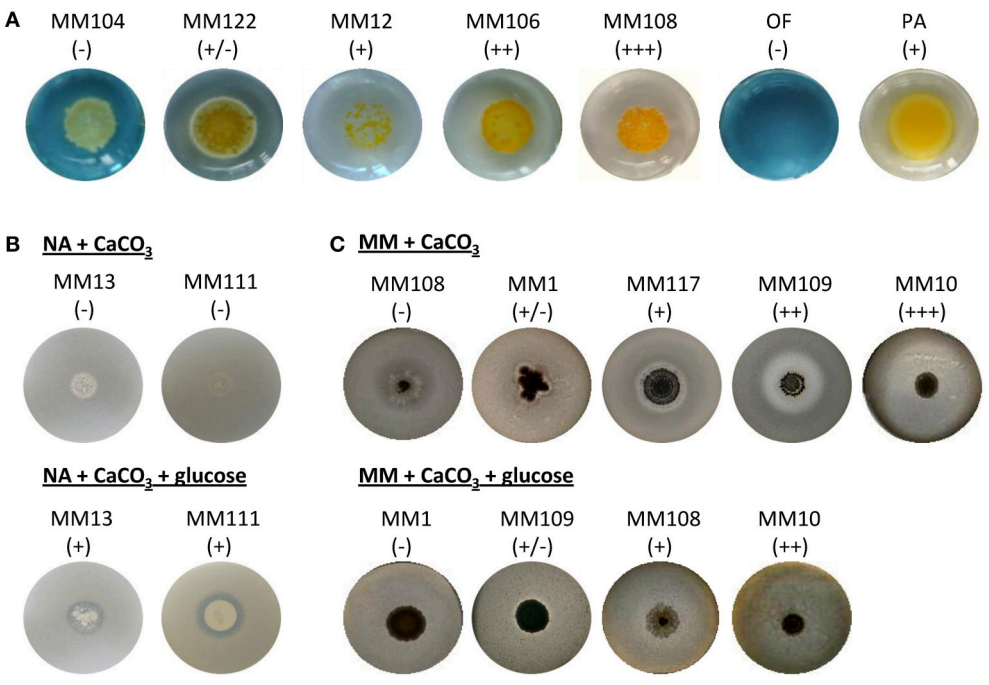

FIGURE 8 | Dissolution-related metabolic activities of the moonmilk *Streptomyces*. Metabolic performance observed for oxidative glucose degradation **(A)**, and calcium carbonate dissolution in diluted nutrient agar **(B)** or minimal media **(C).** *Pseudomonas aeruginosa* ATCC 27853 (PA) was used as positive control in glucose oxidative assay. The metabolic performance is designated through the symbols: (–) lack, (+/–) weak, (+) moderate, (++) good, and (+ + +) strong activity. Media not supplemented with glucose are in the upper line in **(B,C)**, while media with addition of glucose are displayed in the bottom line of the corresponding panels.

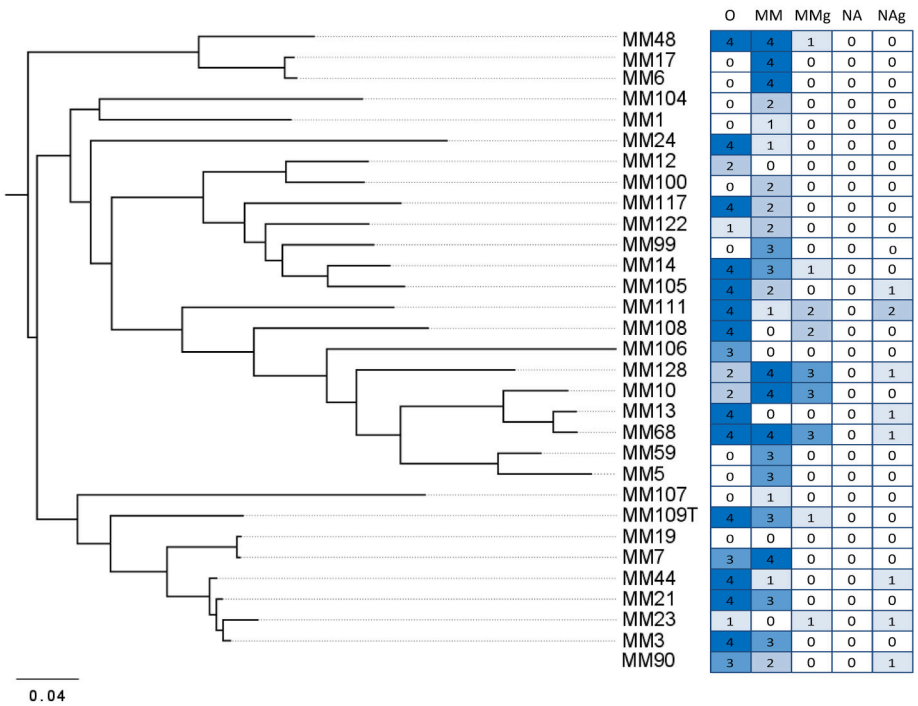

FIGURE 9 | The global pattern of carbonate dissolution-associated metabolic activities of moonmilk *Streptomyces*. The heatmap plot representing metabolic performance of moonmilk *Streptomyces* strains indicated by color scale, correlated with their MLSA-based phylogenetic classification (Maciejewska et al., 2016). O, glucose oxidation; MM, minimal media with $CaCO_3$; MMg, minimal media with $CaCO_3$ and glucose; NA, diluted nutrient agar with $CaCO_3$; NAg, diluted nutrient agar with $CaCO_3$ and glucose.

nutrient agar—supplemented or not with glucose. The clear effect of glucose breakdown was observed in diluted nutrient agar in which the supplementation with the carbohydrate induced $CaCO_3$ dissolution in 26% of the isolates, while none of the phylotypes promoted dissolution in the non-supplemented medium (**Figures 8B**, **9**). In the non-supplemented nutrient agar, rich in organic nitrogen source, the cellular energy comes from amino acid utilization, releasing ammonia as the by-product (**Figure 1**), which increases the pH of the medium and thus promotes precipitation rather than dissolution. The addition of glucose clearly induces the opposite effect but only in a minority of the isolates which suggests the preference toward amino acids as carbon source over glucose in the large majority of the studied strains (**Figures 8B**, **9**). On the contrary, when assays were performed in the minimal medium, $CaCO_3$ dissolution was instead inhibited by the exogenous supply of glucose, as only 29% of isolates showed ability to solubilize $CaCO_3$ under this condition, while in minimal media without glucose supply the dissolution phenotype was characteristic for 81% of isolates (**Figures 8C**, **9**). This might be related to the fact that in minimal medium not supplemented with glucose the only carbon source constitutes the carbonate/bicarbonate from $CaCO_3$, which is probably efficiently scavenged by cave-dwelling bacteria for autotrophic growth resulting in the high dissolution rate observed under this condition. Although *Streptomyces* are mainly heterotrophic microorganisms, autotrophic growth using CO or CO_2 as a sole carbon source within members of this genus has already been reported (Gadkari et al., 1990; Kim et al., 1998). The identification of a high number of carbonic anhydrases together with SulP transporters among moonmilk *Streptomyces* (**Table 1**) could propose a mechanism via which extracellular bicarbonate would be incorporated into the cell and subsequently converted to CO_2 (**Figure 1**). In the moonmilk niche that is deprived of organic carbon, the uptake of inorganic carbon could thus be a possible scenario, which would primarily promote $CaCO_3$ precipitation, and in a second step would lead to $CaCO_3$ dissolution, as a result of organic acids excretion. The availability of glucose also induces a dissolution phenotype via release of organic acids from glucose breakdown, however only in 3 out of the 31 strains tested. This indicates that, although carbohydrate metabolism might somehow play a role in rock weathering, it is probably not the only operating system leading to this phenomenon, particularly in carbon-limited cave environment, where the source of carbon might be the rock itself.

CONCLUSIONS

If Actinobacteria really participate in the genesis of moonmilk deposits which metabolic activities would potentially be involved? This was the main question addressed in our study which used a collection of *Streptomyces* strains isolated from moonmilk in order to provide metabolic and genetic evidences of their presumed role in mediating the formation of these speleothems. Metabolic profiling revealed that all of the isolated

Streptomyces possessed the capacity to promote calcification through at least one pathway involved in biomineralization. Ammonification of peptides/amino acids was found to be the most widespread and the strongest activity. This could be in agreement with the fact that peptides and amino acids can constitute self-sustainable sources of carbon and nitrogen for bacteria and thus support growth of microbial populations irrespectively of allochtonous nutrient input. Interestingly, genome mining extended the possible spectrum of metabolic capacities as it revealed the presence of additional pathways in each phylotype involved in biomineralization processes, either related to CO_2 hydration or active transport of calcium ions. These findings, supported by microscopy observations of bacteria-like filaments in moonmilk deposits and crystals produced by individual moonmilk-originating bacteria, confirm its biogenic origin and the importance of filamentous Actinobacteria in its genesis. However, the metabolic activities evaluated *in vitro* were not always directly related to the genetic predisposition of individual strains as some isolates with great genetic potential remained metabolically silent. This may suggest that they were grown under conditions too different from those encountered in their original niche to trigger the investigated activity or that specific environmental cues are required for their activation. Consequently, whether those processes are indeed active *in situ* also remains an open question. Additionally, our collection of moonmilk Actinobacteria, though being the most significant population from these speleothems generated so far, does not include the large majority of endemic representatives which are viable but not cultivable A metaproteomic analysis of proteins extracted from moonmilk deposits is most likely the only approach that would accurately identify the strains that importantly participate in carbonatogenesis and the metabolic pathways involved. This approach is currently under investigation.

AUTHOR CONTRIBUTIONS

MM, DA, LM, HB, and MoC collected and processed the samples. MM, DA, LM, AN, ET, MaC, MH, DB, PC, and SR performed experiments and/or analyzed the data. MM, AN, MaC, PD, MH, DB, and SR did bioinformatics, and genome mining. All authors participated to the writing and revision of the 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.2017.01181/full#supplementary-material

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Diversity of Bacteria and the Characteristics of Actinobacteria Community Structure in Badain Jaran Desert and Tengger Desert of China

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To assess the diversity of actinobacterial taxa in desert sands and obtain the novel microbial resources, 79 and 50 samples were collected from the Badain Jaran (BJD) and Tengger Deserts (TGD) of China, respectively. High-throughput sequencing (HTS) of environmental 16S rRNA genes within these samples was conducted on an Illumina Miseq platform, using universal bacterial primers targeting the V3–V4 hypervariable region. Based on the HTS analyses, cultivation-dependent (CULD) techniques were optimized to identify the cultivable *Actinobacteria* members. A total of 346,766 16S rRNA gene reads comprising 3,365 operational taxonomic units (OTUs) were obtained from the BJD sands using HTS, while 170,583 reads comprising 1,250 OTUs were detected in the TGD sands. Taxonomic classification indicated that *Actinobacteria* was the predominant phylum, comprising 35.0 and 29.4% of the communities in BJD and TGD sands, respectively. Among the *Actinobacteria,* members of the *Geodermatophilaceae* were considerably abundant in both deserts, indicating that they represent ubiquitous populations within the deserts. At the genus level, *Arthrobacter* spp. and *Kocuria* spp. were dominant, and corresponded to 21.2 and 5.3% of the actinobacterial communities in BJD and TGD deserts, respectively. A total of 786 and 376 actinobacterial strains were isolated and identified from BJD and TGD samples, respectively. The isolates comprised 73 genera of 30 families within the phylum *Actinobacteria*. In addition to the *Geodermatophilaceae*, *Streptomyces* spp. were a prominent component of the isolates, comprising 25% of the isolates from BJD and 17.5% of those from TGD. Comparison of the actinobacterial community structure in other ecosystems indicated that *Geodermatophilaceae* was the main actinobacterial group in desert sands, which is consistent with our results. Additionally, in these desert habits, *Geodermatophilaceae* and some other core groups may promote or inhabit the subsequent members' occurrence or prosper to shape the bacteria community structure. However, it should be noted that a number of other low-abundance bacteria appear to be specific to desert sands, which

are worth further investigation. In antimicrobial activity assays, 10.36 % of the tested isolates showed antimicrobial activities in one or more screens. Importantly, 37 of the newly isolated strains reported here represent novel taxa that could be valuable resources for further research of novel secondary metabolites and their ecological significance in deserts.

Keywords: *Actinobacteria*, diversity, microbial community, desert sands, 16S rRNA

INTRODUCTION

Extremophiles havex generated significant interest in the biological sciences in recent years, due to their unique genotypes, physiological functions, and secondary metabolites that hold great scientific and industrial value (Ciaramella, 1995). Deserts are extreme environments that are characterized by extreme aridity, intense solar UV radiation, and extreme shifts of temperature in day-night cycles. Consequently, deserts harbor numerous extremophiles (Subramani and Aalbersberg, 2013; Júlia et al., 2016). In addition to the above conditions, deserts feature chronic oligotrophy. Thus, sporadic vascular vegetation and microbiological crusts play critical roles in the primary production of this ecosystem, in addition to keeping soils from undergoing further desertification (Hawkes and Flechtner, 2002). Moreover, vegetation and microbiological crusts contribute greatly in shaping soil micro-ecosystem bacterial community structure in desert environments (Evans and Johansen, 1999; Belnap and Lange, 2003; Sun et al., 2015).

Deserts are typical of extreme, harsh ecosystems, where the availability of water is the cardinal parameter affecting organisms. Consequently, xerophilous microorganisms that are adapted to relatively high temperatures and radiation levels are likely to be the dominant populations in these ecosystems, including desiccation and radiation resistant phyla, such as *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* (Vikram et al., 2016).

The Badain Jaran Desert (BJD) is located in the northwest region of the Alxa Plateau of the Inner Mongolia Autonomous Region (39°20′–41°30′ N, 100°–104° E), and comprises an area of 49,000 km^2, making it the third largest desert in China (Yan et al., 2001). This region is known for its extreme continental climate and experiences the East Asian monsoon in its southwestern quadrant. The East Asian monsoon provides most of the precipitation for this area, accounting for 70% of the annualrainfall, which occurs within 10–35 days between July and September. The average annual rainfall is 150 mm while evaporation can reach as much as 1,500 mm. The Tengger Desert (TGD) is located in the southwest region of the Alashan Left Banner in the Inner Mongolia Autonomous Region and along the central border of Gansu Province (34°30′–39°00′ N, 103°–106°E). The TGD comprises a total area of about 43,000 km^2, and is the fourth largest desert in China. A typical continental climate dominates this region, with an annual average temperature of 7–9°C, and annual rainfall of 116–148 mm that primarily occurs in July and August. The annual evaporation of this region is 3,000–3,600 mm, and the average annual wind speed is 3–4 m/s. With increasing grassland degeneration and increases in desertification, the two deserts are almost contiguous with each other in some places (**Figure 1**).

Actinobacteria are a group of bacteria with high G+C content in the genomic DNA, and well-known for producing abundant secondary active metabolites. Since Waksman discovered streptomycin from *Streptomyces* sp. in 1943, actinobacterial members have been considered as an important source of new antibiotic-producing bacteria. Currently, about 70% antibiotics in clinical use are produced by various actinomyces (Doull and Vining, 1990; Jose and Jebakumar, 2013). However, decades later, people found it is increasingly difficult to obtain novel compounds from the actinobacterial strains from normal environments. Therefore, more and more researchers proposed to find potential functional strains from various extreme environments, in which the external stress factors endow the microorganisms the unique defense mechanisms and metabolic systems, which may be more likely to produce novel antibiotics. Consequently, detecting various actinobacteria from extreme environments has become an important strategy for the discovery of new antibiotics (Phoebe et al., 2001; Wilson and Brimble, 2009). Till now, no systematic studies on actinobacteria regarding their potential abilities to produce bio-active substances and their ecological function in deserts have been reported.

The primary goal of the present research was to obtain a systematic understanding of the bacterial community structure and diversity of actinobacteria inhabiting the two immense BJD and TGD deserts of China. Further, we attempted to better characterize the distribution characteristics of actinobacterial population in these environments. Based on the above findings, we intended to optimize the isolation and cultivation strategies to explore the specific actinobacterial strains and to assess their bio-characteristics, potential functions and applications in depth.

MATERIALS AND METHODS

Sample Collection

A total of 79 samples were collected from the BJD (38°20′–41°30′N, 100°–104°E). Sporadic vascular vegetation and small salt lakes characterize this desert. We thus assigned samples to the following three types based on vegetation cover and salt lake presence: sands with sporadic plants (SP), sands without any vegetation (NV), and sands around salt lakes (SL). Detailed information of samples including types, and the specific collection locations are provided in Table S1. Fifty samples were collected from the TGD, where the microbial

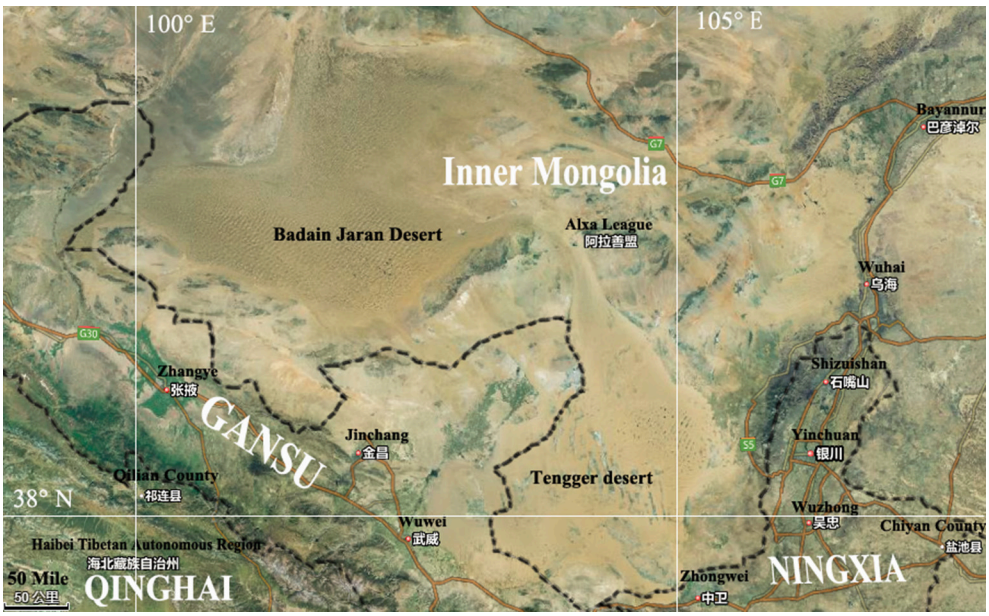

FIGURE 1 | Map showing Badain Jaran desert location and Tengger desert location.

crusts are generally categorized into the following three types: Cyanobacteria-dominated crusts (CC), moss-dominated crusts (MC), and lichen-dominated crusts (LC). Samples from the TGD also included bare sands (BS) (Sun et al., 2015), and sample information is provided in Table S2.

All samples were placed in sterilized envelopes following collection and then taken to the laboratory within 3 days of collection. The samples were immediately processed for cultivation assays and total DNA extraction after arriving at the laboratory.

Isolation and Cultivation Media

Isolation Media

The following seven media were prepared to isolate microbial strains:

M1: 1/5 strength R2A (Difco).

M2: 5 g/L yeast extract, 2 g/L cellobiose, 2 g/L $CaCO_3$, 0.5 g/L MgSO4●7H2O, 1 g/L K_2HPO_4, and 15 g/L agar.

M3: 0.1 g/L NH $_4NO_3$, 2 g/L sodium propionate, 0.05 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L KCl, and 15 g/L agar.

M4: 1 g/L humic acid, 1 g/L asparagine, 0.01 g/L $FeSO_4$, 0.5 g/L Na_2HPO_4, 1.7 g/L KCl, 0.02 g/L $CaCO_3$, and 15 g/L agar.

M5: 2 g/L trehalose, 5 g/L yeast extract, 2 g/L $CaCO_3$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L K_2HPO_4, and 15 g/L agar.

M6: 0.5 g/L K_2HPO_4, 0.25 g/L yeast extract, and 15 g/L agar.

M7: 5 g/L yeast extract, 3 g/L peptone, 10 g/L glycerol, 1.25 g/L sodium pyruvate, 1.25 g/L glycine betaine, and 15 g/L agar.

Media were adjusted to pH 7.2–7.5 using 1M HCl and/or 1M NaOH. 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 as described in Sun et al. (2015). Nystatin (25 mg/L) and potassium dichromate (50 mg/L) were added to the media to inhibit the growth of fungi and Gram negative bacteria.

Cultivation Media

Based the obsevationof the colony diversity growth in the isolation media, the PYG medium was selected to cultivate isolates after initial isolation and consisted of 3 g/L peptone, 10 g/L glycerol, 5 g/L yeast extract, 1.25 g/L sodium pyruvate, 1.25 g/L glycine betaine, and 15 g/L agar, adjusted to pH 7.5. PYG medium was supplemented with compound trace salts solution (0.1% v/v), and compound vitamins (0.1% w/v).

Total DNA Preparation From Sand Samples and PCR Amplification

The 79 sand samples from BJD were pooled into seven composite samples, and the 50 sand samples from TGD were pooled into eight composite samples, according to the environments where the sands were collected. Detailed information of the 15 samples is provided in Table S3. Total genomic DNA from each of the 15 pooled samples was extracted with a PowerSoil DNA isolation kit (MoBio, USA) according to the manufacturer's protocols. Total DNA was then used as template for PCR amplification of 16S rRNA genes. The V3 to V4 hypervariable regions of 16S rRNA genes were PCR amplified using the universal bacterial primers 5′-ACTCCTACGGGAGGCAGCAG-3′ (338F) and 5′-GGACTACHVGGGTWTCTAAT-3′ (806R). PCR amplifications were performed using high fidelity TransStart Fastpfu DNA Polymerase (Transgen, China) in 20 μL reaction mixtures containing 4 μL of 5× FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. The reaction cycling conditions consisted of the following steps: 5 min of an initial denaturation at 94°C

followed by 35 cycles of denaturation at 94°C for 30 s, 45 s of primer annealing at 55°C, 40 s of elongation at 72°C, and then a final 10 min elongation at 72°C.

Illumina MiSeq Sequencing and Raw Data Preprocessing

An AxyPrep DNA Gel Extraction Kit (Axygen, USA) and QuantiFluor™-ST system (Promega, USA) were used to purify and quantify amplicons, respectively. Purified amplicons were pooled in equimolar concentrations and sequenced on an Illumina MiSeq platform with paired-end sequencing (2 × 250 bp). The QIIME software package (version 1.18; White et al., 2009; Caporaso et al., 2010) was used to demultiplex and quality-filter raw reads by removing reads meeting the following criteria: (a) 300 bp reads exhibiting any 50-bp sliding window with an average quality score < 20, and discarding truncated reads that were shorter than 50 bp; (b) any barcode mismatch, greater than two nucleotide mismatches in primers, and reads containing ambiguous characters (c). Lastly, paired sequences were assembled based on their overlapping sequences that were longer than 10 bp. Unassembled reads were discarded.

Statistical Analyses

Chimeras were identified and removed using the UCHIME program (Robert et al., 2001). The Unite database (Release 7.0, http://unite.ut.ee/index.php; Kõljalg et al., 2013) was used as the taxonomic reference database to assess the taxonomic affiliation of each 16S rRNA gene sequence using a confidence threshold of 70% and the RDP Classifier (Wang et al., 2007). Sequences were clustered into operational taxonomic units (OTUs) using a 97% similarity threshold in the UPARSE program (version 7.1, http://drive5.com/uparse/). Alpha diversity and beta diversity metrics were calculated based on OTU abundances within and among samples. Specifically, alpha diversity was measured using the Chao1 richness estimator, and Shannon's diversity index, while measuring the coverage of richness using Good's coverage. Principal co-ordinates analysis (PCoA) was used to assess differences in OTU composition among samples. An analysis of similarities (ANOSIM) test was conducted to determine if sample types contained significantly different bacterial communities. Metastats and LEfSe (LDA effect size) analysis were employed to identify the bacterial groups that were significantly differentiated among sample types. Lastly, co-occurrence networks were constructed to visualize the OTU-based similarities among different communities (White et al., 2009; Caporaso et al., 2010).

Strains Isolation, Purification, Maintenance, and Identification

The dilution plating method was used to isolate microbial strains from the sand samples. Approximately 0.3 mL of a 10^{-4} dilution of a sand sample suspension was spread on each isolation plate. Every kind of media was parallelly spread with the suspension on two same plates and then incubated at 28°C and 45°C for 21 days, respectively. Single colonies were transferred to freshly prepared PYG plates that were supplemented with compound trace salts solution and compound vitamins, followed by subsequent purification of isolates. Purified isolates were maintained on PYG slants at 4°C and also in glycerol suspensions (20%, v/v) at −80°C (Yue et al., 2006). Genomic DNA was extracted from pure cultures and PCR amplification of 16S rRNA genes was conducted as described in Xu et al. (2003). The sequences of the isolates were compared with available 16S rRNA gene sequences from GenBank using the BLAST program and the EzBioCloud (https://www.ezbiocloud.net/) (Yoon et al., 2017).

Antimicrobial Activity Screening

Antimicrobial activities of the isolates were investigated by using media containing *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumonia* subsp. *pneumoniae* ATCC 700603, and *Candida albicans* ATCC 10231, with a concentration of 10^8 colony forming units (CFU) per mL. These assays were performed using Kirby Bauer Method with a culture broth concentration of 1% (v/v).

RESULTS

Sequences and Data Information

The SRA accession number in DDBJ/EMBL/GenBank for the sequences data is SRP134260.

Bacterial Richness and Diversity

A total of 349,374 reads with an average of 23,291 reads per sample remained after quality filtering, and were clustered into 4,298 OTUs at the 97% sequence similarity level. The alpha diversity of each sample estimated by the Chao1 estimator, and Shannon's index, in addition to the coverage estimated by Good's coverage, are provided in **Table 1**.

Rarefaction analyses using the Shannon index as a diversity metric indicated that our sequencing efforts covered nearly all of the diversity that would be expected to be found in these samples (Figure S1). On average, 923 (95% CI: 799–1,029) and 900 (95% CI: 653–1,165) OTUs were found in each of the BJD and TGD samples, respectively. Samples from the desert sands exhibited almost identical Chao1 and Shannon index values, as indicated by ANOVA tests, suggesting no significant difference in the richness or diversity of bacteria between the two deserts (**Figure 2**).

Among the BJD samples, SP samples had the highest Chao1 values, whereas samples from NV, SL and SP did not significantly differ. Among the TGD samples, bacterial richness and diversity was slightly higher in BS samples compared to the others, which was reflected in higher Chao1 and Shannon index values (**Figure 3**).

Bacterial and Actinobacterial Community Structure

A total of 32 phyla, 97 orders, and 470 genera were identified in BJD samples, while 28 phyla, 73 orders, and 302 genera were identified in TGD samples. *Actinobacteria* dominated the bacterial communities of both deserts (35 and 29% relative abundance in BJD and TGD, respectively), followed by *Proteobacteria, Bacteroidetes*, and *Chloroflexi* (**Figure 4**). *Frankiales*, *Micrococcales*, *Micromonosporales*, and

TABLE 1 | Summary of high-throughput sequencing data from the 15 samples.

Sample ID	Sample type	Number of OTUs	Good's coverage wstimator (%)	Chao1	Shannon
BD201610S1	NV	799	98.65	1,046	4.95
BD201610S2	NV	938	98.51	1,145	5.3
BD201610S3	NV	925	98.08	1,190	5.22
BD201610S4	SL	804	99.31	1,051	5
BD201610S5	SL	798	99.19	1,172	4.74
BD201610S6	SP	1,129	99.49	1,294	5.6
BD201610S7	SP	1,067	99.29	1,283	4.91
SPT8001BS	BS	1,155	98.72	1,401	5.42
SPT8002BS	BS	1,165	98.72	1,405	5.67
SPT8003LC	LC	719	98.66	998	4.95
SPT8004LC	LC	824	98.89	1,015	4.34
SPT8005CC	CC	1,039	98.58	1,463	5.57
SPT8006CC	CC	653	99.34	852	4.32
SPT8007MC	MC	892	98.57	1,108	5.34
SPT8008MC	MC	756	98.98	1,063	3.42

BS, Bare sand; CC, Cyanobacteria-dominated crusts; MC, Moss-dominated crusts; LC, Lichen-dominated crusts; SP, sand soil with sporadic plants; NV, sands without any vegetation; SL, sand sample from around salt lakes.

Acidimicrobiales were the most abundant orders within the 15 sand samples. Among these, the families *Geodermatophilaceae*, *Micrococcaceae*, *Micromonosporaceae*, and *Rubrobacteriaceae* were the predominant actinobacterial populations in both deserts. Lastly, the most abundant genera were *Arthrobacter* and *Rubrobacter* in the actinobacterial community, followed by *Blastococcus*, *Modestobacter*, and *Geodermatophilus*, which are members of the *Geodermatophilaceae* (**Figure 5**). In BJD, *Micrococcaceae* was much more prevalent than *Geodermatophilaceae,* while the reverse was observed in TGD samples. *Arthrobacter* was the most abundant genus in BJD samples and the second most abundant genus in TGD samples. *Kocuria* was a dominant genus in BJD samples, but only a few *Kocuria* OTUs were detected in TGD samples. Conversely, *Actinoplanes* were more abundant in TGD samples than in BJD samples. In addition to these dominant genera, a large number of rare bacterial genera were also detected (we defined rare bacterial groups as less than 0.1% abundance of communities), and included the genera *Conexibacter*, *Longispora*, *Dactylosporangium*, *Umezawaea*, *Demequina*, *Janibacter*, and *Motilibacter* in BJD and genera *Ornithinimicrobium*, *Angustibacter*, *Nakamurella*, *Aquiluna*, *Williamsia*, *Amycolatopsis*, and *Kineosporia* in TGD (**Figure 5**).

Metastats analysis indicated that the composition of actinobacterial genera in the two deserts were not significantly different ($P = 1$) (Table S4). However, different sand types represented distinct ecosystems, and generally, bacterial community composition was more strongly correlated with sampling site rather than micro-ecosystem type (**Figure 6**).

In particular, ANOSIM ($R = 0.328$, $p = 0.001$) indicated that community structures were not different between samples taken from sampling sites that were close together. LEfSe analyses indicated that there were some significant differences in community composition among the three sample types from BJD. For example, the abundances of the genera *Virgisorangium*, *Nocardiopsis*, *Brachybacterium, Kineococcus, Georgenia*, and *Streptosporangium* were highly differential among sample types within BJD (**Figure 7**). However, no significant differences in the abundances of these genera were observed for samples from the TGD. The *Geodermatophilaceae* and *Micrococcaceae* were the dominant families in NV, SL, SP, and BS samples, whereas *Micromonosporaceae* were more abundant in the LC, CC, and MC samples. Further, *Pseudonocardiaceae* were more abundant in NV, SP, BS, LC, CC, and MC samples, but not in SL samples. *Arthrobacter* was the predominant genus in NV, SL, SP, and BS samples, but not in LC, CC, or MC samples. Lastly *Rubrobacter* were abundant in LC, CC and MC samples, but not NV, SL, SP, or BS samples (Figure S2).

We used co-occurrence network analysis to identify correlations between different genera among samples. A total of 27 nodes and 84 correlation edges were used to represent the dominant genera (>1% relative abundance). Correlation analysis indicated 77 positive and seven negative correlations. The genera *Solirubrobacter* and *Actinoplanes* exhibited the most correlations (12 edges for each), followed by the *Pseudonocardia*, *Marmoricola*, *Saccharothrix*, and *Friedmanniella* which each comprised 10 edges. These results indicated that the six genera were likely to be the key actinobacterial members in the sand communities of the two deserts. Among the family *Geodermatophilaceae*, three genera appeared to also be important with *Geodermatophilus* exhibiting six edges, *Modestobacter* with five, and *Blastococcus* with two (**Figure 8**).

Isolation of Actinobacterial Strains

A total of 786 actinobacterial strains were isolated and identified from the BJD samples. 16S rRNA gene sequencing indicated that these 786 strains belonged to 30 families and 73 genera (Figure S3A). In addition, 376 actinobacterial strains were isolated from TGD samples, comprising 18 families and 29 genera (Figure S3B). 16S rRNA gene sequencing indicated that members of *Geodermatophilaceae* were the major cultivated actinobacterial taxa in both of these deserts, which is consistent with the cultivation-independent analyses. Moreover, the taxonomic distributions of isolates were highly similar to those of the cultivation-independent analyses.

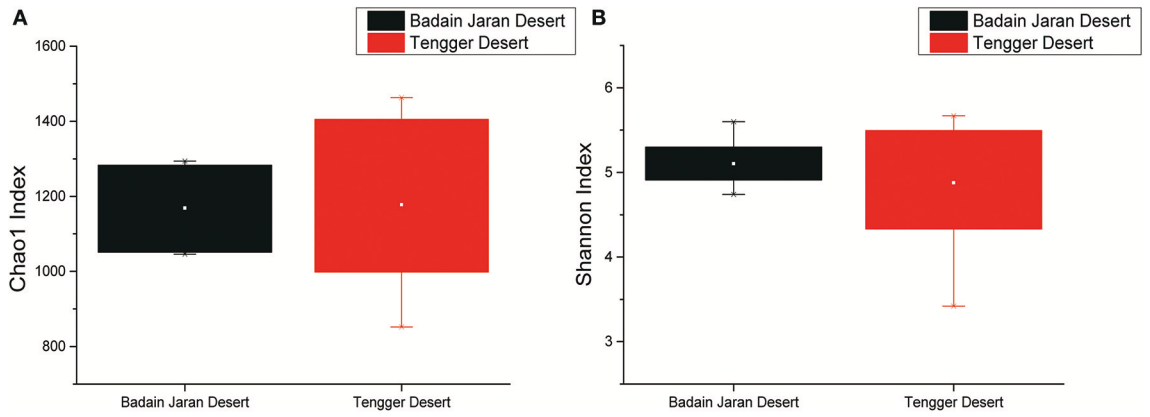

FIGURE 2 | Statistical comparisons of Chao1 and Shannon indices between two desert sand samples. **(A)** Values of Chao1 index; **(B)** values of Shannon index.

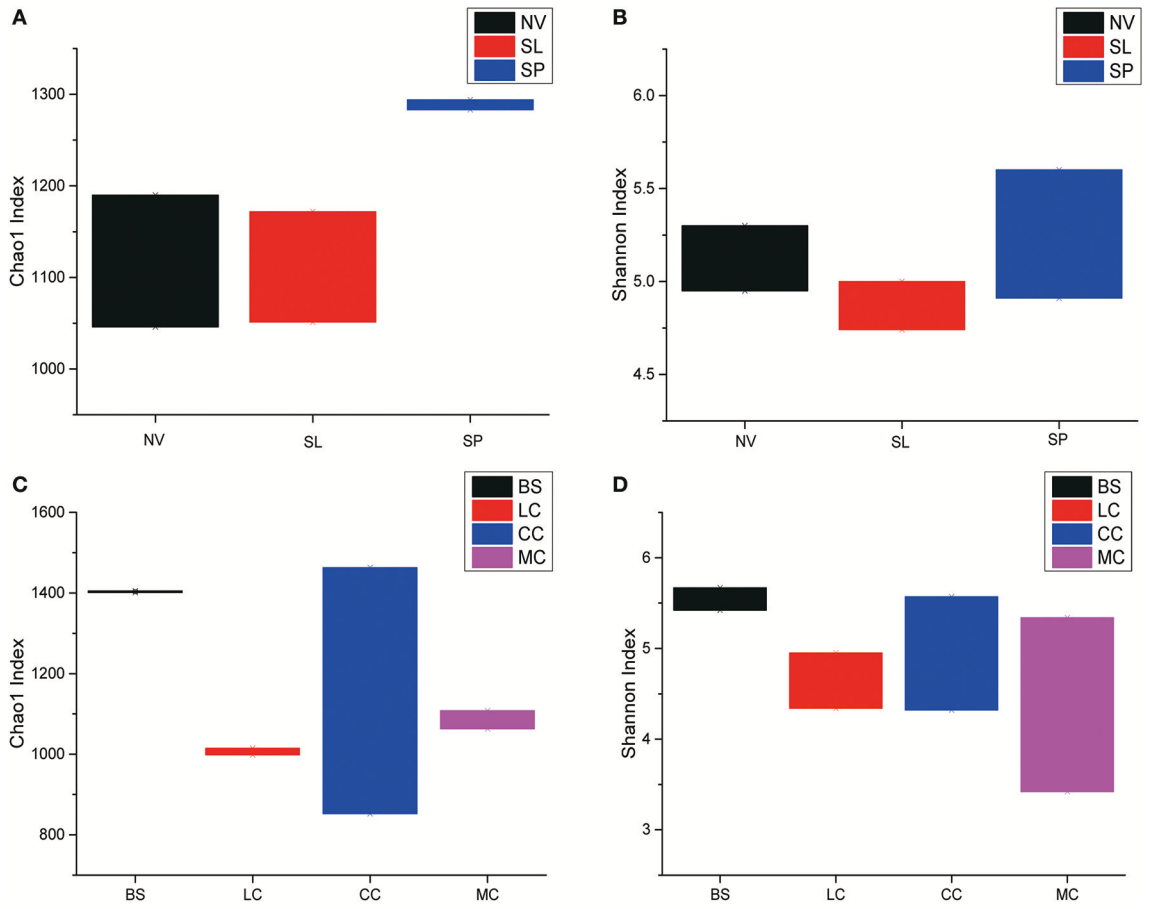

FIGURE 3 | Statistical comparisons of Chao1 and Shannon indices among three types of Badain Jaran desert sand samples (NV, SP, and SL) and four types of Tengger desert sand samples (BS, LC, MC, and CC). **(A)** Values of Chao1 in of BJD; **(B)** values of Shannon index of BJD; **(C)** values of Chao1 of TGD; **(D)** values of Shannon index of TGD.

Arthrobacter spp. and *Kocuria* spp. were the primary cultivated genera, and comprised 13.4 and 4.1% of the actinobacterial community isolates in BJD, respectively (Figure S4; **Figure 9**). *Streptomyces* spp. were also dominant among the isolated genera, comprising 25% of BJD isolates and 17.5% of TGD isolates. In addition, some low abundance taxa that were

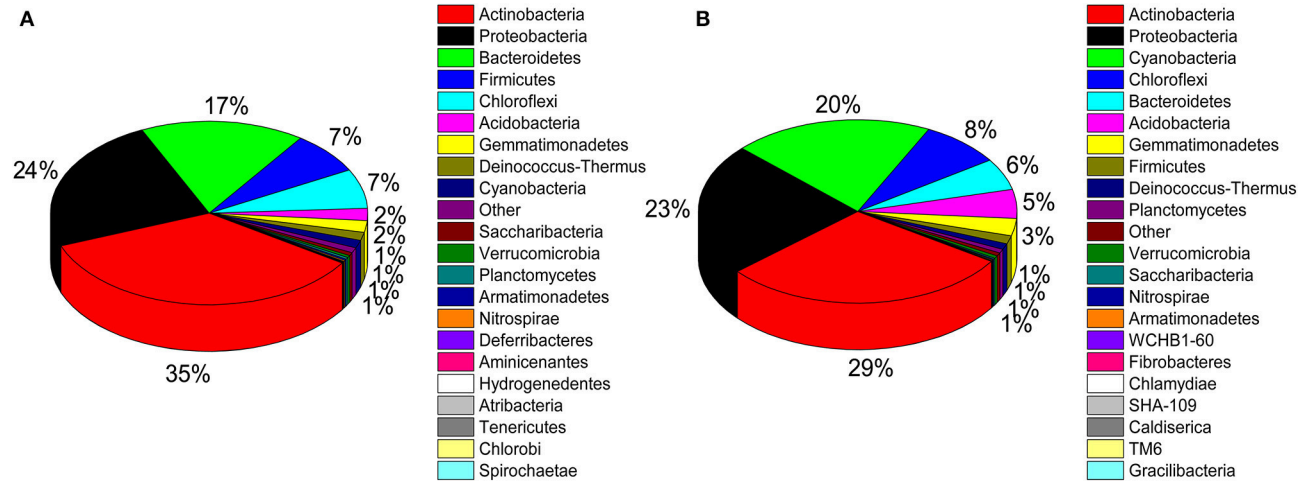

FIGURE 4 | Pie chart showing the percentage of relative abundance of bacterial communities from two desert sand samples in HTS method. **(A)** Badain Jaran desert sands; **(B)** Tengger Desert sands.

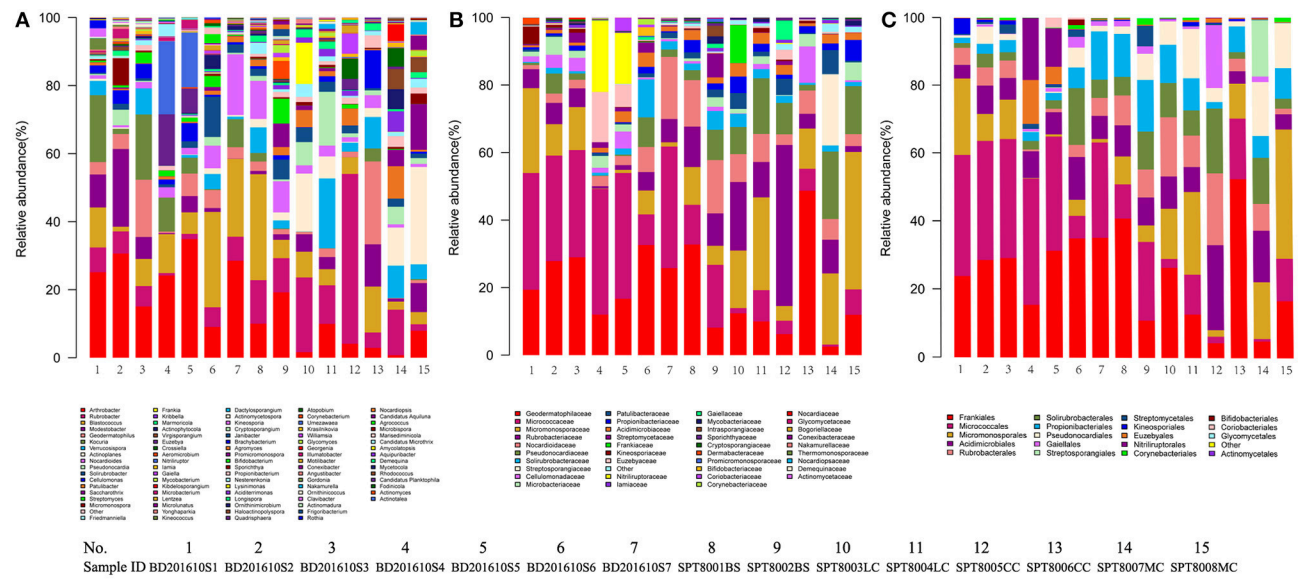

FIGURE 5 | The relative abundances of different actinobacterial taxon level of two desert sand samples in HTS method. **(A)** Genus taxon level; **(B)** family taxon level; **(C)** order taxon level.

detected using cultivation-independent analysis including *Deinococcus* spp., *Skermanella* spp., *Actinophytocola* spp., *Patulibacter* spp., *Rufibacter* spp., and *Geminicoccus* spp. were also isolated.

Comparative Analysis of HTS and CULD Results

Of the dominant genera (>1%), 4.3 and 32.2% were detected using the two methodologies in BJD and TGD samples, respectively (**Figure 10**). In addition, 14.0 and 17.1% of the rare genera (<0.1%) were identified by both methodologies in BJD and TGD, respectively. However, there were some genera that were only detected by either cultivation-dependent (CULD) or high-throughput sequencing (HTS) analysis (**Figure 10**). These results indicate that the composition of the native microbial communities are not only driven by the dominant groups, but also rare taxa that are active and integral members of community structure.

Actinobacterial Members in Desert Sands

Geodermatophilaceae populations were the dominant group in both the BJD and TGD deserts, comprising 27.4 and 18.2% of the *Actinobacteria* according to cultivation-independent analyses. The genera *Blastococcus*, *Modestobacter*, and *Geodermatophilus* exhibited nearly equivalent diversity in the two deserts (Figure S4). However, *Blastococcus* was more ubiquitous and

abundant in all samples, compared to the other two genera. *Modestobacter* was more abundant in NV, MC, and CC samples, while OTUs associated with this genus were rare in SL, SP, and BS samples. *Geodermatophilus* was abundant in NV, SP, and CC samples, but not in LC or MC samples. Generally, NV, SP, BS, and CC samples harbored the most abundant *Geodermatophilaceae* communities (**Figure 11**). Futher, more *Geodermatophilaceae* isolates were obtained form the NV and BS samples, which could be regarded as more oligotrophic environments. In total, 52 *Geodermatophilaceae* strains were obtained from BJD, including 36 *Blastococcus* spp., six *Geodermatophilus* spp. and 10 *Modestobacter* spp. In addition, 34 *Blastococcus* spp., 11 *Geodermatophilus* spp., and 25 *Modestobacter* spp. were obtained from TGD. Importantly, 21 actinobacterial isolates from BJD representing novel species within the following 15 genera were obtained: *Actinokineospora*, *Actinomycetospora*, *Auraticoccus*, *Blastococcus*, *Cellulomonas*, *Kibdelosporangium*, *Mariniluteicoccus*, *Microbacterium*, *Nesterenkonia*, *Nocardia*, *Nocardioides*, *Pseudonocardia,* and *Solirubrobacter*. Likewise, 16 novel taxa were isolated from TGD that were distributed among 11 genera (**Figure 12**). These novel actinobacterial taxa are worthy of further bioprospecting studies.

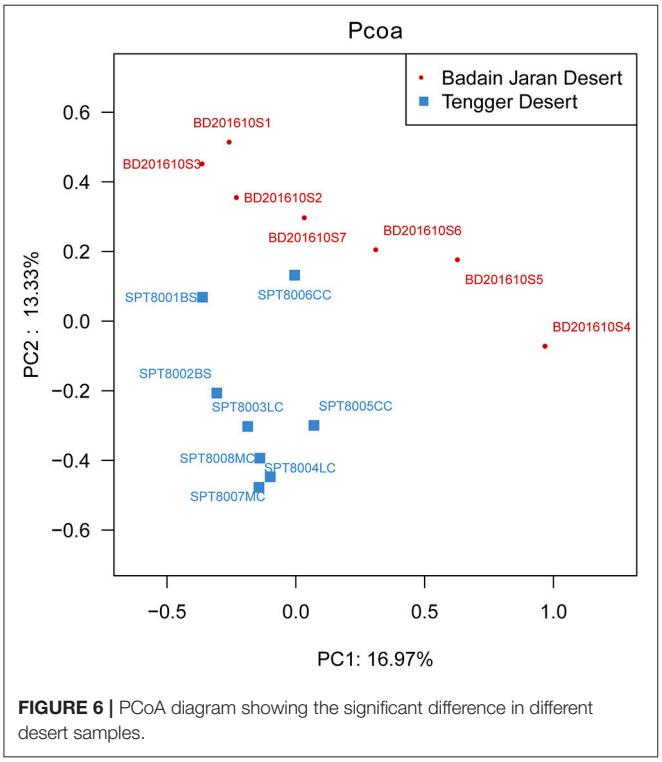

FIGURE 6 | PCoA diagram showing the significant difference in different desert samples.

Antimicrobial Activities

Five-hundred actinobacterial isolates from BJD were screened for antimicrobial activities. Of these, 1.6% of the isolates exhibited antimicrobial activity against *Escherichia coli* ATCC 25922, 3.5% against *Enterococcus faecalis* ATCC 29212, 1.7% against *Klebsiella pneumonia* subsp. *pneumoniae* ATCC 700603, 1.2% against *Pseudomonas aeruginosa* ATCC 27853, and 2.2% against *Candida albicans* ATCC 10231. Overall, 10.36% of the tested isolates from BJD exhibited antimicrobial activity in one or more screens.

DISCUSSION

With the rapid technological innovation in molecular biology in recent years, various new techniques, and in particular high-throughput cultivation-independent approaches, have been extensively applied in microbial ecology research. These new methodologies have greatly expanded our knowledge of bacterial community composition. Consequently, numerous studies have shown that microbial diversity is far higher and more complex than previously thought. 16S rRNA gene cloning and denaturing gradient gel electrophoresis (DGGE) have been traditionally used to identify uncultured microbial community structures in the past. However, these methods are particularly susceptible to 16S rRNA gene copy numbers, and the methods are both complicated and costly. High-throughput sequencing precludes the need to build clone libraries, and the efficiency associated with HTS sequencing approaches and the widespread adoption of the Illumina Miseq sequencing platform has accelerated analytical capabilities. Consequently, HTS analysis on the Illumina Miseq platform has provided comprehensive and efficient analyses of environmental microbial communities. (Navarro-Noya et al., 2013; Poulsen et al., 2013; Sul et al., 2013; Yu et al., 2013; Wu et al., 2014). Claesson et al. (2010) conducted a comprehensive

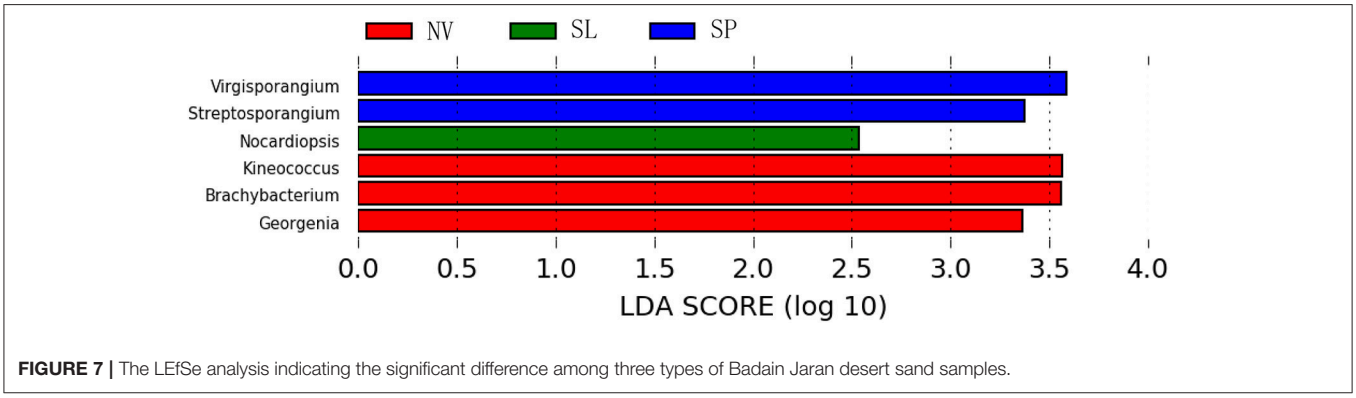

FIGURE 7 | The LEfSe analysis indicating the significant difference among three types of Badain Jaran desert sand samples.

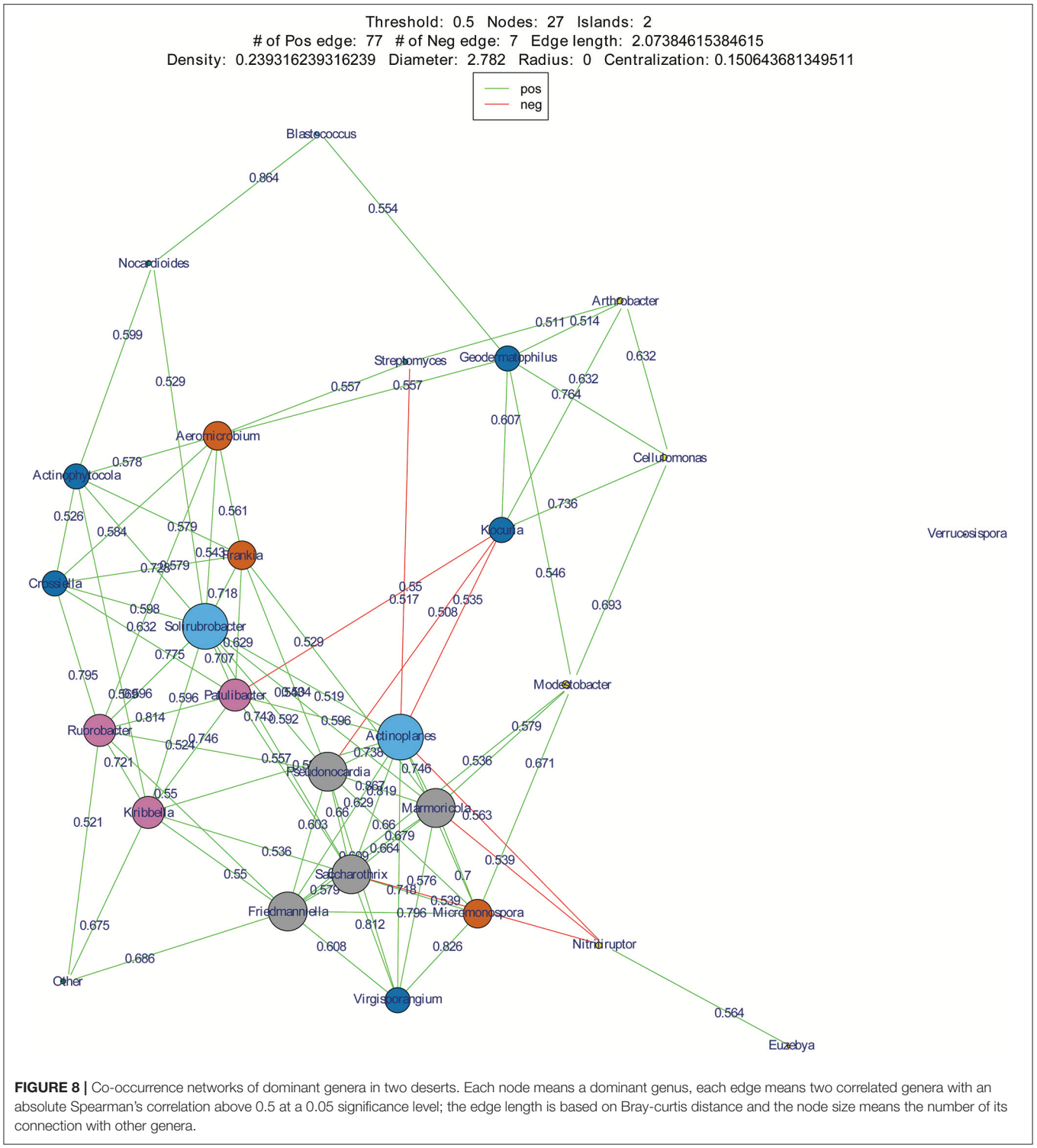

FIGURE 8 | Co-occurrence networks of dominant genera in two deserts. Each node means a dominant genus, each edge means two correlated genera with an absolute Spearman's correlation above 0.5 at a 0.05 significance level; the edge length is based on Bray-curtis distance and the node size means the number of its connection with other genera.

analysis of 9 variable regions of the 16S rRNA gene. The results showed that V1 and V9 had the worst effects, followed by V7 and V8 regions, while V3, V4, and V5 regions were relatively better. In addition, by comparing the regions of V1/V2, V2/V3, V3/V4, V5/V6, and V7/V8 in series, the accuracy of the tandem region V3/V4 and V4/V5 were the highest. Considering the classification efficiency (CE), the V3 / V4 region had significant amplification preference relative to other regions (Claesson et al., 2010). Here, we analyzed the bacterial communities of two Chinese deserts using HTS, and accordingly, modified the

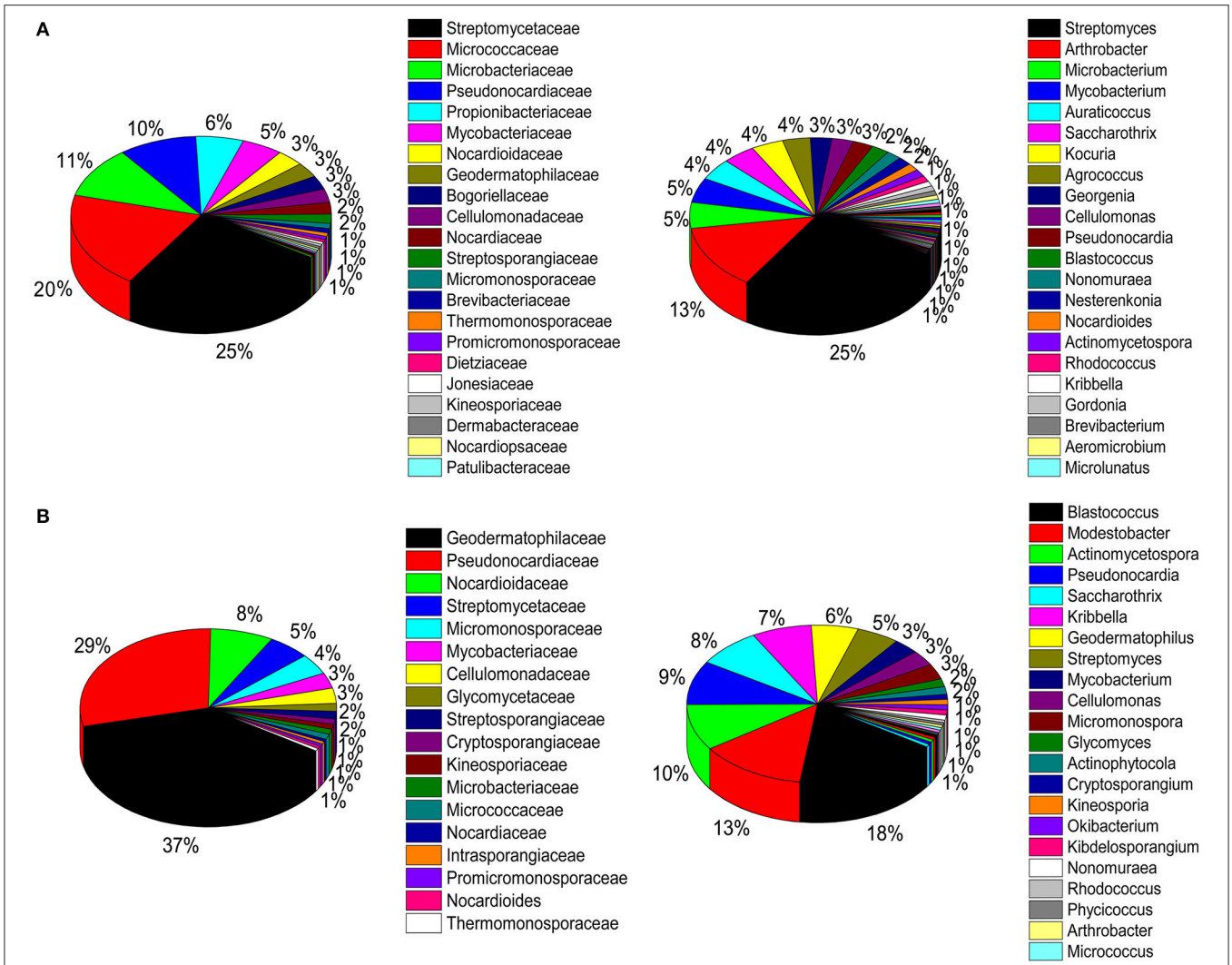

FIGURE 9 | Pie chart showing the percentage of relative abundance of bacterial communities from two desert sand samples in CULD method. **(A)** Badain Jaran desert sands; **(B)** Tengger desert sands. The sequences of the bacteria were assigned to family (1/3) and genus level (2/4).

media and temperature for isolation and cultivation the targeted actinobacterial strains. Our results provide evidence for a number of actinobacterial strains that are specific for these desert sands and were isolated and identified via cultivation-dependent and -independent analyses. Importantly, these results describe the diversity and composition of actinobacterial communities in different sand types, which has not been systematically investigated in these two deserts (Sun et al., 2015; Li et al., 2016).

Comparative results from the two deserts and the different mico-ecosystems indicated that geographical barrier contributed much more in shaping the bacterial communities than micro-ecological types. On the other side, even in a certain same terrain, the indigenous microorganisms' ecological service function resulted in discriminating micro-ecosystem.

Deserts are typical of extreme, harsh ecosystems, where the availability of water is the cardinal parameter affecting organisms. Consequently, xerophilous microorganisms that are adapted to relatively high temperatures and radiation levels are likely to be the dominant populations in these ecosystems. *Actinobacteria* are a ubiquitously distributed bacterial phylum inhabiting diverse terrestrial ecosystems. Based on this observation, we approved that *Actinobacteria* is the predominant microorganisms in desert sand microbial communities using HTS and CULD data. Generally, *Actinobacteria*, characterized by high genomic GC content, complex peptidoglycan layer containing in the thick cell wall, motility with spore-bearing cells and pigmentation in colonies, may have evolved into some genetic and metabolic functions to help them inhabit in the deserts with drought and high ultraviolet radiation (Mohammadipanah and Wink, 2012).

A total of 4,298 OTUs were obtained using cultivation-independent HTS, indicating a high level of bacterial diversity in these desert environments. Importantly, the cultivation-independent and -dependent analyses provide evidence for valuable microbial resources that warrant further research.

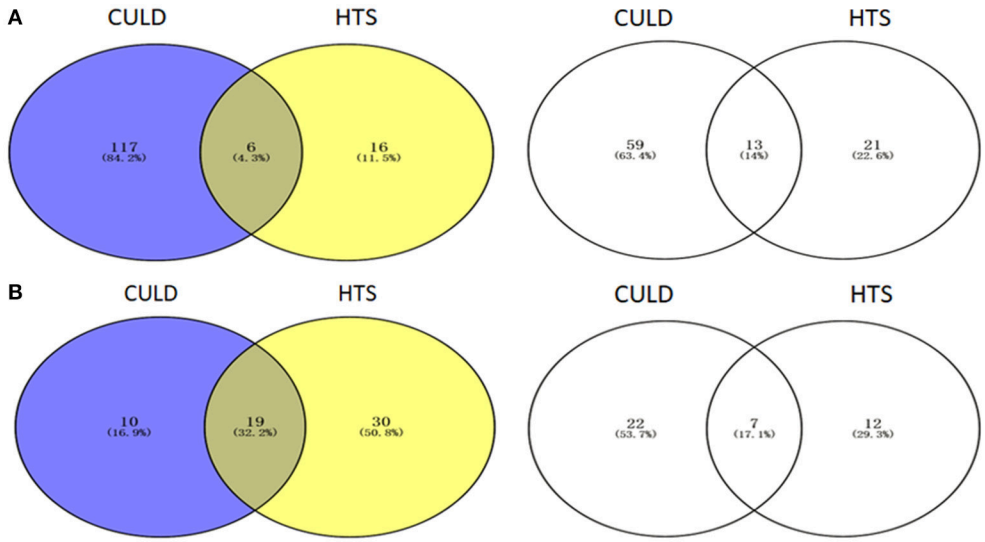

FIGURE 10 | Venn diagrams illustrating the number of genera between High-throughput sequencing analysis and Cultivation-dependent method. **(A)** Badain Jaran desert sands; **(B)** Tengger desert sands. The Venn diagrams were compared by dominant genera (1/3) and rare genera (2/4).

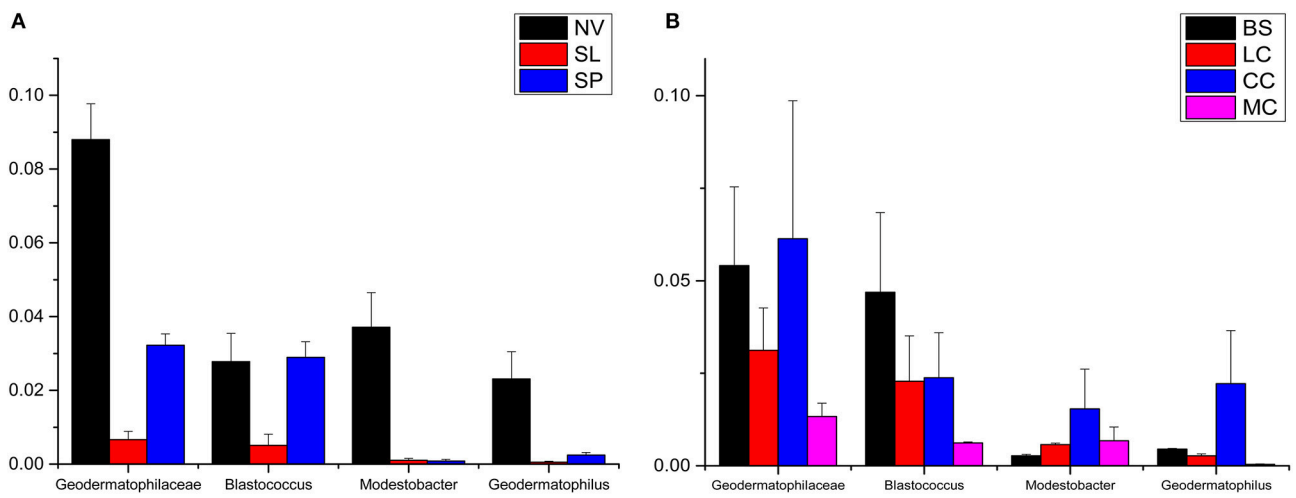

FIGURE 11 | The relative abundances of Geodermatophilaceae in different types of the two deserts. **(A)** Badain Jaran Desert sand samples; **(B)** Tengger Desert sand samples.

In particular, some specific, novel *Actinobacteria* members and rare microbial groups were identified that could serve as highly valuable microbial resources. OTUs affiliated with the *Geodermatophilaceae* were frequently detected among the 15 sample types analyzed here, which is consistent with our previous supposition that *Geodermatophilaceae* are ubiquitous within arid deserts (Sun et al., 2015). *Geodermatophilaceae* have been detected from various relatively extreme environments, including stone habitats (Salazar et al., 2006; Chouaia et al., 2012; Gtari et al., 2012; Normand et al., 2012), dry hot valleys (Nie et al., 2012), and arid sand from deserts (Montero-Calasanz et al., 2012; Montero-Calasanz C. et al., 2013; Montero-Calasanz M. C. et al., 2013; Saul-Tcherkas et al., 2013), among others. Thus, *Geodermatophilaceae* strains may be pioneer microorganisms that play a key role in shaping the bacterial community structure in these harsh environments. Furthermore, to some extent, in these desert habits, the microbial community structures may always be doomed from some pioneer population's colonization, where the core groups may promote or inhabit the subsequent members' occurrence and prosper.

Actinoplanes and *Kocuria* were abundant members of their communities and were also two key genera of the desert sand communities based on co-occurrence network analyses. Interestingly, *Actinoplanes* and *Kocuria* were negatively correlated in the co-occurrence network. A large number of

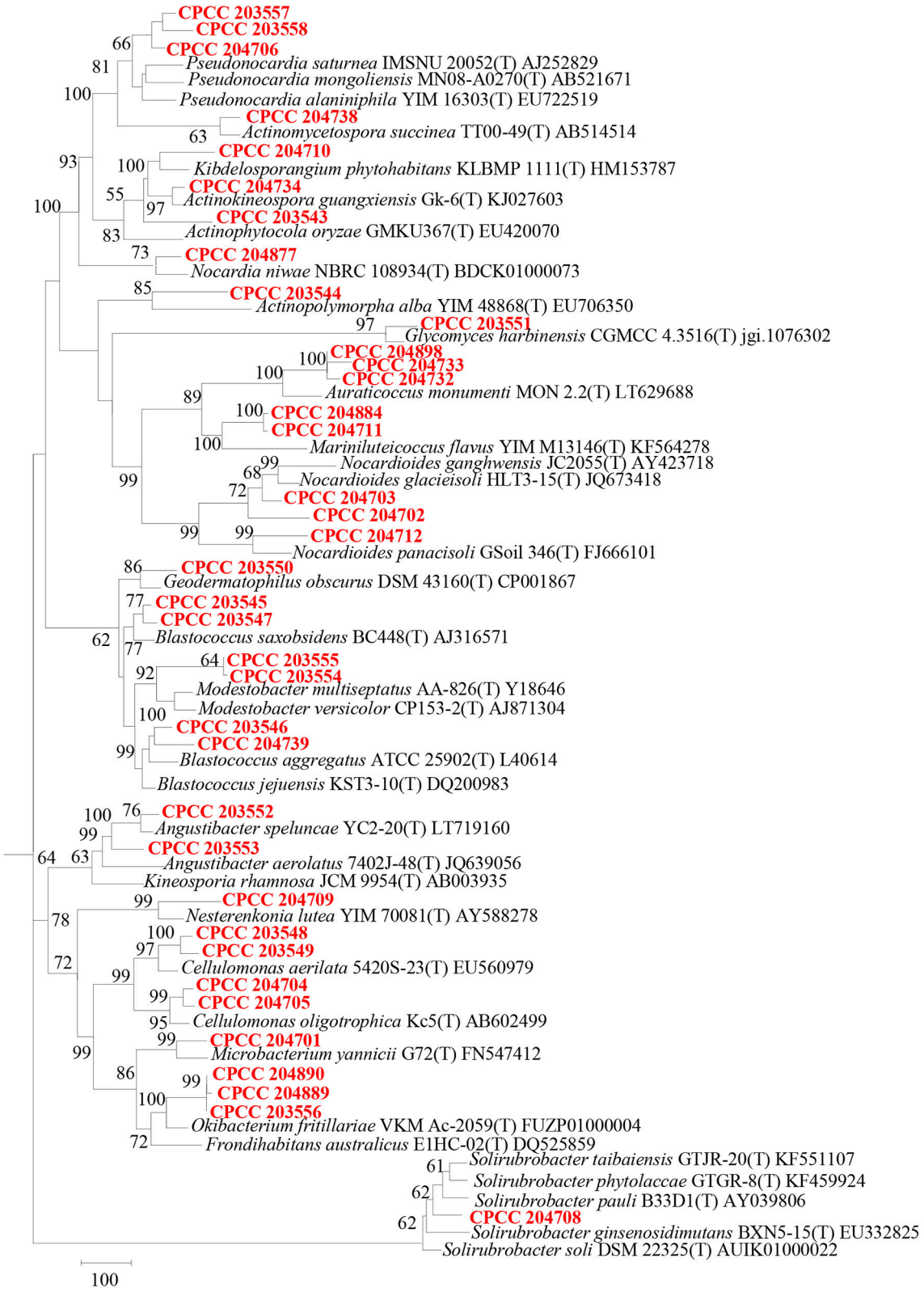

FIGURE 12 | Dendrogram based on 16S rRNA gene sequences analysis of 37 novel species and some relevant strains in the two deserts. Bar, 100 substitutions per nucleotide position.

Kocuria spp. were isolated from our cultivation assays (21 strains) from sand samples, yet only a few *Actinoplanes* strains were obtained. In addition, *Streptomyces* was an abundant component of both the HTS and cultivation assays. However, *Streptomyces* and *Actinoplanes* were negatively correlated in the co-occurrence network analysis. These results point to a potentially intriguing negative interaction between *Streptomyces*, *Kocuria* and *Actinoplanes* that may explain the paucity of *Actinoplanes* isolates that were obtained. Future research should investigate the potential for supplements that inhibit the growth of *Streptomyces* and *Kocuria* in order to promote the isolation of *Actinoplanes*. Regardless, our results suggest the presence of valuable microbial strains, particularly rare community members that are present within these desert sands, and which may hold great significance in understanding the ecology of these systems or in novel microbiological drug research. The analyses here, particularly the co-occurrence networks, provide a framework from which to design isolation media that may promote the growth of these potentially valuable taxa.

AUTHOR CONTRIBUTIONS

YS carried out the experiments and prepared the manuscript. Y-LS helped prepare some experiments. TZ, L-YY, and HW contributed in sampling from deserts. HS helped correct the writing. Y-QZ is responsible for designing the research and preparing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01068/full#supplementary-material

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Genome Mining of the Marine Actinomycete *Streptomyces* sp. DUT11 and Discovery of Tunicamycins as Anti-complement Agents

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Marine actinobacteria are potential producers of various secondary metabolites with diverse bioactivities. Among various bioactive compounds, anti-complement agents have received great interest for drug discovery to treat numerous diseases caused by inappropriate activation of the human complement system. However, marine streptomycetes producing anti-complement agents are still poorly explored. In this study, a marine-derived strain *Streptomyces* sp. DUT11 showing superior anti-complement activity was focused, and its genome sequence was analyzed. Gene clusters showing high similarities to that of tunicamycin and nonactin were identified, and their corresponding metabolites were also detected. Subsequently, tunicamycin I, V, and VII were isolated from *Streptomyces* sp. DUT11. Anti-complement assay showed that tunicamycin I, V, VII inhibited complement activation through the classic pathway, whereas no anti-complement activity of nonactin was detected. This is the first time that tunicamycins are reported to have such activity. In addition, genome analysis indicates that *Streptomyces* sp. DUT11 has the potential to produce novel lassopeptides and lantibiotics. These results suggest that marine *Streptomyces* are rich sources of anti-complement agents for drug discovery.

Keywords: marine streptomycetes, genome mining, secondary metabolites, tunicamycin, anti-complement activity

INTRODUCTION

Marine environments cover more than 70% of the surface of the Earth, and are habitat of diverse microorganisms. Marine actinomycetes are rich sources for a myriad of bioactive natural products. Among various marine actinomycetes, marine streptomycetes have received the most attention due to their biosynthetic potential for producing novel bioactive compounds (Subramani and Aalbersberg, 2012; Agustina et al., 2016; Prietodavó et al., 2016).

Genome sequences of marine streptomycetes not only provide insights in mechanisms of marine environmental adaptation (Ian et al., 2014; Tian et al., 2016), but also benefit discovery of their biosynthetic potential (Zhao and Yang, 2011). Various useful metabolites were identified based on genome mining of *Streptomyces* (Xu et al., 2016; Ye et al., 2017; Yu et al., 2018). However, so far studies on genome mining of marine *Streptomyces* remain limited (Zhang et al., 2015; Chen et al., 2016; Jin et al., 2018).

Complement system is one of the important human immune defense systems, and it plays an important role in eliminating foreign microorganisms, clearance of damaged cells, adaptive immunity, inflammation, tissue regeneration, and tumor growth (Barnum, 2017). However, the improper activation of complement system may lead to a variety of diseases, such as rheumatoid arthritis and Alzheimer's disease (Morgan and Harris, 2015). Chemical synthesis of anti-complementary agents has the limitations such as high cost, low selectivity, and unwanted side effects. Therefore, natural products with anti-complement activities have received increasing attention. Up to now, various anti-complementary compounds were isolated from algae and plants (Jin et al., 2015; Wen et al., 2017), whereas compounds with such activities have been poorly studied in microorganisms. The only known microbial-derived anti-complement compounds are complestatin and its analogs, which are produced by *Streptomyces lavendulae* SANK 60477 (Kaneko et al., 1989). Microbial production of anti-complement agents has the advantages to reduce production cost by rapid accumulation of metabolites and easy scale-up fermentation. In addition, novel compounds can also be obtained by genetic modifications of the microbial producers. Therefore, exploration of microbial strains with the potential to produce anti-complement compounds is of great interest, and the information of related genome sequences of the producer will promote rapid discovery of such active compounds.

During the screening of marine streptomycetes producing anti-complement compounds, we found that *Streptomyces* sp. DUT11 has superior anti-complement activities. In this study, the anti-complement compounds of *Streptomyces* sp. DUT11 were studied by genome mining of *Streptomyces* sp. DUT11, and its biosynthetic potential was also explored. Our current report indicates that marine *Streptomyces* strains are valuable sources of anti-complement compounds for drug discovery.

MATERIALS AND METHODS

Strains and Culture Conditions

Strain *Streptomyces* sp. DUT11 was isolated from the marine sediment about 10 m below sea level in Xinghai Bay (39° 52′N, 121° 35′E), Dalian, China. This strain was deposited in the China General Microbiological, and the strain number was CGMCC 14581. The strain was maintained on ISP4 agar slants at 4°C and in 20% (v/v) glycerol stock at −80°C. For plate culture, strain DUT11 was grown at 28°C on Bennett's agar. For seed culture, tryptic soy broth (TSB) medium was used.

To prepare samples for bioactivity assays, *Streptomyces* sp. DUT11 was inoculated into 20 mL TSB medium in 50 mL tubes to culture at 28°C for 2–3 days. Then, the seed culture was inoculated into the M33 fermentation media with an inoculum size of 2% (v/v) to culture at 28°C for 7 days. To prepare metabolites from agar culture for molecular networking analysis, 50 μL seed culture was spread over the 6 cm plate containing A1 agar medium to culture at 28°C for 7 days. The compositions of all the cultural media used in this study were provided in Supplementary Table S1.

Genome Sequencing, Assembly, Annotation, and Mining

The genome of *Streptomyces* sp. DUT11 was sequenced through Pacbio RS technology (English et al., 2012) in State Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, and the acquired data were assembled by Canu v1.5 (Berlin et al., 2015). The open reading frames (ORFs) prediction and genome annotation were acquired by RAST (Rapid Annotation using Subsystem Technology) (Brettin et al., 2015). The Clusters of Orthologous Groups (COGs) of gene functions and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway prediction was performed through WebMGA (Altschul et al., 1990; Ogata et al., 1999).

Identification of potential biosynthetic gene clusters (BGCs) for secondary metabolites were proposed by bioinformatics tool website antiSMASH (Weber et al., 2015). Further gene function analysis was through manual BLAST on NCBI and viewed on visual software Artemis Release 16.0 (Carver et al., 2012). The alignment of the genomes was performed by the website Double ACT v2[1] and viewed through software Artemis Comparison Tool (ACT) (Carver et al., 2005). The genome sequence data have been deposited in the GenBank database and was assigned the accession number PRJNA351245 (CP025511).

Sequence Analyses and Genome-Wide Comparative Analysis

16S rRNA PCR amplification was performed using the methods described previously (Lee et al., 2003; Li et al., 2007). The universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1429R (5′-AAGGAGGTGATCCAAGCCGCA-3′) were used to amplify the 16S rRNA sequence. The amplified 16S rRNA gene was sequenced by TaKaRa and uploaded to web-based EzTaxon-e program[2] (Kim et al., 2012) for comparison. A 16S rRNA phylogenetic tree was created by software Geneious (Kearse et al., 2012) based on the EzTaxon-e database and partially on BLAST of some closely related strains.

The whole-genome based phylogenetic trees with peptide sequences or DNA sequences were generated with Composition Vector Tree v3 (CVtree v3) (Xu and Hao, 2009), and

[1]http://www.hpa-bioinfotools.org.uk/pise/double_actv2.html

[2]http://eztaxon-e.ezbiocloud.net/

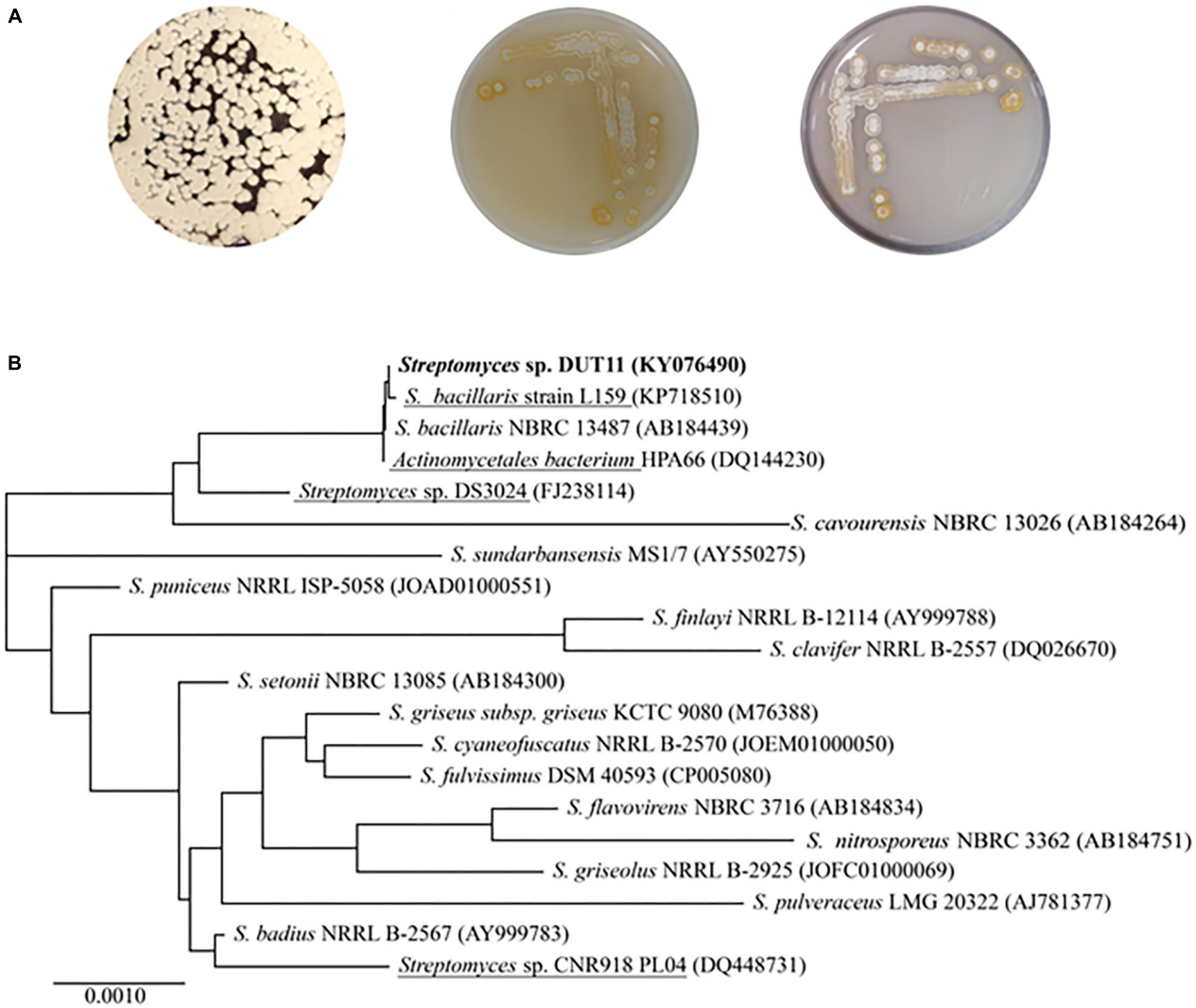

FIGURE 1 | Morphological characteristics and phylogenetic analyses of *Streptomyces* sp. DUT11. **(A)** Phenotype of DUT11 cultured on A1 (left), M33 (middle) and ISP4 medium (right), respectively. **(B)** Neighbor-joining tree showing the phylogenetic relationships of DUT11 and related strains based on 16S rRNA sequences. The strains underlined were isolated from marine environment. The accession numbers of the 16S rRNA sequences were provided in the parentheses.

OrthoANI was used to generate OAT heat map (Ouk Kim et al., 2016). The genomes used for analysis were obtained from GenBank[3]. Nine closely related genomes were chosen for analysis. Due to the poor quality of the genome of *S. bacillaris* NBRC 13487, it was excluded in the genome alignments.

Anti-complement Activity Test

Seven media, namely TSB, TSBY, TYDM, M3, M9, M33, and A1 medium (Supplementary Table S1) were chosen as fermentation media to test the production of anti-complement agents. The fermentation broth was centrifuged at 6,000 rpm for 15 min to obtain supernatant and mycelia. Subsequently, the supernatant was extracted by EtOAc and the mycelia was extracted by MeOH. Then the extracts were dried and re-dissolved into 1 mL 5% dimethyl sulfoxide (DMSO) solution to acquire crude extracts for anti-complement test.

A hemolytic assay was used to determine the inhibition of complement activation in the classical pathway (CP) (Morgan and Harris, 2015) with minor modifications. 1 × BBS (barbital buffer solution) buffer was used for solution buffer. The fresh sheep red blood cell (SRBC) was diluted to 2% for the test. Heparin was used as a positive control, which was dissolved in 1 × BBS. After preliminary test, 1/160 serum was chosen to be submaximal lysis in the absence of complement inhibitors. Sensitized erythrocytes (EAs) were prepared by incubating 2% SRBS with equal volumes of 1:1000 hemolysin. The samples were dissolved in 1 mL 5% DMSO, which were diluted 10 times in 5% DMSO to test anti-complement activities. For the

[3]http://www.ncbi.nlm.nih.gov/genome/

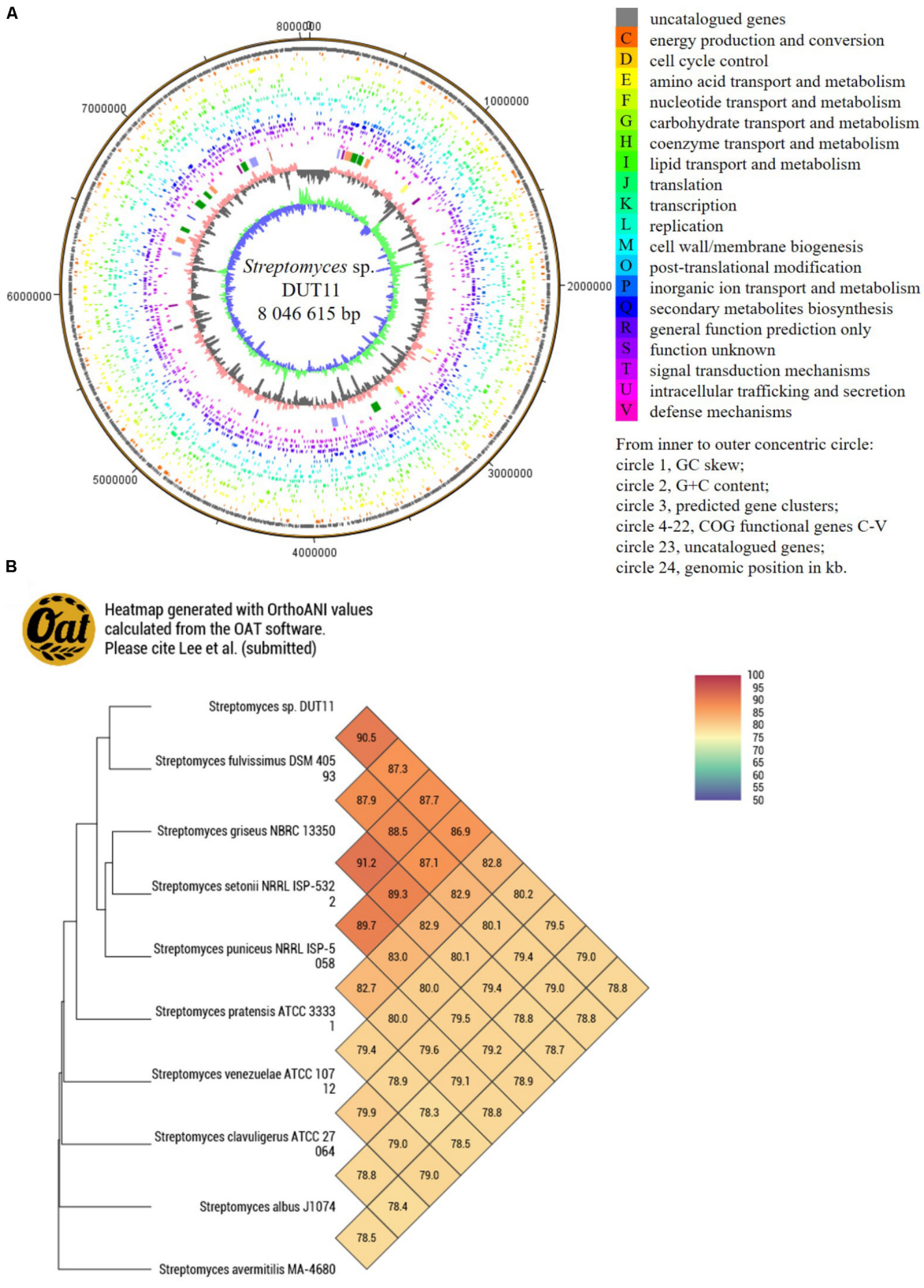

FIGURE 2 | Circular map of *Streptomyces* sp. DUT11 genome and comparative genome analysis. **(A)** Circular map of the genome. **(B)** Heat map generated with OrthoANI values between strain DUT11 and nine closely related strains.

anti-complement activity test, every 100 μL diluted sample and 100 μL serum solution (SS) were mixed and incubated at 37°C for 10 min, after which the mixture was cooled down on ice. Subsequently, 200 μL of EAs was added and the volume was filled to 600 μL with 1 × BBS. The mixtures were then incubated at 37°C for 30 min. Control groups were incubated under the same conditions, which include: (1) standard control: 200 μL EAs and 100 μL serum in 300 μL 1 × BBS; (2) 100% lysis: 200 μL EAs in water (400 μL); (3) sample blank: 100 μL dilution of each sample in 500 μL 1 × BBS; (4) sample test: 200 μL SS with 200 μL EAs in 200 μL 1 × BBS (Zhu et al., 2008). After incubation, the reacted mixtures were centrifuged at 5,000 rpm, 4°C for 10 min immediately. Optical density of the supernatants (200 μL) was measured at 405 nm with a spectrophotometer (Multiskan GO 1510, Thermo Fisher Scientific, Finland). The inhibition percentage of each sample was calculated by excluding the controls and blanks and then divided by a standard value, and the anti-complementary activity (%) is calculated from the formula (Wang et al., 2008):

$$\frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Extraction, Analysis, and Purification of the Compounds With Anti-complement Activity

For analysis the secondary metabolites in strain DUT11, the cultured plates were cut into small pieces and extracted with 1:1 MeOH/H_2O or EtOAc. The samples were dried and re-dissolved into MeOH to analyze by UPLC-MTQ MS (Agilent1290- Bruker MicroTOF-Q II) using the gradient of 5–100% ACN/H_2O containing 0.1% formic acid for 9 min, and 100% ACN with 0.1% formic acid for 1 min with a 0.5 mL/min flow rate (Agilent Infinity 1290 HPLC, Supelco Discover C18 250 mm × 4.6 mm, 5 μm column and Bruker MTQ MS system). The acquired LC-MS

TABLE 1 | List of the BGCs in *Streptomyces* sp. DUT11.

No.	Type	Size (kb)	Most similar cluster (accession no., gene number)	Similarity (%)
1	Butyrolactone	1.9	Gamma-butyrolactone (AL645882, 2)	100
2	Terpene	22.1	–	
3	Other KS	62.2	Abyssomicin (MG243704)	10
4	NRPS	29.1	Griseobactin (FN545130, 17)	64
5	NRPS	20.6	Coelichelin (AL645882, 10)	72
6	T3PKS	2.7	Naringenin (CM000913, 2)	100
7	Lantipeptide	4.9	Venezuelin (HQ328852, 4)	100
8	Terpene	21.2	Steffimycin (AM156932)	19
9	Ectoine	4.6	Ectoine (AY524544, 4)	100
10	Lantipeptide	23.0	–	
11	Siderophore	6.5	Desferrioxamine B (AP009493, 5)	80
12	Thiopeptide	32.5	–	
13	Ectoine	10.4	–	
14	NRPS	67.5	Oxazolomycin (EF552687)	9
15	Ectoine-butyrolactone	15.4	Pristinamycin (FR681999)	17
16	Lantipeptide	7.6	Amf S (AP009493, 5)	100
17	Lassopeptide	5.0	SRO15-2005 (NZ_DS999644, 5)	100
18	Nucleoside	11.1	Tunicamycin (HQ172897, 14)	85
19	Terpene	21.1	–	
20	Siderophore-terpene	37.0	Kinamycin (AH012623)	8
21	T2 PKS	24.1	Medermycin (AB103463, 36)	66
22	NRPS	45.3	FD-594 (AB469194)	4
23	Bacteriocin	11.1	–	
24	NRPS	60.9	Asukamycin (GQ926890)	11
25	NRPS	43.8	Viomycin (AY263398)	9
26	T2 PKS	41.3	Nonactin (AF074603, 14)	92
27	Terpene	16.1	Hopene (AL645882, 13)	69
28	NRPS	55.0	Friulimicin (AJ488769)	6
29	T1 PKS-NRPS	21.7	SGR_PTMs (AP009493, 6)	100
30	Melanin	3.0	Melanin (AP009493, 2)	100
31	T3 PKS	1.7	Alkylresorcinol (AP009493, 3)	100
32	Terpene	8.1	Isorenieratene (AP009493, 7)	71
33	NRPS-thiopeptide	45.7	Daptomycin (AY787762)	10

Similarity, the percentage of genes that are similar to genes in reference BGC.

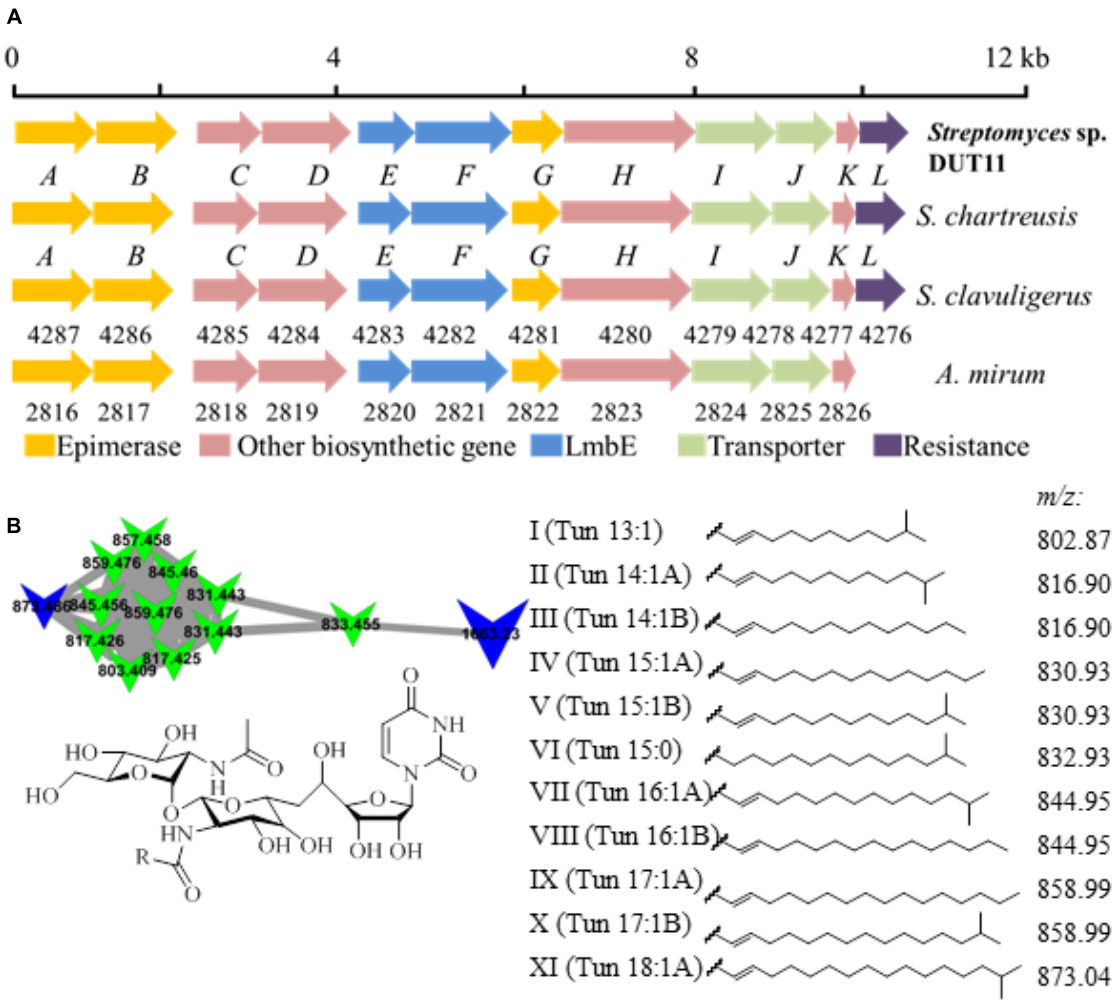

FIGURE 3 | Organization of the tunicamycin BGC and identification of tunicamycins in *Streptomyces* sp. DUT11. **(A)** The tunicamycin BGC from strain DUT11. **(B)** Molecular networking map generated by GNPS and corresponding structures of the tunicamycin analogs.

data was uploaded to Global Natural Products Social Molecular Networking (GNPS) website (Wang et al., 2016) to generate the molecular networking map and the map is viewed by Cytoscape v3.4. The open accession of the GNPS data in this study via MassIVE is ftp://massive.ucsd.edu/MSV000082328.

To isolate active components from fermentation broth and mycelia, *Streptomyces* sp. DUT11 was cultured in modified M33 medium at 30°C with 200 rpm agitation for 7 days for extraction and purification of the active compounds. After 7 days cultivation, the fermentation broth (45 L) was centrifuged at 6,000 rpm, after which the mycelia were extracted by MeOH and the supernatant was extracted by EtOAc. The resultant organic extracts were concentrated in vacuo until dried. The crude extracts of supernatant and mycelia were separated by C18 column with H_2O/MeOH (v/v, 100:0, 70:30, 50:50, 30:70, 0:100), to afford five fractions for each samples. Fractions 4 and 4′ (70% MeOH) with high anti-complement activity were purified by HPLC on the C18 column (XDB-C18, 4.6 mm × 250 mm, 5 μm at a flow rate of 0.4 mL/min using a gradient solvent from 5 to 95% CH_3CN with UV detector set at 260 nm, and compound A was isolated. The active fractions 5 and 5′ (100% MeOH) were purified using the same method to isolate compound B and compound C.

LC/MS (Agilent1290-MS6230 Trap system) was used for further detection of the compounds. LC/MS analysis was performed on a XDB-C18 Column (Agilent, 150 mm × 4.6 mm, 5 μm column) at a flow rate of 0.4 mL/min using a gradient solvent from 5 to 95% ACN over 50 min.

A Waters ACQUITY^{TM} series mass spectrometer equipped with an electrospray ionization source was used for UPLC-Q/TOF MS analysis of compounds. MS/MS detection mode was set as: mass range, from *m/z* 100 to 2,000 in positive mode; capillary, 3 kV; sample cone voltage, 35 V; desolvation gas temperature, 350°C; flow rate of desolvation gas, 600 L/h. The column temperature was maintained at 40°C. Mobile phase A (H_2O, 0.1% formic acid) and B (ACN, 0.1% formic acid) were used; and the gradient program was as follows: 0–5 min 10% B,

TABLE 2 | Anti-complementary activities of *Streptomyces* sp. DUT11 in different fermentation media*.

Sample type	Medium						
	TSB	TSBY	TYDM	M3	M9	M33	A1
S	44.0 ± 2.9	28.0 ± 5.0	12.0 ± 2.1	36.5 ± 3.6	30.2 ± 2.9	55.3 ± 1.5	39.9 ± 3.4
ES	45.3 ± 3.3	34.1 ± 4.7	13.1 ± 2.3	38.4 ± 3.8	31.1 ± 2.8	56.5 ± 2.0	44.2 ± 3.0
EM	47.2 ± 4.6	36.3 ± 4.0	14.1 ± 2.7	39.5 ± 3.0	32.9 ± 2.5	60.8 ± 2.2	46.2 ± 2.8

*S, supernatant; ES, extract of the supernatant; EM, extract of the mycelia. The values represent the percentages of inhibition (%); sample concentration was 0.06 mg/mL; the anti-complement activity of the positive control heparin at 0.06 mg/mL was 40.2%.

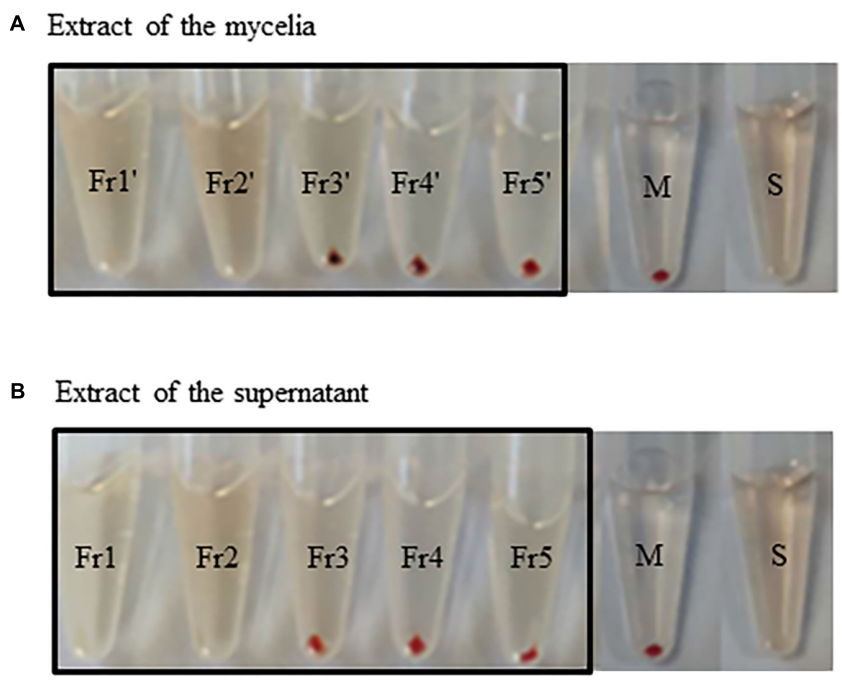

FIGURE 4 | Anti-complement activity of the extracts of *Streptomyces* sp. DUT11. **(A)** The anti-complement activity of fractions from the extracts of mycelia obtained with different MeOH concentrations: Fr1′: H_2O/MeOH (v/v, 100:0); Fr2′: H_2O/MeOH (v/v, 70:30); Fr3′: H_2O/MeOH (v/v, 50:50); Fr4′: H_2O/MeOH (v/v, 30:70); Fr5′: H_2O/MeOH (v/v, 0:100); S: full hemolysis; M: mechanical hemolysis. **(B)** The anti-complement activity of fractions from the extracts of supernatant. Fr1: H_2O/MeOH (v/v, 100:0); Fr2: H_2O/MeOH (v/v, 70:30); Fr3: H_2O/MeOH (v/v, 50:50); Fr4: H_2O/MeOH (v/v, 30:70); Fr5: H_2O/MeOH (v/v, 0:100); S: full hemolysis control; M: mechanical hemolysis control.

30–31 min 40–10% B, 40 min 10% B; flow rate 0.35 mL/min; and injection volume was 1 μL.

RESULTS

Comparative Analysis of the 16S rRNA and Genome of *Streptomyces* sp. DUT11

Streptomyces sp. DUT11 was selected due to its superior anti-complement activity during our primary assay of the marine strain library. This strain grows well on A1, M33, and ISP4 agar, generating white spores (**Figure 1A**). DUT11 tolerates up to 10% NaCl in TSB media, and achieved the highest biomass with 3% NaCl (data not shown). The 16S rRNA sequence of *Streptomyces* sp. DUT11 is the same with that of the type strain *S. bacillaris* NBRC 13487 (100% similarity), and is the most closest to that of *S. globisporus* NBRC 12867 (Waksman and Lechevalier, 1953) (99.65% similarity). However, so far the genome sequences of these two type strains are still not available (**Figure 1B**).

Due to the complicate procedures of anti-complement assay, we decided to use genome mining to facilitate the discovery of anti-complement agents from *Streptomyces* sp. DUT11. The genome of this strain was sequenced and analyzed. The genome size of *Streptomyces* sp. DUT11 is 8,027,164 bp with 71.83% GC content. There are 7,745 ORFs covering 6.76 Mb with 72.22% GC content. The coding percentage is 84.7% and the average length of ORFs is 872 bp. Totally 63 tRNA and 9 rRNA genes were also predicted. From the genome circle view, it was observed that the majority of the secondary metabolic BGCs were located near the ends of the genomes (**Figure 2A**). By blasting the COG database, two third of the annotated genes were classified, and there are 291 genes

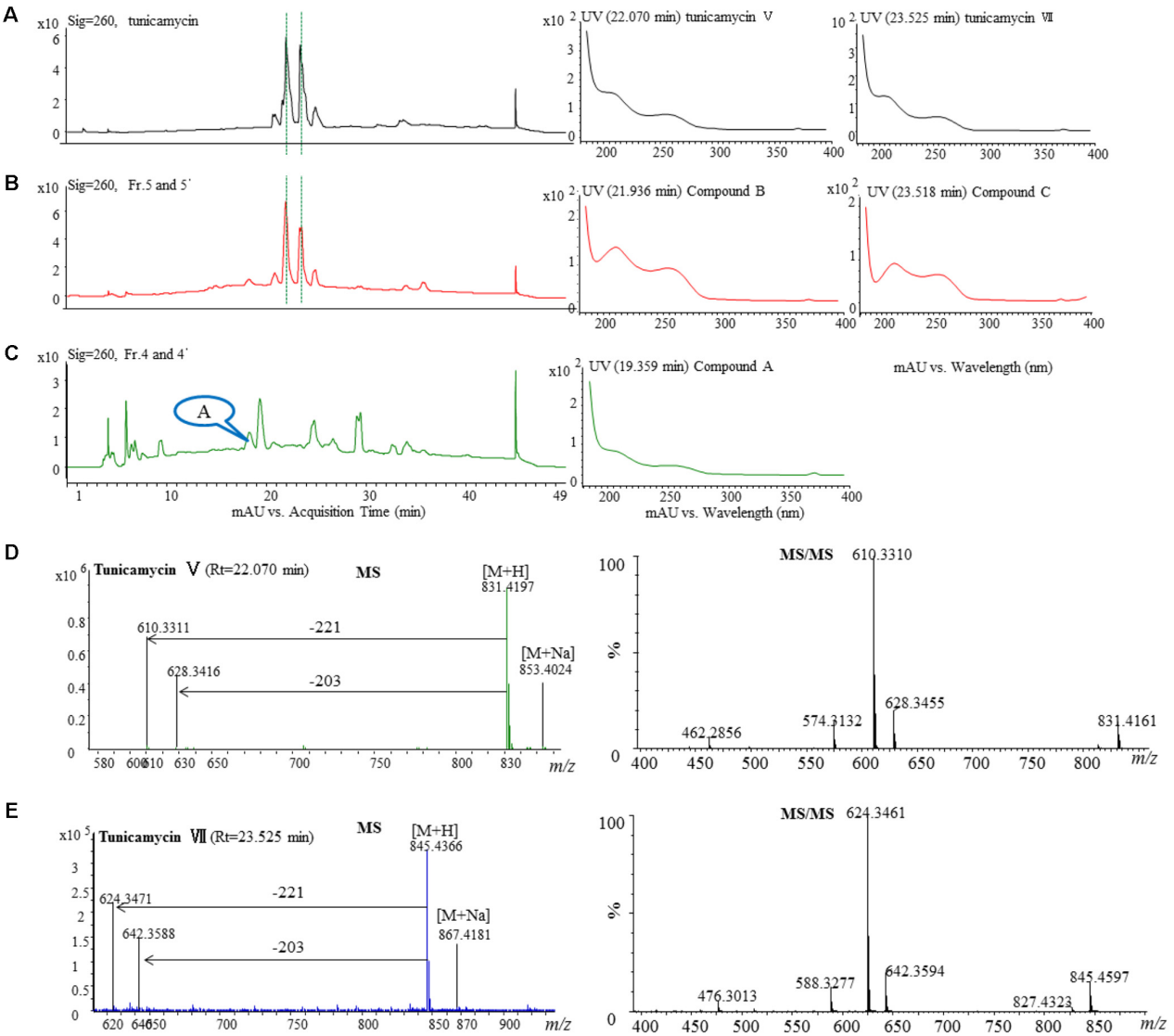

FIGURE 5 | HPLC, MS, and MS/MS analysis of the tunicamycin standard and HPLC analysis of the active fractions. Each sample was purified by HPLC on the C18 column (XDB-C18, 4.6 mm × 250 mm, 5 μm at a flow rate of 0.4 mL/min using a gradient solvent from 5 to 95% CH_3CN with UV detector set at 260 nm. **(A)** HPLC analysis of the tunicamycin standard under 260 nm and the UV-Vis spectra of the tunicamycin standard. **(B)** HPLC analysis of the active fractions 5 and 5′ under 260 nm and UV-Vis spectra of compounds B and C which were isolated from the fractions. The HPLC profiles of fraction 5 and 5′ are the same. **(C)** HPLC analysis of the active fractions 4 and 4′ under 260 nm and UV-Vis spectra of compound A which was isolated from the fractions. The HPLC profiles of fraction 5 and 5′ are the same. **(D,E)**, MS-MS analysis of the standard tunicamycin V and VII, respectively.

annotated to related with secondary metabolites (Supplementary Figure S1).

The genome sequence reported in this study thus provide basis to study related genomes. Among the closely related strains of DUT11, there are three marine actinomycetes, namely, *Streptomyces* sp. DS3024 (Hao et al., 2009), *S. bacillaris* strain L159 (Petříčková et al., 2015), and *Actinomycetales* bacterium HPA66 (Zhang et al., 2006). According the CVTree based on global protein or DNA sequences (Supplementary Figure S2), the most related strain with DUT11 is *S. fulvissimus* DSM 40593 (Myronovskyi et al., 2013), which shared a 16S rRNA similarity of 99.38% with DUT11. Furthermore, the OAT heat map (**Figure 2B**) also showed that *S. fulvissimus* DSM 40593 is the closest strain from genome scale, and the ANI (average nucleotide identity) score is 90.4% between the two strains. The second closest strain is *S. griseus* NBRC 13350 (87.9% similarity). The genomes of the rest strains are 78–83% similar to that of *Streptomyces* sp. DUT11.

Analysis of the BGCs and Genome Mining for Discovery of Tunicamycins

There are 33 BGCs including at least seven non-ribosomal peptide synthases (NRPSs), five polyketide synthases (PKSs), six post-translationally modified peptides (PTMPs), and

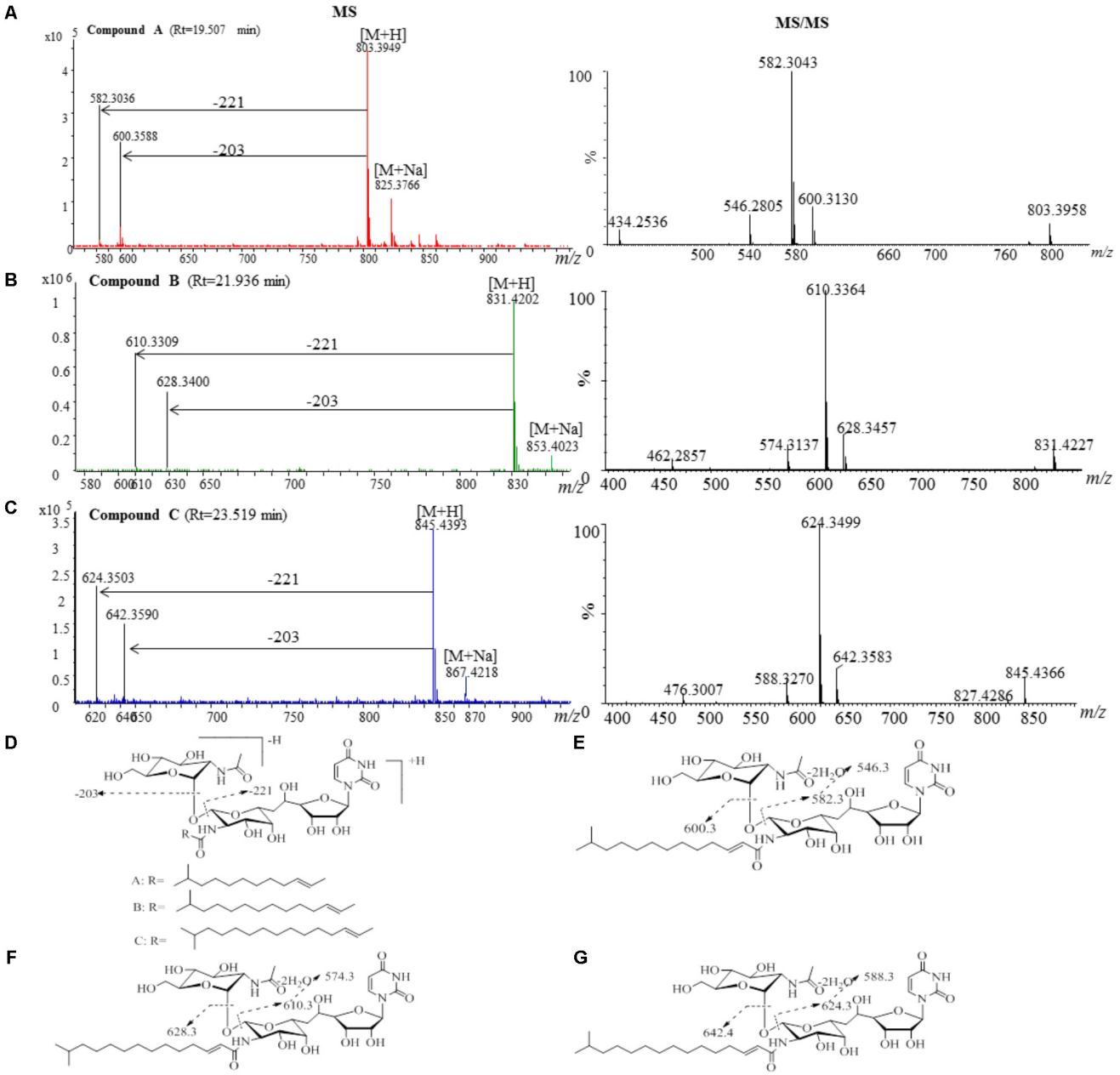

FIGURE 6 | MS and MS/MS analysis of the purified compounds A, B and C. **(A)** The component of compound A generates a $[M + H]^+$ ion at *m/z* 803.3926, which is fragmented into main ions of 600.3, 582.3, 546.3, etc. **(B)** The component of compound B generates a $[M + H]^+$ ion at *m/z* 831.4141, which is fragmented into main ions of 628.3, 610.3, 574.3, etc. **(C)** The component of compound C generates a $[M + H]^+$ ion at *m/z* 845.4366, which is fragmented into main ions of 642.4, 624.3, 588.3, etc. **(D)** Voltage gradient-induced ions at $[M + H - 221]^+$ and $[M + H - 203]^+$ indicate fragmentations across the compound A, B and C. MS/MS fragmentation pattern of the compound A **(E)**, compound B **(F)**, and compound C **(G)**.

five terpene BGCs in the genome sequence. Furthermore, BGCs involve in the biosynthesis of ectoine, siderophore, bacteriocin, and butyrolactone which are common in most of the actinobacteria were also identified in DUT11 (**Table 1**).

Biosynthetic gene clusters potentially involved in biosynthesis of three known compounds, namely, tunicamycin, nonactin, and medermycin were focused. Among these BGCs, Cluster 18 was annotated as tunicamycin BGC by analyses with antiSMASH. Through further comparison with other tunicamycin BGCs in different strains, the cluster in DUT11 has the closest similarity to the gene cluster in *S. chartreuis* (Chen et al., 2010) (**Figure 3A**), and all the genes share around 94–98% identities with the corresponding genes in *S. chartreuis* (Supplementary Table S2). Strain DUT11 also lacks *tunM* and *tunN* which encodes methyltransferase and NUDIX hydrolase, respectively,

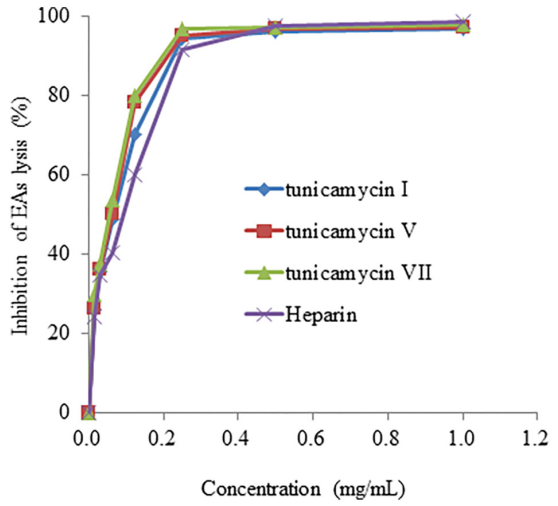

FIGURE 7 | The anti-complement activity of tunicamycin I, V and VII. A hemolytic assay was used to determine the inhibition of complement activation in the classical pathway.

but these genes are not essential for tunicamycin biosynthesis (Chen et al., 2010).

We further analyzed tunicamycin production in both agar culture and liquid fermentation. Firstly, we identified a cluster of tunicamycin analogs in GNPS map which has been annotated by GNPS (Wang et al., 2016). Depending on the reported structures and molecular weight range, at least eleven tunicamycin analogs with different lengths of alkane tails ranging from 13 to 18 carbons were identified from the A1 agar culture of DUT11 (**Figure 3B**) by comparing the MS spectrum with *m/z* shifts (Supplementary Figure S3).

Discovery of Tunicamycins as Anti-complement Agents From *Streptomyces* sp. DUT11

Anti-complement activities of the extracts from both mycelia and fermentation broth of *Streptomyces* sp. DUT11 were detected. As shown in **Table 2**, when different culture media were compared, the highest anti-complement activities were observed using the M33 medium. In addition, similar anti-complement activities were observed for the extracts of supernatant and mycelia grown in M33 fermentation medium.

We further isolated active fractions from the liquid culture using the M33 medium. The extracts of mycelia and supernatant were concentrated to yield 10 g and 8.5 g. The two organic extracts were separated by C18 column with H_2O/MeOH (v/v, 100:0, 70:30, 50:50, 30:70, 0:100), to afford five fractions. The fractions 3, 4, and 5 from the crude extract of supernatant eluted using 50%, 70%, and 100% methanol showed good anti-complement activities, which were 58.9%, 77.1%, and 70.4%, respectively. Similarly, the fractions 3′, 4′, and 5′ eluted using 50%, 70%, and 100% methanol from the crude extracts of mycelia showed better anti-complement activity, which were 50.1%, 86.03%, and 58.6%, respectively. We deduced that the strain DUT11 can produce a variety of substances with anti-complement activity (**Figures 4A,B**).

The active fractions 4, 4′, 5, and 5′ were further analyzed by HPLC, and tunicamycins were detected. The profile of tunicamycin standard was presented in **Figure 5A**. Tunicamycin analogs compound A (retention time, Rt: 19.5 min), compound B (Rt: 21.9 min), and compound C (Rt: 23.5 min) were detected (**Figures 5B,C**) in the active fractions. In the tunicamycin standard we purchased, only two tunicamycin analogs, namely tunicamycin V and VII, which are corresponding to compounds B and C, were present (**Figure 5A**). MS/MS analysis of tunicamycin V and VII was shown in **Figures 5D,E**. We further purified the three tunicamycin analogs in the active fractions. Compound A gives $[M + H]^+/[M + Na]^+$ ions at *m/z* 803.3949/825.3766 (calcd. for $C_{36}H_{59}N_4O_{16}$: 803.3921, ppm: 3.49; $C_{36}H_{58}N_4O_{16}Na$: 825.3740, ppm: 3.15). Compound B gives $[M + H]^+/[M + Na]^+$ ions at *m/z* 831.4202/853.4023 (calcd. for $C_{38}H_{63}N_4O_{16}$: 831.4234, ppm: −3.84; $C_{38}H_{62}N_4O_{16}Na$: 853.4053, ppm: −3.52) and compound C gives $[M + H]^+/[M + Na]^+$ ions at *m/z* 845.4393/867.4218 (calcd. for $C_{39}H_{65}N_4O_{16}$: 845.4390, ppm: 0.35; $C_{39}H_{64}N_4O_{16}Na$: 867.4210, ppm: 0.92), respectively. The peaks generated by MS/MS analysis showed that the main fragment ions are at 600.3, 582.3, and 546.3; 628.3, 610.3, and 574.3; as well as 642.4, 624.3, and 588.3, respectively (**Figures 6A–C**). Fragmentation of molecular adduct ions give rise to $[M + H\text{-}221]^+$ and $[M + H\text{-}203]^+$ ions across the tunicamycin α β-1, 1′-glycosidic bonds to generate stable deglycosylated species, the relative masses of which are diagnostic of the attached *N*-acyl group (-R) (**Figure 6D**). Comparing with the structural spectroscopic data in the literature (Tsvetanova and Price, 2001; Chen et al., 2010) and the tunicamycin standard, we confirmed that compounds A, B, and C are tunicamycin I, V, and VII (**Figures 6E–G**), respectively. The other compounds in these active fractions are being investigated in our ongoing work.

We further confirmed anti-complementary activities of the isolated tunicamycins produced by *Streptomyces* sp. DUT11. As shown in **Figure 7**, the concentrations that resulted in 50% hemolysis inhibition (CH_{50}) of tunicamycin I, tunicamycin V, and tunicamycin VII were 0.071 ± 0.01 mg/mL (0.088 ± 0.012 mM), 0.060 ± 0.009 mg/mL (0.072 ± 0.011 mM), and 0.045 ± 0.009 mg/mL (0.053 ± 0.011 mM), respectively. The CH_{50} of the positive control heparin was 0.115 ± 0.05 mg/mL. The CH_{50} of tunicamycin standard V and VII were 0.059 ± 0.008 mg/mL (0.071 ± 0.009 mM), and 0.046 ± 0.009 mg/mL (0.054 ± 0.011 mM), respectively. This is the first time that tunicamycins are proved to have anti-complement activities.

Other BGCs in *Streptomyces* sp. DUT11

Cluster 26 was originally identified as type II PKS gene cluster by antiSMASH. With further manual comparison, we found that the gene cluster is close to that of nonactin gene cluster from *S. griseus* ETH A7796 (Supplementary Figure S4A) (Walczak et al., 2000). Most of the genes in Cluster 26 shared 85–90% identities with that in the nonactin BGC (Supplementary Table S3). However, there is no counterpart of *non*I (AAD37458.1), which is a putative acetoacetate reductase in this gene cluster. In the

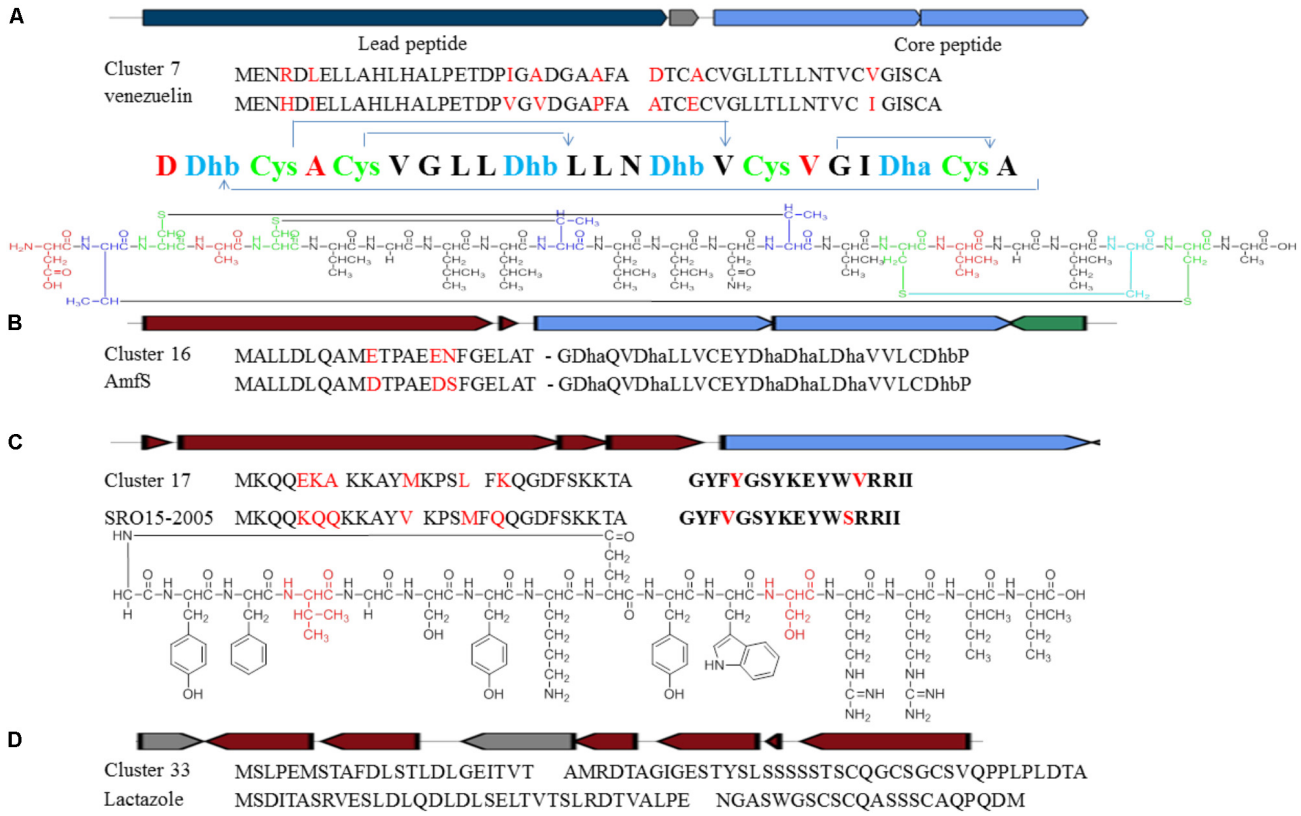

FIGURE 8 | Identification of post-translationally modified peptide class BGCs in *Streptomyces* sp. DUT11. **(A)** Comparison of Cluster 7 with the venezuelin peptide gene cluster. **(B)** Comparison of Cluster 16 with the AmfS gene cluster. **(C)** Comparison of Cluster 17 with the lassopeptide SRO15-2005 gene cluster. **(D)** Comparison of Cluster 33 with the lactazole gene cluster.

primary search in the GNPS annotation, only monactin was found annotated in one molecular cluster. Subsequently, based on the information from GNPS and MS spectra comparison, seven cyclized nonactin analogs were found around the monactin node, including nonactin, dinactin, trinactin, and tetranactin (Smith et al., 2000) (Supplementary Figures S4B–D). Besides, several hydrolyzed liner nonactin analogs were also observed in the same molecular cluster. However, no anti-complement activity of nonactin was detected under our assay conditions in this study (data not shown).

Cluster 21 is another type II PKS BGC in DUT11, which has 66% similarity to that of the medermycin BGC from *Streptomyces* sp. K73 (Ichinose, 2003). The organization of Cluster 21 is also similar to the medermycin BGC. Nevertheless, the significant difference between the two BGC is that the two genes encoding phosphopantetheinyl transferase and putative carbohydrate kinase, respectively, are not present in Cluster 21. The other parts of the genes shares around 70–80% similarities between Cluster 21 and medermycin BGC (Supplementary Table S4). Due to the inability to get the medermycin standard sample, we did not test the anti-compliment activity of this compound.

Cluster 7 probably produces a lantibiotic peptide with four S-S cycles. The peptide sequence including leader peptide and core peptide is quite similar to the reported venezuelin peptide sequence (Kersten et al., 2011). However, five amino acid in the leader peptide and three in the core peptide are different (**Figure 8A**). The putative product of *ven*L in the reference BGC includes both protein kinase C (PK C) like superfamily domain and lanC-like superfamily domain. Nevertheless, DNA sequences encoding such two domains are divided into two separate genes in Cluster 7. The domains are highly conserved with around 72% similarities although the intergenic region is quite different.

Cluster 16 is a class III lantipeptide BGC and quite similar to AmfS BGC (**Figure 8B**), the product of which is a morphogen, a biological surfactant which positively regulates the formation of erect aerial mycelium (Ueda et al., 2002; Willey and Gaskell, 2011). The five genes involved in the biosynthesis share around 80% similarities with the reference ones. Compared to AmfS BGC, the core peptide encoded by related gene in this cluster is the same but the leader peptide contains three mutations.

Cluster 17 is a lassopeptide BGC and close to SRO15-2005 BGC (Maksimov et al., 2012) (**Figure 8C**). There are only two amino acid changes in the core peptide, but there are six amino acid differences in the leader peptide. The gene construction of Cluster 33 is partially close to lactazole BGC and might be

TABLE 3 | The anti-complementary activities of other compounds on the classical pathway (CH_{50})*.

Compound	Anti-complementary activity (CH_{50} mM)	Source	Reference
Stigmasta-4-ene-3β, 6β-diol	0.060 ± 0.020	*Viola kunawarensis*	Wang et al., 2017
Saringosterone	0.080 ± 0.030		
Aurantiamide acetate	0.020 ± 0.010		
Solalyratin B	0.050 ± 0.020		
Machicendonal	0.040 ± 0.009	*Helicteres angustifolia*	Yin et al., 2016
(*7S*,*8R*)-dihydrodehydrodiconiferyl Alcohol	0.009 ± 0.002		
Fifteen cycloartane triterpenes	0.120 to 0.467	*Beesia calthaefolia*	Mu et al., 2016
Eleven phenolic compounds	0.113 to 1.210	*Viola tianshanica*	Qin et al., 2015
Isomangostanin	0.032 ± 0.009	*Garcinia mangostana*	Quan et al., 2010
Garcinone E	0.012 ± 0.012		
(*3S*)-falcarinol	0.087	*Dendropanax morbifera*	Chung et al., 2011
(*3S*, *8S*)-falcarindiol	0.015		
(*3S*)-diynene	0.040		
Complestatin	0.0003	*Streptomyces lavendulae* SANK 60477	Kaneko et al., 1989
Tunicamycin I	0.088 ± 0.012	*Streptomyces* sp. DUT11	This study
Tunicamycin V	0.072 ± 0.011		
Tunicamycin VII	0.053 ± 0.011		

**CH*$_{50}$ *means the concentrations that resulted in 50% hemolysis inhibition.*

a lassopeptide BGC hybrid with an additional NRPS and PKS (Hayashi et al., 2014) (**Figure 8D**). It is still not clear how the PKS and the NPRS affect the formation of the final products. The precursor peptides are quite different between the two BGCs, indicating that strain DUT11 has the potential to produce novel lassopeptides.

In addition, there are quite abundant BGCs of PTMPs in the DUT11 genome. Two of the tree lantibiotic BGCs and one lassopeptide found their close reference sequences which would help to identify the structures. Two more thiopeptide BGCs were identified as novel ones and the main backbone could be predicted.

Cluster 3 was supposed to be PKS I-NRPS hybrid BGC. There should be 7 PKS modules in this gene cluster and three A-domains of NRPS. This is the biggest PKS related BGC in the DUT11 genome. Through comparison and analysis, we found that there might be several key domains lost in the modules, and the backbone based on the function prediction was speculated. Another type III PKS BGC Cluster 6 might only include 2 genes and shared 71% similarities to naringenin BGC (Alvarez-Alvarez et al., 2015). The rest one was Cluster 29 and is a small type I PKS-NRPS hybrid gene cluster. This BGC only contains one PKS module including KS, AT, DH, KR, and ER domains and one A-domain responsible for ornithine. The BGC construction is quite close to the BGC class of polycyclic tetramate macrolactams including SGR PTMs BGC (Luo et al., 2013) and frontalamides BGC (Blodgett et al., 2010). Furthermore, eight proposed NRPS related BGCs were observed in the DUT11 genome, even though most of those only contained small NRPS genes. Cluster 4 and Cluster 5 were found to be quite close to BGC of griseobactin (Patzer and Braun, 2009) and coelichelin (Bentley et al., 2002), respectively. The predicted structures should be the same with reference compounds. Cluster 14 was found to be able to synthesize a four amino acid peptide backbone, namely, gly-ser-ser-ser. Cluster 24 was supposed to be a NRPS-PKS hybrid BGC which contains five A-domains coding leu-ser-dhb-cys-gly and one putative PKS sets. Further analysis of expression of these BGCs and their corresponding metabolites will provide functional insights of the *Streptomyces* sp. DUT11 genome.

DISCUSSION

Marine streptomycetes are rich sources of various secondary metabolites for drug discovery. In this study, we report the biosynthetic potential of the marine strain *Streptomyces* sp. DU11, which was revealed by analysis of its genome sequence. We found that genome sequences of many species having close phylogenetic relationship with stain DUT11 are still not available. Therefore, the available genome information reported in this study will assist the studies of these closely related species.

Complement system plays important roles in defense of invading microorganisms, clearance of damaged cells, adaptive immunity, and tissue regeneration (Hourcade et al., 2016). However, inappropriate and excessive activation of complement system will cause tissue damage diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic inflammatory response syndrome (SIRS), and acute respiratory distress syndrome (ARDS) (Qu et al., 2009). Anti-complement compounds are potential drugs to cure disorder of complement system, but so far such agents are mainly from plants rather than microbial source (Kaneko et al., 1989). Tunicamycins are a family of nucleoside antibiotic consisting of uracil, *N*-acetylglucosamine, a unique 11-carbon 2-aminodiadose sugar (tunicamine), and an *N*-acyl chain with variable lengths (Tsvetanova and Price, 2001; Price and Tsvetanova, 2007). Tunicamycins target bacterial cell wall biosynthesis by inhibiting

early stage of peptidoglycan biosynthesis (Winn et al., 2010). They are inhibitors of eukaryotic protein *N*-glycosylation to induce endoplasmic reticulum stress and consequently become drugs to study apoptotic cell death related diseases (Cóppola-Segovia et al., 2016; Guo et al., 2017). The antagonistic activity of tunicamycin against *Bacillus subtilis* was known (Chen et al., 2010). In the previous study, tunicamycin was proved to be able to block the glycosylation on the synthesis of pro-C4, C2 and factor B and inhibited the secretion of these proteins, which led to decrease of the activity of the complement system, and the study was performed using tissue culture of guinea-pig peritoneal macrophages (Matthews et al., 1982), where the authors incubated for 6 h in the experiment. However, in our current study, we added tunicamycins directly to combine complement proteins *in vitro* to inhibit the complement complex activity, and our assay is more rapid (less than 1 h). We found that tunicamycin I, V and VII have anti-complement activity comparable to, if not stronger, than other natural anti-complementary agents (**Table 3**). Although complestatins show better anti-complement activity, these compounds have very poor solubility. Therefore, searching for new microbial-derived anti-complement agents are of great interest. Our results suggest that other compounds with known structures may also be explored to their anti-complement activities.

Nonactin and related analogs are a central class of macrocyclic ionophores consisting of 32-membered ring (a cyclodotricontane) built with 24 carbon and 8 oxygen atoms (Martinez-Haya et al., 2017). These compounds show antibiotic, antitumor and anti-virus activities and are also widely used for the preparation of ion-selective electrodes and sensors (Zhan and Zheng, 2016). Although production of nonactin has been attempted through chemical synthesis and optimization of biosynthesis, the yields are not satisfied so far (Zhan and Zheng, 2016). Therefore, manipulation of strain DUT11 may provide alternative source of nonactin and its analogs.

Medermycin is featured with a fused three-ring structure composed of a benzene ring, a quinone and a stereospecific pyran ring, and possesses significant antitumor activities against many types of cancer cells as well as antibacterial activity (He et al., 2015; Lü et al., 2015). Our studies indicate that DUT11 is a promising producer of medermycin for drug discovery.

Genome mining and metabolite analysis indicates that strain DUT11 has great potential in secondary metabolite production. Besides the production of tunicamycin, nonactin and medermycin analogs with known structures, many other BGCs in strain DUT11 may produce novel metabolites. For example, there are quite abundant PTMP BGCs in the genome sequence of DUT11. PTMPs are ribosomal peptides with various activities including antimicrobial, antitumor and antivirus activities (Arnison et al., 2012). We have found that the extracts of mycelia and supernatant of strain DUT11 have good antifungal and antibacterial activities (Supplementary Figure S5), and the novel PTMPs would be further explored to produce such bioactive compounds. Furthermore, up to four NRPS BGCs, three PKS BGCs and four terpene BGCs are quite different from the known BGCs in the literature, and novel metabolites may be produced by further genome mining.

From the phylogenetic tree based on 16S rRNA sequences, strain DUT11 is close to *S. bacillaris*, and up to now, the only active compound was reported in *S. bacillaris* strain L159 (Hu and Macmillan, 2012), which revealed a novel peptide with autophagy inhibitory activity. There is no other report about the genome analysis and potential analysis of secondary metabolites produced by the closely related strains of DUT11. Therefore, exploration of the novel metabolites in DUT11 will provide more insights on the biosynthetic potential of marine microorganisms.

Streptomyces sp. DUT11 tolerates up to 10% NaCl and grows well in 3% NaCl in the liquid TSB medium (Supplementary Figure S6). Its fast growth and ability of producing various secondary metabolites make it suitable to be further explored to produce useful metabolites using sea water. Our studies here indicate that marine streptomycetes are valuable sources for developing anti-complement agents and other novel metabolites for biotechnological applications.

AUTHOR CONTRIBUTIONS

CS, F-WB, and X-QZ conceived the project. X-NX and L-YC performed the study. X-NX, L-YC, and CC analyzed the data and drafted the manuscript. X-QZ, CS, and Y-JT critically revised 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: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01318/full#supplementary-material

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Anti-microfouling Activity of *Glycomyces sediminimaris* UTMC 2460 on Dominant Fouling Bacteria of Iran Marine Habitats

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Discovery of environmentally safe anti-fouling agent is currently of considerable interest, due to the continuous impact of biofoulers on the marine habitats and the adverse effects of biocides on the environment. This study reports the anti-adhesion effect of marine living Actinobacteria against fouling strains isolated from submerged panels in marine environments of Iran. The extract of *Glycomyces sediminimaris* UTMC 2460 affected the biofilm formation of *Kocuria* sp. and *Mesorhizobium* sp., as the dominant fouling agents in this ecosystem, up to 93.2% and 71.4%, respectively. The metabolic activity of the fouler bacteria was reduced by the extract up to 17 and 9%, respectively. This indicated the bactericidal potency of the extract on cells in the biofilm state that enables the compound to be effective even once the biofilms are established in addition to the inhibition of biofilm initiation. Moreover, extra polymeric substance (EPS) production by fouling bacteria was reduced by 60–70%. The absence of activities against fouling bacteria in suspension and also the absence of toxic effect on *Artemia salina* showed the harmless ecological effect of the anti-microfouling extract on the prokaryotic and eukaryotic microflora of the studied Iran marine ecosystem. Metabolic profiling of *G. sediminimaris* UTMC 2460 revealed the presence of compounds with molecular formulae matching those of known anti-fouling diketopiperazines as major components of the extract. These results suggest that the extract of *Glycomyces sediminimaris* UTMC 2460 could be used as a potentially eco-friendly viable candidate in comparison to the synthetic common commercial anti-microfouling material to prevent the fouling process in marine habitats of Iran.

Keywords: surface microlayer, antifouling substances, fouling organisms, marine Actinobacteria, secondary metabolites, marine sediment

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INTRODUCTION

Colonization and successive overgrowth of epibiotic organisms on submerged surfaces is a natural process, known as biofouling. It causes ecological and economic problems for water-immersed man-made structures and marine sectors such as the loss of productivity in aquaculture, toxicity and side effects of chemical anti-fouling treatments on non-target organisms

(Thomas and Brooks, 2010), increased fuel costs for shipping, corrosion of metal pipes, ship and filtration system blockage; an increase in the weight of off-shore sea platforms, in addition to the costs associated with ongoing prevention and control (Garrett et al., 2008). Current anti-fouling strategies inhibit or eradicate the fouling organisms, by releasing high amounts of toxic chemicals into the environment. Therefore, compounds with high inhibitory performance and low side effects have a high priority for biofouling control.

The phenomenon of biofouling is preceded by biofilm formation. Microbial adhesion corresponding to the first stages of biofilm formation (microfouling) is a critical step in biofouling. Although the initial adhesion of microfoulers in primary steps is reversible and achieved by physical forces such as Brownian motion, gravity, and water flow, following the maturation of the biofilm their presence becomes permanent (Cao et al., 2011). Formation of slime layers comprising surface-attached bacteria (mainly *Proteobacteria*) and their extracellular polymeric substances provide the prerequisite conditions for further attachment and increases the settlement of macrofouling organisms (Donlan and Costerton, 2002).

Detailed knowledge about biofilms is crucial for understanding and preventing biofouling. However, it is more practical to inhibit fouling formation at the initial phase. To discover non-toxic and eco-friendly benign anti-foulants, organisms such as typical free fouling organisms with natural chemical defense mechanisms have been investigated (Abarzua et al., 1999; Piazza et al., 2011; Dobretsov et al., 2013).

The most serious problem encountered in the development of derivatives of natural anti-fouling agents is producing large amounts of anti-fouling compounds using these organisms (Fusetani, 2011). To overcome this problem, other sources such as easily cultivable terrestrial and marine microorganisms including bacteria, fungi, and cyanobacteria have been explored in recent years (Qian et al., 2009). Among various habitats, marine environments provide great opportunity for discovery of novel bioactive compounds, due to intact, underexplored and highly bio-diverse ecosystems, compared to terrestrial environments.

The Persian Gulf's marine environment is characterized by environmental extremes due to its location and bathymetry (Sale et al., 2011). The extreme salinity and temperature fluctuations of the Persian Gulf waters, which is one of the semi-enclosed marine systems, have created unique marine ecosystems (Bayani, 2016).

The phylum Actinobacteria is extraordinarily diverse and widely distributed in the marine environment (Maldonado et al., 2005; Pathom-Aree et al., 2006). Due to their large genome size and proven ability to produce structurally and functionally novel bioactive compounds, they hold a prominent position in biotechnological industries as sources of substitutes for commercial synthetic materials (Subramani and Aalbersberg, 2013). Therefore, marine Actinobacteria are a rich source of novel and biologically active products, with the potential to impede the adhesion of fouling organisms on artificial or natural surfaces. Herein, we investigated new marine Actinobacteria as a potential source of eco-friendly metabolites with anti-microfouling activity.

MATERIALS AND METHODS

Isolation of the Marine Microfouler Bacteria

Experimental Field for Biofilm Formation on Artificial Surfaces

In order to isolate dominant fouling bacteria from the Oman sea, four different artificial substrates including wood, aluminum, steel and fiberglass were mounted on polyethylene holders and exposed to seawater at 4 m depth and left for 14 days at 31°C to form microfouling films. Each structure was collected after a 1-day interval and transferred to the laboratory under sterile conditions. Physicochemical parameters such as temperature, salinity, pH and dissolved oxygen of the water sample were also measured during the formation of films.

Isolation of Initial Fouling Bacteria From Submerged Artificial Platforms

Each panel was washed with sterile artificial seawater to rinse the loosely attached cells, mud, and clay. Surface-attached bacteria (biofilm slime samples) were obtained through swabbing and scraping surfaces of the material, using sterile cotton swabs and razors. Media including Vaatanen nine salt solution (VNSS), Zobella marine agar (ZMA), and nutrient agar were used to cultivate microfouling bacteria at the early stage of the fouling process. Plates were incubated at 28°C for 5 days in the dark. The morphologically distinct biofilm bacterial colonies were purified and stored at 8°C for further analysis.

Assessment of Biofilm Formation by Crystal Violet Assay

The ability of the prevalent microfouling bacteria in the Oman sea to form static biofilms was determined in 96 well flat-bottom polystyrene microtiter plates (Biofil, China) as described (Stepanović et al., 2000). Each well of the microplates was filled with 300 µl of the test bacterial inoculums in nutrient broth supplemented with 1% glucose and 1% sucrose with a cell density of 0.2 at 600 nm, and incubated for 72 h at 37°C. Culture medium and planktonic cells, as well as loosely adhered bacteria, were removed by dual washing with sterile physiological saline. Firmly attached bacteria firstly were fixed with 250 µl methanol for 15 min, then stained with 250 µl crystal violet (0.05%, v/v). After 15 min, the stain was withdrawn and the wells were washed twice with sterile distilled water. Plates were air-dried at room temperature and the stain was ultimately solubilized by adding 250 µl of glacial acetic acid (33% v/v) for 15 min and its absorbance was recorded at 545 nm. Growth medium was included for sterility check. *Staphylococcus aureus* UTMC 1403 was used as positive control. Experiments were run in triplicate.

Selection of Optimum *in vitro* Condition for Biofilm Formation by Model Fouling Marine Bacteria

The impact of additive sugars in enhancing biofilm formation of two potent fouling bacteria was investigated by adding different concentrations (0.5 and 1% v/v) of three types of saccharide (glucose, sucrose, and fructose) alone and also in combined

regimes. Then, glucose and sucrose 1% regimes as the biofilm formation enhancers were included in two different nutrient growth media of BHI broth (Merck, Germany) and nutrient broth (Merck, Germany) and the biofilm formation support of these two modified media were compared.

Isolation of Marine Actinobacterial Strains

Sampling Sites and Sediment Samples Collection

The sediment samples were collected using a grab sampler from different Iranian sites of the Persian Gulf and the Oman sea, from a depth of 8–70 m between December 2013 and September 2014. Sediment samples were placed in sterile plastic bags and transported with ice pieces to the lab and were stored at 8°C for further studies.

Sample Preparation

Samples were heated at 50°C for 72 h to select heat-resistant spore-producing bacteria (including Actinobacteria) and decrease the colonization of unwanted fast-growing bacteria. Dried samples were then ground, sieved and transferred to sterilized falcon tubes for further analysis.

Isolation Condition of Actinobacterial Strains

Serial dilutions of 10^{-1} to 10^{-3} of each treated sediment sample were homogenized in 3% natural sea salt solution and 200 μl of each sample was spread evenly on modified oligotrophic isolation media (**Table 1**). The isolation media were appended with Oman sea salt to select indigenous marine dwelling bacteria. Moreover, all media were prepared in 1/5 diluted form to simulate the oligotrophic conditions of the marine ecosystem. Isolation of colonies was conducted from the 14th to the 28th day of incubation at 28°C. Individual colonies observed on plates were subcultured on ISP2 agar medium (malt extract, 10 g; yeast extract, 4 g; glucose, 4 g; calcium carbonate, 2 g; agar 14 g in 1 L of distilled water, pH 7.5) until pure colonies were obtained. Ultimately, purified isolates were stored at −20°C in half strength ISP2 media containing 30% glycerol for further studies.

Fermentation and Extraction of Secondary Metabolites

Bacterial precultures were prepared by inoculating patches (1 cm × 1 cm) of well-grown colonies in 50 ml ISP2 broth culture medium, followed by incubation at 28°C with shaking at 160 rpm for 48 h. The fermentation medium was then inoculated with 5% of actively growing seed culture. After 7 days of incubation at 28°C, extraction was performed by shaking with the same ratio of ethyl acetate for 1 h, followed by supernatant separation using centrifugation at 4,000 rpm for 10 min. The organic phase was evaporated to dryness and concentrated using a rotary evaporator (Heidolph, Germany) at 35°C to obtain the bacterial extract.

Anti-adhesion Activity in Presence of Crude Extract

To evaluate the inhibitory effect of the extracts on bacterial adhesion, 50 μl of each crude extract dissolved in methanol to a final concentration of 100 and 300 μg ml^{-1} was added to each well. 250 μl of fresh test bacterial cell suspension was then added to each well (final optical density of 0.2 at 600 nm) in nutrient broth media supplemented with 1% glucose and 1% sucrose, and the resulting mixtures were incubated at 37°C. After an optimal adherence time of 72 h, non-adherent bacteria in the spent medium were removed and the wells were washed twice with physiological saline. Fixation and staining steps were performed in the same manner using the crystal violet assay. The relative rate of adhesion to the bottom of wells in the presence of crude extract was calculated using the following equation

TABLE 1 | The modified media used in isolation of marine-derived Actinobacteria from sediment samples.

Medium	Components	Reference
*1/5 Glycerol asparagine agar (GA)	Glycerol, 2 g; asparagine, 0.5 g; K_2HPO_4, 1 g; NaCl, 0.5 g; $MgSO_47H_2$ O, 0.5 g; $FeSO_47H_2O$, 0.01 g; $CuSO_4$, 0.001 g; $MnSO_4$, 0.001 g; $ZnSO_4$, 0.001 g; Agar, 14 g and 1 L of seawater, pH 7.5	Chen et al., 2012
1/5 Vaatanen Nine-Salt Solution (VNSS)	Glucose, 0.1 g; peptone, 0.2 g; yeast extract, 0.1 g; soluble starch, 1 g; $FeSO_47H_2O$, 0.01 g; $Na_2HPO_4 \cdot 2H_2O$, 0.007 g; sea salt, 17.6 g; Na_2SO_4, 1.47 g; $NaHCO_3$, 0.08 g; KBr, 0.04 g; KCl, 0.25 g; $MgCl_2 \cdot 6H_2O$, 1.87 g; $CaCl_2 \cdot 2H_2O$, 0.41 g; $SrCl_2 \cdot 6H_2O$, 0.01 g; H_3BO_3, 0.01 g; agar, 14 g and 1 L of distilled water, pH 7.5	Heindl et al., 2012
1/5 "Reasoner's 2A (R2A)	Yeast extract, 0.1 g; bacteriologic peptone, 0.1 g; casein hydrolysate, 0.1 g; glucose, 0.1 g; soluble starch, 0.1 g; sodium pyruvate, 0.06 g; agar, 14 g and 1 L of seawater, pH 7.2–7.4	Heindl et al., 2012
Modified HV agar (Natural sea water)	Fish powder, 0.1 g; $CaCO_3$, 0.02 g; Na_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 1.7 g; $FeSO_4.7H_2O$, 0.01 g; Vitamin solution, 1 ml; agar, 14 g; 1 L of sea water, pH 7–7.5 Vitamin solution consist of (biotin, 200 mg; pyridoxine HCl, 500 mg; thiamine HCl, 500 mg; riboflavin, 1 g; nicotinamide, 1 g; P-aminobenzoic acid, 100 mg 1 L of distilled water	Sharma, 2014
Modified HV agar (Soil extract)	Fish powder, 0.1 g; $CaCO_3$, 0.02 g; Na_2HPO_4,0.5 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 1.7 g; $FeSO_4.7H_2O$, 0.01 g; Vitamin solution, 1 ml; agar, 14 g; 1 L of soil extract, pH 7–7.5	Ramesh and Mathivanan, 2009
Modified Zobella marine agar	Peptic digest of animal tissue extract, 30 ml; yeast extract, 1 g; ferric citrate, 0.1 g; NaCl, 19.45 g; $MgCl_2$, 8.8 g; Na_2SO_4, 3.240 g; sea salt, 1.8 g; KCl, 0.55 g; $NaHCO_3$, 0.16 g; KBr, 0.08 g; $SrCl_2$, 0.034 g; boric acid, 0.022 g; NH_4NO_3, 0.0016 g; Na_2HPO_4, 0.008 g; sodium silicate, 0.004 g; sodium fluorate, 0.0024 g; agar, 14 g; 1 L of distilled water, pH 7.2+_0.2	Satheesh et al., 2012

**All media are prepared in one-fifth diluted form.*

(Bakkiyaraj and Karutha Pandian, 2010).

$$\text{Adhesion (\%)} = \frac{(\text{Control OD} - \text{Treated OD})}{\text{Control OD}} \times 100$$

Microtiter wells containing bacterial cell suspension in nutrient broth medium without added extract were used as bacterial adhesion controls. In addition, the maximum percentage of methanol used for dilution of extracts and sterile nutrient broth medium was used as negative controls. Diuron (Sigma-Aldrich, United States), which is a commercially available anti-fouling agent, was used as a positive control (1 and 10 μg ml^{-1}).

Quantification of Extra Polymeric Substance (EPS) Content for Potent Biofilm-Forming Bacteria

Determination of EPS content of the selected marine fouling bacteria in the presence and absence of the extract at concentrations of 100 and 300 μg ml^{-1} was conducted by using the total carbohydrate assay, as follows. The culture broth from 72 h cultures of each microfouling bacterium was centrifuged at 14,000 × *g* and 4°C for 15 min to obtain the culture supernatant. The supernatant was passed through a 0.2 μm filter and the filtrate was mixed with ethanol at a ratio of 1:3, then left to precipitate at 4°C overnight. Settled EPS was collected by centrifugation at 10,000 × *g* and 25°C for 5 min, then dissolved in PBS and the concentrations of carbohydrates were determined with the modified phenol-sulphuric acid method using glucose as the standard (DuBois et al., 1956).

Anti-bacterial Activities of the Anti-microfouling Extract Against the Marine Biofilm Forming Strains

The most effective extract with biofilm inhibition activity was evaluated for its inhibitory effect on marine biofilm forming bacteria using the agar disk diffusion method as described by Kirby-Bauer (Hudzicki, 2009). Firstly, 20 μl of the extract stock solution in 1 ml of methanol was applied on a sterile disk and allowed to dry such that the final concentrations on each disk were 5, 15, and 100 μg ml^{-1}. The nutrient agar plates were seeded by spreading 100 μl of each test cell suspension (cell density of 0.2 at 600 nm), onto which the disks were placed and incubated at 35°C for 24 h. Standard antibiotic-containing disks [cefotaxime (30 μg), imipenem (10 μg), and ticarcillin (20 μg)] and filter paper disks soaked in methanol were used as positive and negative controls, respectively. Anti-microbial efficacy was determined by measuring the growth inhibition zone around the disks after 24 h.

Bacteriostatic and Bactericidal Activity of Anti-microfouling Extracts

The minimum inhibition concentration value of the effective extract with the highest anti-adhesion and anti-bacterial activity was determined by the microdilution method following the CLSI procedure (Wayne, 2017). Briefly, 20 μl of each microfouling strain suspension with a cell density of 0.2 at 600 nm was added to each well containing extract at concentrations of 6.25–400 μg ml^{-1}, along with an appropriate growth medium. Ciprofloxacin (0.25, 0.5, 1, 2, and 4 μg ml^{-1}) was used as a positive control and well plates containing only bacterial suspensions were considered as the negative control. Following incubation at 35°C for 24 h, the lowest concentration at which the bacterial growth was inhibited and the one at which there was no visible bacterial growth on the agar plate was recorded as the MIC and MBC values, respectively.

Quantification of Microfouler Metabolic Activity in Presence of Anti-microfouling Extract

The XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay was used to determine the bactericidal effects of the extract on single species biofilm populations as described (Padmavathi et al., 2014) with minor modifications. After biofilm formation of the tested bacteria in nutrient broth medium, the cell density was adjusted to 0.2 at 600 nm and combined with 50 μl extract solution to provide final concentrations of 50, 100, 200, and 300 μg ml^{-1} in a total volume of 300 μl per well. The wells were washed twice with 300 μl of sterile physiological saline. Then biofilms were incubated with 200 μl of XTT solution containing 150 mg XTT and 10 mg of phenazine methosulfate (Sigma-Aldrich) for 6 h in the dark, at 37°C at 120 rpm. The absorbance due to the formazan formed was measured at 490 nm using a MRP4+ microplate reader (Hyperion, England). Ciprofloxacin (2 and 4 μg ml^{-1}) was used as a positive bactericidal reference. Wells containing plain culture medium without biofilms were used as blanks and biofilm containing wells without extracts were used as negative controls. The results were expressed as a percentage of inhibited activity.

Eukaryotic Cell Toxicity Assay

Brine shrimp *Artemia salina* larvae were used as a model for detecting the toxicity of the extract. The anti-crustacean assay was performed in 24 well clear polystyrene plates (Meyer et al., 1982) in artificial seawater (3%) as the hatching solution. The extract was added at different concentrations (5, 10, 20 μg ml^{-1}) and incubated at 25°C in the dark with 15–20 larvae in each well. Artificial seawater containing 1% DMSO and potassium dichromate solution (0.5 M), were used as the negative and positive controls, respectively. The Mortality rate was calculated after 24 h according to the following formula:

$$M = \frac{A - B - N}{G - N} \times N - 0 \qquad \textit{Viability} = 100 \times (1 - M)$$

Where A = dead larvae number after 24 h, N = dead larvae number initially, M = dead larvae percentage after 24 h, G = total number of larvae, and B = average number of dead larvae in negative control after 24 h.

Hemolytic Activity Assay

The lytic activity of the extract was measured against human red blood cells. The red blood cells (10%) were separated by

centrifugation at 3,500 rpm for 15 min and washed with PBS buffer three times. The most potent biofilm inhibiting extract was prepared at final concentrations of 50, 100, 200, 300, 400 $\mu g\ ml^{-1}$ and added to the red cell suspension in PBS buffer. Mixtures of blood and extract were incubated at 37°C for 1 h. The supernatants were collected by centrifugation at 2,500 rpm for 5 min and optical densities were measured at 545 nm. Ferrous sulfate solution at a concentration of 65 mM was used as a positive control.

Evaluation of Anti-microfouling Activity of the Actinobacterial Extracts in Field Experiments

The anti-adhesion activity of the extracts on two dominant fouling bacteria of the ecosystem was tested in field conditions following a modification of a literature method (Schwartz et al., 2017). Gels were prepared by adding 0.75 g of Gelrite® (Dokhefa, Poland) to 50 ml of distilled water and the pH was adjusted to 7.1. The mixture was sterilized and then cooled to 70°C. Extracts were added to the liquid gel solution at a final concentration of 100 $\mu g\ ml^{-1}$ and poured into sterile 48-well flat-bottom polystyrene microtiter plates. The same amount of methanol alone was added to Gelrite in the negative control well and Diuron (1 $\mu g\ m^{-1}$) was used as a positive control. Bacterial strains were grown on nutrient broth supplemented with 1% glucose and 1% sucrose at 28°C and 120 rpm. The cells were collected once stationary phase had been reached. After centrifugation, cells were suspended in sterile artificial seawater at an optical density of 0.5 at 600 nm. Wells were seeded using 1 ml of fouling bacterial suspension and the resulting cultures were grown for 7 days at 32°C. Non-adherent bacteria were removed and the adhering bacteria were washed twice using physiological saline. The attached bacteria were moved from the gel surface into aqueous solution by sonicating at 40 KHz for 1 min using Elmasonic P sonicator (Elma, Germany). Finally, the adhesion rate of attached bacteria on the gel surface (n = 3) of each treatment and control were quantified spectrophotometrically at 600 nm.

Characterization and Identification of Actinobacterium With Anti-microfouling Activity

The potent anti-adhesion producing strain was characterized using morphological, physiological, and molecular approaches.

Morphological Identification

Cultural characteristics based on observation of macro-morphology of the strain grown on ISP2 medium after incubation at 28°C for 14 days were recorded. Furthermore, the spore-bearing hypha and spore chains were directly examined using bacteria grown on ISP2 medium using the cover-slip technique and a Zeiss Merlin field emission scanning electron microscope (SEM), applying the SEM Smart software version 5.05.

Biochemical and Physiological Characteristics

Physiological characteristics, including decomposition of xenobiotics, enzyme activity, and utilization of different sole carbon and nitrogen sources were determined as described (Goodfellow et al., 2012; Stackebrandt, 2014). Growth at different temperatures (15, 28, 37, and 42°C), and pH values (4.0–12.0 at intervals of 2 pH units), and NaCl tolerance at concentrations ranging from 0 to 10% with intervals of 2.5% NaCl (w/v) were assessed after incubation at 28°C for 7–14 days on ISP2 medium. In addition, the chemotaxonomic marker diaminopimelic acid (DAP) in cell wall hydrolysates was analyzed using the thin layer chromatography (Staneck and Roberts, 1974).

Molecular Identification of the Most Potent Anti-microfouling Actinobacteria and Selected Dominant Biofilm Forming Bacterial Strains

The 16S rRNA genes were amplified using a set of universal primers (**Supplementary Table 1**). Amplified chromosomal DNA obtained from PCR reactions was purified using a PCR purification kit (NucleoSpin® Gel and PCR Clean-up). The 16S rRNA gene sequences were blasted against the EzBioCloud database (Yoon et al., 2017).

Metabolic Profiling of the Most Potent Strain With Anti-microfouling Activity

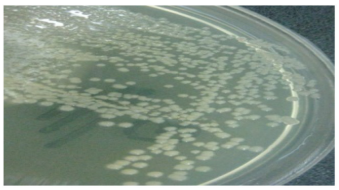
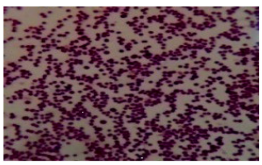
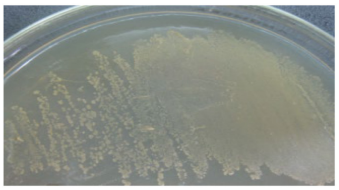
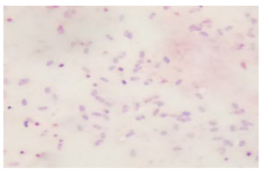
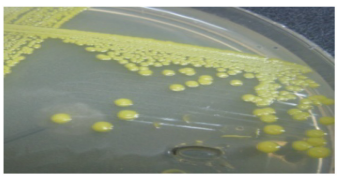
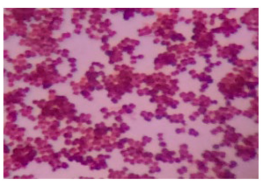
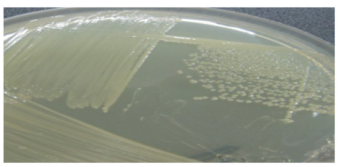
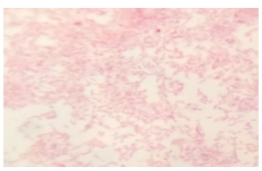
The identification of specialized metabolites in the extract of *Glycomyces sediminimaris* UTMC 2460 was performed using a UHPLC-ESI-Q-TOF instrument with the following specifications: a Dionex UltiMate 3000 UHPLC connected to a Zorbax Eclipse Plus C18 column (100 × 2.1 mm, 1.8 μm) coupled to a Bruker MaXis IMPACT mass spectrometer. The mass spectrometer was operated in positive ion mode with a scan range of 50–3,000 *m/z*. The solvent system of water (A)-acetonitrile (B), each supplemented with 0.1% formic acid, was used for chromatography. A gradient of 5–100% B over 30 min was applied at a flow rate of 0.2 mL min^{-1}. Calibration was performed with 1 mM sodium formate at the start of each run.

RESULTS

Isolation of Marine Fouling Bacteria From Submerged Platforms in Oman Sea

Marine fouling bacteria of the region were isolated from biofilms or microfouling shaped on four types of immersed panels. A slime layer was visually observed on the submerged surfaces from 24 h and reached approximate 99% coverage in the second week of the immersion. Among various apparent colonies on three different nutritious media, four prevalent macro-morphological strain types were selected based on repetition on different platforms and successive detection on the isolation medium. Microscopic and macroscopic images of the prevalent initiators of the fouling phenomena in the Oman sea are shown in **Table 2**.

TABLE 2 | Microscopic and macroscopic characteristic of the dominant marine biofilm bacteria obtained from Oman sea.

Strain	Macroscopic image	Microscopic image	Artificial surface types
Psychrobacter sp. UTMC 2516			Woody panel
Bacillus sp. UTMC 2517			Steel panel
Kocuria sp. UTMC 2449			Fiberglass panel
Mesorhizobium sp. UTMC 2518			Aluminum panel

Biofilm Forming Ability of the Selected Fouling Bacteria

The biofilm formation intensity of the isolated fouling strains was evaluated via the crystal-violet colorimetric assay. To compensate for background absorbance, the OD of the sterile medium with fixative and dye was recorded and subtracted from the results. The absorbance values of the crystal-violet served as an indicator of the total biomass of attached bacteria at the bottom of microplate wells. Bacterial strains didn't show significant differentiation in biofilm formation ability at a low inoculum concentration (5 μl), while their ability to form biofilms varied at higher levels of inoculation (15 and 50 μl). Among four dominant fouling bacteria, two strains of *Kocuria* sp. UTMC 2449 and *Mesorhizobium* sp. UTMC 2518 showed the highest biofilm formation ability, which corresponded to an increase in optical density up to 0.7 at 600 nm (**Figure 1**). These two strains were selected as the primary fouling bacteria and used as the test microfoulers in further experiments. In this experiment *Staphylococcus aureus* UTMC 1403 was used as positive control strain due to its strong ability to form biofilms.

Optimum *in vitro* Conditions for Biofilm Formation of Main Fouling Bacteria

The effects of growth medium supplementation with glucose, sucrose, and fructose were evaluated on the enhancement of biofilm formation by fouling bacteria. The results indicate that glucose and sucrose were more efficient sugars in comparison to fructose (**Supplementary Figure 1**), and the combination of glucose and sucrose 1% was more effective in the formation of the biofilm than each of them alone (**Supplementary Figure 2**). In addition, there was no significant difference between BHI and nutrient broth medium supplemented with 1% glucose and sucrose (**Supplementary Figure 3**). Hence, nutrient broth medium supplemented with 1% glucose and sucrose was selected as the medium supporting biofilm formation by *Kocuria* sp. UTMC 2449 and *Mesorhizobium* sp. UTMC 2518 for *in vitro* growth.

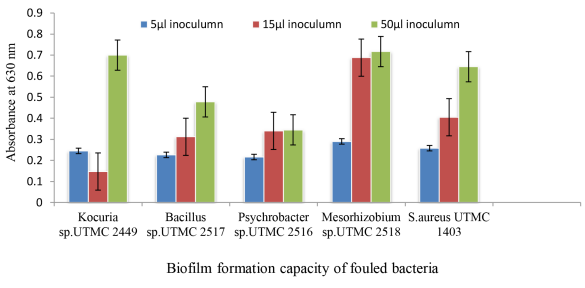


FIGURE 1 | The biofilm formation of dominant isolated fouling bacteria from immersed artificial platforms in Oman sea detected by crystal violet assay.

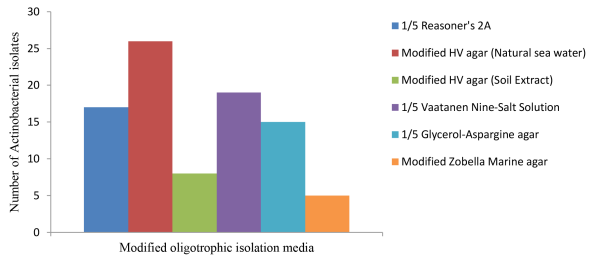


FIGURE 2 | Comparison of the isolation media versus the number of recovered strains. Modified HV medium containing artificial seawater supported the highest number and diversity of the Actinobacteria.

Sediment Sample Collection and Isolation of Marine Actinobacteria

A total of 50 sediment samples were collected from different sites between December 2013 and August 2014. The seawater physicochemical conditions at the test site were recorded. The average temperature, salinity level, pH and dissolved oxygen level at the sampling site were 32°C, 27.72 psu, 7.15 and 7.12 μmol l^{-1}, respectively. A total of 90 strains were isolated from five oligotrophic isolation media. Modified HV medium containing artificial seawater provided the highest number and diversity of recovered strains with the minimum contaminants, followed by 1/5 Vaatanen Nine-Salt Solution and 1/5 R 2A (**Figure 2**).

Production and Extraction of Metabolites From Marine Actinobacterial Strains

Among a total of 90 isolated actinobacterial strains from the sediment samples, 10 morphologically distinct isolates were selected for evaluation of the anti-microfouling activity of their metabolite extracts. Concentrated crude extracts were preserved in the University of Tehran bio-compound Collection.

Anti-adhesion Activity of the Extracts

The ability of the selected marine actinobacterial extracts to attenuate biofilm formation by potent fouler bacteria from the Oman sea (*Kocuria* sp. and *Mesorhizobium* sp.) is shown in **Figures 3A,B**. The final results are expressed as the percentage of the reduction of adhered cells compared to a control that was not exposed to the extracts. Extracts with the highest anti-adhesion property at low concentration were selected for further analysis. All five initially selected extracts showed a concentration-dependent decrease in biofilm formation with the maximum biofilm inhibition activity at the lowest concentration value of 100 μg ml^{-1}. The selected extracts caused 18–91% and 21–71% reduction in biofilm biomass production by *Kocuria* sp. UTMC 2449 and *Mesorhizobium* sp. UTMC 2518, respectively, at the minimum concentration of 100 μg ml^{-1}. Most of the extracts exhibited stronger biofilm formation suppression against both fouling bacteria at the higher concentration of 300 μg ml^{-1}, as shown in **Figures 3A,B**. Among five potent extracts, the extract from *Glycomyces* sp. UTMC 2460 showed the maximum anti-adherence activity against the fouling bacteria.

Effect of Bacterial Extract on EPS Production of Microfouling Bacteria

A considerable decrease in EPS production by fouling bacteria in the presence of the extract from *Glycomyces* sp. UTMC 2460 was detected. The carbohydrate concentrations of the EPS extracted from *Kocuria* sp. UTMC 2449 suspensions were 92.19 μg ml^{-1} and 62.05 μg ml^{-1} in the presence of 100 and 300 μg ml^{-1} of the extract, respectively, while those in EPS isolated from *Mesorhizobium* sp. UTMC 2518 were 52.15 and 55.32 μg ml^{-1}, respectively. The maximum reduction in EPS production (approximately 70%) was observed in *Kocuria* sp. UTMC 2449 in the presence of crude extract (**Figure 4**). The extract could diminish the EPS production in fouling bacteria (62–73%) with a similar efficiency of 1 and 10 μg ml^{-1} diuron on *Mesorhizobium* sp. and *Kocuria* sp., respectively.

Antibacterial Effect of the Extract Against Main Fouling Bacteria

The antibacterial activity of the extract against the pelagic form of fouling bacteria was investigated due to its probable effect on the balance of the ecosystem. The zone of inhibition produced by the extract from *Glycomyces* sp. UTMC 2460 against *Kocuria* sp. UTMC 2449 at concentrations of 15 and 100 μg ml^{-1} was 3 mm and 8 mm, respectively (**Supplementary Figure 4**). No anti-bacterial activity for the extract was detected against *Mesorhizobium* sp. UTMC 2518 at concentrations up to 100 μg ml^{-1}.

Determination of the MIC and MBC Values of the *Glycomyces* sp. Extract

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the extract from *Glycomyces* sp. UTMC 2460 were determined for the fouler strains. The extract did not show growth inhibitory activity on fouling bacteria at concentrations less than 400 μg ml^{-1} and in fact the extract could not inhibit the fouler strains at low concentrations which has its anti-fouling effect. 100% bacteriostatic activity of the positive control against *Kocuria* sp. and *Mesorhizobium* sp. was exhibited at a concentration of 1 μg ml^{-1} for ciprofloxacin. The MBC value for ciprofloxacin was 2 μg ml^{-1}. The *Glycomyces* sp. UTMC 2460 extract shows no bactericidal activity against two potent fouler bacteria in the planktonic phase.

Bactericidal Effect of the Most Potent Extract Against the Fouling Bacteria in the Biofilm State

An XTT reagent incubation time of 6 h was found to be optimal since the color intensity did not change further after this period (data not shown). Evaluation of the viability and metabolic activity of *Kocuria* sp. UTMC 2449 and *Mesorhizobium* sp. UTMC 2518 in the presence of the *Glycomyces* sp. extract in the biofilm state revealed that *Kocuria* sp. UTMC 2449 was more susceptible to the extract at the concentration examined.

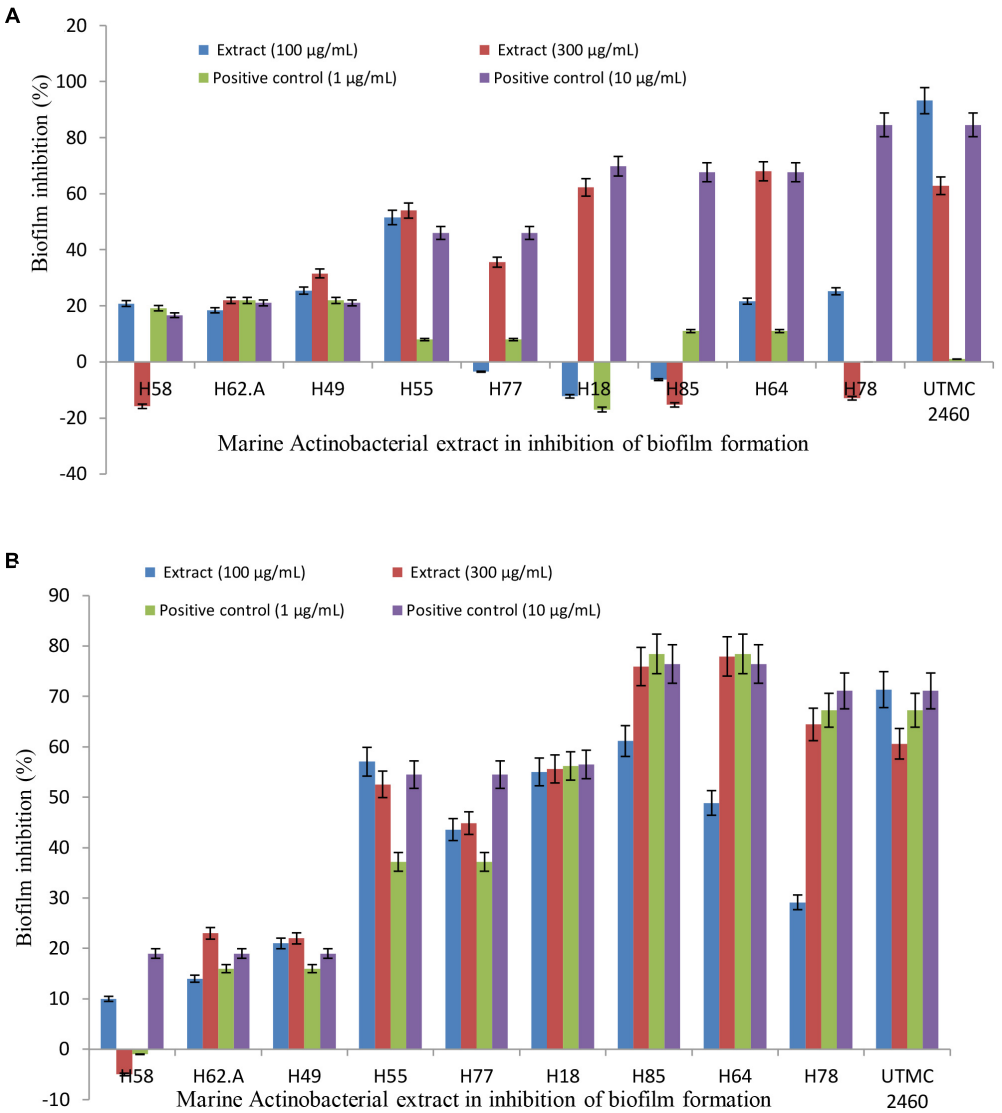


FIGURE 3 | Anti-adherence activity of *Glycomyces* sp. UTMC 2460 metabolite against dominant marine fouled bacteria. The graph illustrating the potential of selected marine Actinobacterial extracts (100 and 300 µg ml^{-1}) on the adhesion prevention of marine fouling bacteria: **(A)** *Kocuria* sp. UTMC 2449 and **(B)** *Mesorhizobium* sp. UTMC 2518 in crystal violet assay.

The metabolic activity at the minimum biofilm inhibition concentration of the extract (100 µg ml^{-1}) reduced by up to 17% and 9%, respectively (**Figure 5**), after 6 h incubation with XTT reagent. The bactericidal effect of the extract was observed during the early phase of growth, with a 1.23 and 0.95 log reduction for *Kocuria* sp. UTMC 2449 and *Mesorhizobium* sp. UTMC 2518, respectively. Furthermore, *Kocuria* sp. UTMC 2449 and *Mesorhizobium* sp. UTMC 2518 biofilms subjected to the positive control presented 1.43 and 1.17 log metabolic reduction, respectively.

Cytotoxicity Effect

The anti-crustacean assay provides an indication of the ecological safety of the anti-microfouling extracts. From the results obtained, it was inferred that all extracts expressing high anti-biofilm activity were not toxic toward *Artemia salina* larvae, because all exposed larvae were alive and motile after 24 h of incubation.

Hemolytic Activity of the *Glycomyces* Extract

The extract from the strain *Glycomyces* sp. UTMC 2460 had almost no or minimal (up to 8.8%) lytic effect at concentrations of 50, 100, and 200 µg ml^{-1} on human red blood cells (**Figure 6**). The biological safety of the most potent extract was inferred from its non-toxic effect on the blood cells. At the high concentrations of the extract (300 and 400 µg ml^{-1}) toxicity was observed, presumably due to minor components of the extract.

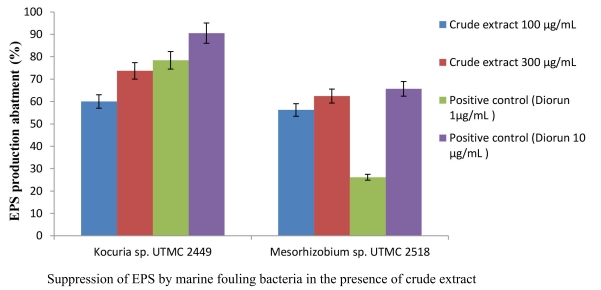


FIGURE 4 | Inhibitory effect of *Glycomyces* sp. UTMC 2460 extract on EPS production of fouling bacteria. Respective samples incorporated with "Diorun" (1 and 10 µg ml^{-1}) serve as controls.

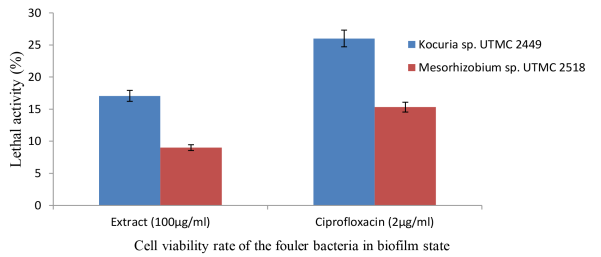


FIGURE 5 | The effect of extract on the viability of *Kocuria* sp. UTMC 2449 and *Mesorhizobium* sp. UTMC 2518 cells in biofilm state. The lethal activity of the *Glycomyces* sp. UTMC 2460 metabolite at the lowest biofilm inhibition concentration (100 µg ml^{-1}) was resembeled to ciprofloxacin as the control compound with bactericidal activity.

Anti-microfouling Activity of the Crude Extract in Simulated Field Conditions

The effect of the *Glycomyces* extract on the adhesion of fouling bacteria was investigated using *in vitro* conditions that mimic the natural conditions of the Oman sea in terms of pH, salinity and temperature. The cell density of *Kocuria* sp. UTMC 2449 on the gels in the presence of the extract and in the positive control did not differ from each other and cell attachment was reduced up to 37%. The extract exhibited lower suppression of the adhesion of *Mesorhizobium* sp. UTMC 2518 (31%) than Diuron (1 µg ml^{-1}). While the positive control showed a higher inhibitory effect on *Mesorhizobium* sp. UTMC 2518 (43%) than *Kocuria* sp. UTMC 2449 (36%) (**Figure 7**), the converse was observed for the extract.

Molecular Identification of the Most Prevalent Marine Fouling Bacteria From Artificial Platforms

Results obtained from BLAST analyses of the 16S rRNA sequences from the fouling isolates against the GenBank and EzBioCloud databases showed that they belong to four genera, including *Psychrobacter*, *Kocuria*, *Bacillus* and *Mesorhizobium* (**Table 3**). These sequences were deposited in the GenBank database under the accession numbers shown in **Table 3**.

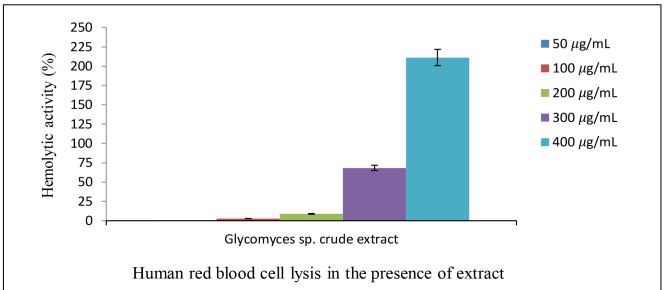


FIGURE 6 | Hemolytic activity of *Glycomyces* sp. UTMC 2460 crude extract on human red blood cell. Almost no or a low hemolytic effect was detected at low concentrations of the extract (50, 100, and 200 µg ml^{-1}). Hemolysis activity was evaluated in comparison to $FeSO_4$ solution (65 mM) as a positive control.

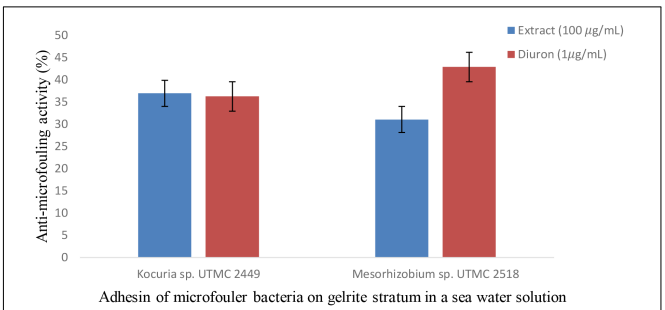


FIGURE 7 | Anti-microfouling activity of *Glycomyces* sp. UTMC 2460 crude extract on two fouler bacteria in a simulated field condition. The extract exhibited 31% and 37% microfouling inhibition activity against *Mesorhizobium* sp. UTMC 2518 and *Kocuria* sp. UTMC 2449, respectively, in a mimicked field condition at *in vitro* experiment.

TABLE 3 | Molecular identification of marine fouling bacteria based on 16S rRNA sequence.

UTMC number	Strain name	Similarity (%)	Accession number
2449	*Kocuria* sp.	*Kocuria atrinae* 99%	MH201002
2516	*Psychrobacter* sp.	*Psychrobacter faecalis* 95.5%	MH201003
2517	*Bacillus* sp.	*Bacillus atrophaeus* 99.8%	MH201004
2518	*Mesorhizobium* sp.	*Mesorhizobium amorphae* 98.5%	MH201005

Polyphasic Identification of the Strain Producing the Anti-microfouling Activity

Observation under a microscope indicated that the strain was Gram-positive with branching substrate mycelium and thus belongs to the phylum Actinobacteria (**Figure 8B**). Rod-like spores on aerial sporophores were observed under scanning electron microscopy (**Figure 8A**) and the strain formed small smooth colorless surface colonies on ISP2 medium. The optimum temperature and pH required for growth of the strain were 28°C and 7.5°C, respectively. The strain showed NaCl tolerance up to 5% (w/v). The physiological and biochemical characteristics of the strain are illustrated in **Supplementary Table 2**. The 16S rRNA was sequenced and BLAST analysis against the GenBank

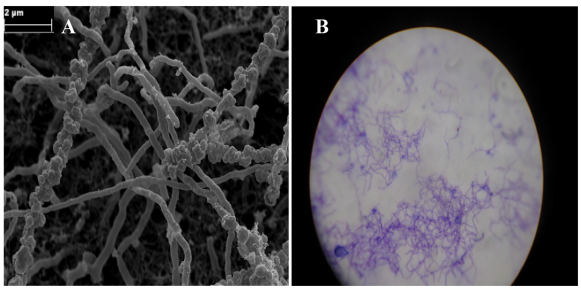


FIGURE 8 | Micro-morphological characteristic of *Glycomyces sediminimaris* UTMC 2460 on ISP2 medium after 10 days' incubation at 28°C. **(A)** Scanning electron micrograph of spore chains of the strain (Bar, 2 µm). **(B)** Filamentous vegetative mycelia under light microscopy.

and EzBioCloud databases showed the highest similarity with *Glycomyces phytohabitant* (97.01%). The sequence of its 16S rRNA was deposited in the GenBank database under accession number KU1741966. The strain has been identified as a new species in the genus under the epithet of *Glycomyces sediminimaris* UTMC 2460, using a polyphasic assessment (Mohammadipanah et al., 2018).

Characterization of the Metabolites in the *Glycomyces* Extract

UHPLC-ESI-Q-TOF-MS analyses indicated that the majority of the metabolites in the *Glycomyces sediminimaris* UTMC 2460 extract have molecular formulae and retention times corresponding to the diketopiperazine class of natural products (**Supplementary Table 3** and **Supplementary Figure 5**). The major constituents are proposed to be cyclo-(leucyl-prolyl) (**1**), cyclo-(isoleucyl-prolyl) (**2**), cyclo-(phenylalanyl-prolyl) (**3**), cyclo-(prolyl-valyl) (**4**), cyclo-(alanyl-leucyl) (**5**), cyclo-(prolyl-tyrosyl) (**6**), and cyclo-(prolyl-tryptophyl) (**7**), cyclo-(alanyl-phenylalanyl) (**8**), and cyclo-(leucyl-valyl) (**9**) (**Figure 9**).

DISCUSSION

The establishment of micro- and macro-organism communities either on living or non-living substrate (biofouling) generally involve a sequence of succession started by the colonization of surfaces by bacterial biofilm formation (Wahl, 1989) and macrofouler settlement is controlled by prevention of biofilm formation (Salta et al., 2013). Therefore, bacterial adhesion is probably one of the most critical steps to target in the search for an efficient antifouling. Although, the majority of commonly used anti-fouling paints are based on biocidal agents that induce general toxic responses in the marine environment associated with heavy metal toxicity and antibiotic toxicity, some natural anti-fouling compounds from diverse source have been identified (Fusetani, 2011). However, bacterial sources are preferred as their reproducible process of cultivation and scale up that ensure product supply for commercialization (Dobretsov et al., 2006; Gademann, 2007; Qian et al., 2007). Actinobacteria pose a dominant status in the production of novel bioactive compounds including antifouling agents such as lobocompactol (Cho and Kim, 2012), quercetin (Gopikrishnan et al., 2016) and diketopiperazines (Li et al., 2006).

The majority of Iran marine ecosystems are poorly explored. Until the beginning of 2018, less than 1% of previous studies in Iran marine habit was conducted on marine bacteria and fungi and several less known/unknown taxa are still waiting to be identified (Maghsoudlou et al., 2017). For this reason, Actinobacterial strains were isolated from subtidal sediment samples of the Iranian waters in the Persian Gulf and the Gulf of Oman and their anti-adhesion activities was evaluated versus commercial and natural anti-fouling products against dominant microfouling initiator of this ecosystem.

The investigation of the structure of pioneer fouling communities on four kinds of submerged artificial substrate (wood, fiberglass, aluminum, and steel) in Oman Sea revealed different dominant species compared to the other ecosystems. Unlike other previous studies which illustrated γ-Proteobacteria such as *Pseudoalteromonas*, *Shewanella*, and *Vibrio* (Lee et al., 2003) predominant in marine biofilms, in this study the prevalent initial marine fouling bacteria belonged to *Mesorhizobium, Kocuri*a, *Bacillus* and *Psychrobacter* genera in which *Mesorhizobium* sp. *and Kocuri*a sp. formed strict biofilm structures in *in vitro* conditions and were selected as the potent microfouler strains in the complementary analysis. The composition of bacterial biofilm communities during the first stage of biofouling period, not only differs based on the seawater in which the substrata of the biofilms were submerged but also depends on the time course and surface type. Despite the isolation of *Kocuria* members from different marine sources Dastager et al. (2014) and Pote and Bhadekar (2014), there hasn't been any reports demonstrating the biofilm forming abilities of this genus rather than their degradation activity. Hence, this result can trigger further studies in the field of biofilm formation and quorum sensing investigations of this genus. Based on different reports, (Prasad et al., 2014; Yong and Chan, 2014), *Mesorhizobium* species have been isolated from seawater and natural surfaces which were immersed in the aqua environment. In addition, they found that *Mesorhizobium* species have the quorum sensing abilities; hence our reported data about the strict biofilm formation ability of the isolated *Mesorhizobium* sp. UTMC 2518 from an aluminum platform is in line with the previous report on this genus (Yong and Chan, 2014).

In this research, the criteria for selection of artificial substrates (fiberglass, steel wood, and aluminum) were based on the frequency of usage and application of materials in the marine infrastructure equipment in Persian Gulf ports provenance of Iran. As mentioned, the surface type of immersed panels in which biofilms formed on has a fundamental role in the composition and accumulation rate of bacterial communities in biofilms sampled during the first stage of biofouling. The observed data in field experiments and former studies demonstrate that surface roughness aids the physical retention of cells by enhancing adhesive contact, protecting from hydrodynamic forces, grazing activity and desiccation (Fletcher and Callow, 1992), and settling on hydrophobic compared to

hydrophilic self-assembled monolayers in static assays is higher (Callow et al., 2000). The highest distribution rates of fouling population on different experimental panels were in the order of wood > fiberglass > steel > aluminum which was in line with the settled diversity. Low fouling rate on steel and aluminum panels can be originated from hydrophilic properties of the panel or viable toxicity of metal surface. As a result of this, due to high amounts of fouling on the second week immersed panels, isolation of primary marine fouling bacteria from these panels was replaced by 24-h, and 72-h immersed panels were considered. Despite higher fouling rates on wood panels, a variety of initial marine biofilm forming bacteria were obtained in the following isolation order fiberglass > steel > aluminum > wood.

In the second part of this study, in the Actinobacterial strains isolation process, modifications were applied to the isolation media to simulate oligotrophic conditions. Amongst the retrieved Actinobacteria, approximately 30% were separated from modified HV medium (containing NSW) which was the most oligotrophic in comparison with other media. In respected studies, ½ GYA medium and HV-agar medium were used, respectively, for isolation of Actinobacteria from sediment and seawater samples, demonstrating the effectiveness of oligotrophic conditions in strain isolation (Gebreyohannes et al., 2013; Sharma, 2014).

Deterrence of marine fouling is linked to the control of attachment and development of microfouling communities. The inhibition of adhesion may result from two mechanisms, i.e., a specific inhibition of the adhesion process or a consequence of toxicity toward the strain(s). In the present study, the anti-microfouling activity of the ten marine Actinobacteria from deep-sea sediments of the Persian Gulf was screened through anti-attachment assay, of which the crude extract of five strains demonstrated distinct anti-adherence activity against the settlements of potent biofilm-forming bacteria. The extract from *Glycomyces sediminimaris* UTMC 2460 strain exhibited the adherence inhibition in range of 72–95% with no or negligible antibacterial effect on two potent fouler bacteria in the planktonic state. This indicated limited bacterial toxicity and growth inhibition activity of the extract on the dominant microflora of this ecosystem. In accordance with the present study, the anti-microfouling activity of the crude extract of *Streptomyces filamentosus* R1 and *S. fradiae* against the marine fouling bacterial strains, such as *Bacillus* sp., *Serratia* sp., and *Alteromonas* sp. is reported by Bavya et al. (2011) and Prakash et al. (2015), respectively. Despite the lower antibacterial effect of *G. sediminimaris* UTMC 2460 extract on the planktonic form of the microfoulers in comparison to similar aforementioned studies, its mechanism of action has the privilege to be delivered by the biofilm formation inhibition. The effect of any anti-fouling agent on the proliferation of adhered strains has the advantage of effect on planktonic cells as they will not harm the balance of the microflora in the marine habitats.

Extra polymeric substance as a crucial and integral part of a biofilm plays a pivotal role in the durability of a biofilm through the adhesion and aggregation of other microfoulers. Therefore, abating EPS production can arrest the biofilm formation which holds promise for the development of functional anti-fouling agents. It can be concluded that, the high potent anti-adherence activity of Actinobacterial extract against *Kocuria* sp. UTMC 2449

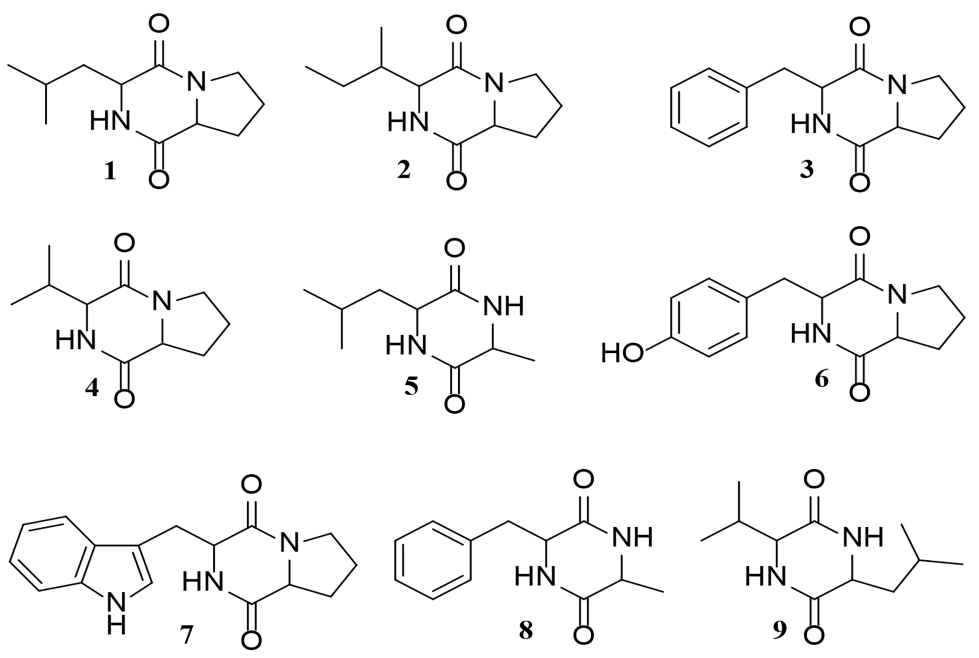


FIGURE 9 | Structure of the putative diketopiperazines identified in the extract of *Glycomyces sediminimaris* UTMC 2460: 2,5-diketopiperazines cyclo-(leucyl-prolyl) (**1**), cyclo-(isoleucyl-prolyl) (**2**), cyclo-(phenylalanyl-prolyl) (**3**), cyclo-(prolyl-valyl) (**4**), cyclo-(alanyl-leucyl) (**5**), cyclo-(prolyl-tyrosyl) (**6**), and cyclo-(prolyl-tryptophyl) (**7**), cyclo-(alanyl-phenylalanyl) (**8**), and cyclo-(leucyl-valyl) (**9**).

and *Mesorhizobium* sp. UTMC 2518 biofilm can also be attributed to the considerable inhibition in EPS production.

Besides anti-microfouling efficacy, toxicity is a major concern for marine coating industry as effective marine natural compounds are sometimes as toxic as heavy metals. Thus, the toxicity profiles of the five potent anti-microfouling extracts were also determined in this study. The absence of mortality effect on the larvae of *Artemia salina* mitigating their ecological concern. Additionally, compared with the LC_{50} of tributyltin, which is generally less than 0.00001 $\mu g\ ml^{-1}$, (Cardwell et al., 1999) these results indicate that these extracts are much less toxic than tributyltin. The extract of *Glycomyces sediminimaris* UTMC 2460 as the most effective extract on fouling inhibition of *Kocuria* sp. UTMC 2449 and *Mesorhizobium* sp. UTMC 2518, exhibit not only no toxic effect on *Artemia salina* but also has a negligible hemolytic activity which is an indication of acute toxicity effect. Therefore, it can be concluded that the crude extract of *Glycomyces sediminimaris* UTMC 2460 can be considered as an environmentally safe anti-fouling agent.

Analysis of the crude extract of from *Glycomyces sediminimaris* UTMC 2460 identified nine compounds corresponding to the family of diketopiperazines. Diketopiperazines are known to possess potent anti-fouling activity (Li et al., 2006; Cho et al., 2012) and the anti-fouling properties of seven of the nine diketopiperazines tentatively identified in this study have already been investigated. These include compounds **1**, **3**, **4**, **7**, and **9** (Li et al., 2006), and metabolites **5** and **8** (Cho et al., 2012). The anti-fouling activity of *Glycomyces sediminimaris* UTMC 2460 is therefore most likely attributed to the production of diketopiperazines **1–9**.

CONCLUSION

The overall results of the present study clearly emphasized the strain *Glycomyces sediminimaris* UTMC 2460 as a competent producer of the anti-microfouling natural product and substantiated its low toxic nature. Furthermore, the physiological, molecular and chemotaxonomical analysis, allowed its introduction as a new species in *Glycomyces* genus called *Glycomyces sediminimaris*. The sufficient studies to explore the bioactivity of the *Glycomyces* strains have not been performed and only a single report exists on the anti-tumor and antibiotic activity of *G. harbinensis* secondary metabolite (Lee et al., 1986). Hence, this report adds new valuable biological activity to this genus capability and can accelerate the investigation of other novel bioactivities from this genus and the species of *G. sediminimaris*. Further work on the anti-microfouling activity of the putative diketopiperazines produced by *G. sediminimaris* and field trial experiments are demanding to development a novel eco-friendly compound that is introduced as a potential substitute for commercial chemical biocide in the maritime industry.

AUTHOR CONTRIBUTIONS

SH performed the experiments and analysis and wrote the draft of the manuscript. FM and AM contributed as supervisors. FM defined the approach, designed the experiments, and edited the manuscript content. AM managed the water/sediment sampling. YD and GC performed the metabolite profiling, wrote the related sections, and contributed to the editing of the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Comparative Genomics Provides Insights Into the Marine Adaptation in Sponge-Derived *Kocuria flava* S43

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Sponge-derived actinomycetes represent a significant component of marine actinomycetes. Members of the genus *Kocuria* are distributed in various habitats such as soil, rhizosphere, clinical specimens, marine sediments, and sponges, however, to date, little is known about the mechanism of their environmental adaptation. *Kocuria flava* S43 was isolated from a coastal sponge. Phylogenetic analysis revealed that it was closely related to the terrestrial airborne *K. flava* HO-9041. In this study, to gain insights into the marine adaptation in *K. flava* S43 we sequenced the draft genome for *K. flava* S43 by third generation sequencing (TGS) and compared it with those of *K. flava* HO-9041 and some other *Kocuria* relatives. Comparative genomics and phylogenetic analyses revealed that *K. flava* S43 might adapt to the marine environment mainly by increasing the number of the genes linked to potassium homeostasis, resistance to heavy metals and phosphate metabolism, and acquiring the genes associated with electron transport and the genes encoding ATP-binding cassette (ABC) transporter, aquaporin, and thiol/disulfide interchange protein. Notably, gene acquisition was probably a primary mechanism of environmental adaptation in *K. flava* S43. Furthermore, this study also indicated that the *Kocuria* isolates from various marine and hyperosmotic environments possessed common genetic basis for environmental adaptation.

Keywords: marine sponge, *Kocuria flava*, environmental adaptation, comparative genomics, third generation sequencing (TGS)

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INTRODUCTION

Marine sponges are well known to be a rich source of diverse actinomycetes such as *Streptomyces*, *Salinispora*, *Micromonospora*, *Kocuria*, etc. (Taylor et al., 2007). Sponge-derived actinomycetes are distributed randomly in sponge hosts, and they are thought to be taken up from the flowing sea water by filtration and dwell in the mesohyl matrix of sponge hosts (Abdelmohsen et al., 2014). Accordingly, the actinomycetes are endowed with the ability to survive in both nutrient-poor sea water and nutrient-rich sponge mesohyl. They are physiologically or metabolically distinct from their terrestrial counterparts owing to their adaptation to marine environments. A number of isolates grow and differentiate faster on the medium prepared with sea water (Jiang et al., 2007). Some *Streptomyces* strains strictly require salt for their growth (Khan et al., 2011). A few isolates own several secondary metabolite biosynthesis gene clusters absent in their closely related terrestrial relatives. For instance, sponge-derived *Streptomyces* sp. GBA 94-10 and PVA 94-07

share 10 identical secondary metabolite gene clusters not present in their terrestrial relative *S. albus* J1074, most of which are responsible for encoding non-ribosomal peptide synthetases and polyketide synthases (Ian et al., 2014). To date, research interest has been largely focused on the diversity and natural products of marine sponge-derived actinomycetes, however, the mechanisms behind their environmental adaptation are still not well understood. With the rapid development of the second/third next generation sequencing techniques, genomics may provide new insights into the genetic basis for environmental adaptation in sponge-derived actinomycetes.

Over the past decades, great progresses have been made in understanding the genetic basis for marine adaptation in Gram-negative bacteria. The discovery of the sodium-pumping NADH dehydrogenase Nqr (Unemoto and Hayashi, 1993) and the associated genes *nqrA-F* (Mulkidjanian et al., 2008) provided the genetic link to sodium dependence in Gram-negative marine bacteria. Nqr can create an ionic motive force to generate ATP and drive other cellular processes (Schäfer et al., 2008). The gene acquisition of certain transporters represents another important marine adaptation mechanism. High abundance of ABC branched chain amino acid (BCAA) transporters was found in several marine *Roseobacter* strains (Moran et al., 2007), and the associated genes were detected in marine metagenomes as well (Morris et al., 2010). BCAAs can be converted into L-glutamate, which would help acidify the basic cytoplasm (Takami et al., 2002). Marine cyanobacterium *Synechococcus*, with greater capacity to transport Na^+ than freshwater species, might adapt to the oligotrophic environment by using more sodium-dependent transporters than a model freshwater cyanobacterium (Palenik et al., 2003). In addition, intracellular accumulation of compatible solutes seems to be an alternative marine adaptation strategy. The marine adaption in *Novosphingobium* was suggested to be based on the organic osmolyte (ectoine) mechanism that was different from those reported in Gram-negative marine bacteria that exported Na^+ via the sodium-pumping NADH dehydrogenase Nqr (Gan et al., 2013).

Initial attempts to provide the evidence of marine adaptation in actinomycetes concentrated in the obligate marine genus *Salinispora* (Penn and Jensen, 2012). Marine adaptation genes (MAGs) were identified from *S. tropica* and *S. arenicola* by comparative genomic analysis, and the loss of a mechanosensitive channel gene *mscL* was thought to result in the inability of *Salinispora* strains to grow in low osmotic environment. The finding was later confirmed by genetic complementation of *S. tropica* with *mscL* (Bucarey et al., 2012). By the genomic comparison of two sponge-derived *Streptomyces albus* isolates with their terrestrial relative *S. albus* J1074, several putative MAGs linked to electron transport and potassium uptake were identified (Ian et al., 2014). In addition, marine-derived *Streptomyces* subgroup was found to possess some common characteristics of marine adaptation on the basis of comparative genomics (Tian et al., 2016). In specific, marine streptomycetes possessed more functional genes and transporters than other streptomycetes to adapt to the cold, hyperosmosis, oligotrophy, and other marine environments (Tian et al., 2016). Nonetheless, among diverse actinomycete groups, only a few representative members belonging to *Salinispora*, *Streptomyces*, *Janibacter*, *Aeromicrobium*, *Rhodococcus*, and one unclassified species "marine actinobacterium PHSC20C1" have been studied on their marine adaptation to date (Penn and Jensen, 2012; Tian et al., 2016). To improve our knowledge of the mechanisms for environmental adaptation in marine actinomycetes, it is significant to investigate a wider range of actinomycete lineages.

Taxonomically, *Kocuria* belongs to the family Micrococcaceae and comprises a group of coccoid actinomycetes with high environmental adaptability. *Kocuria* species have been recovered from various habitats, particularly marine environments such as sediments (Bala et al., 2012; Jiang et al., 2015), seawater (Seo et al., 2009), sponges (Abdelmohsen et al., 2014), and corals (Mahmoud and Kalendar, 2016). In our efforts of isolating actinomycetes from sponges collected from South China Sea (Li and Liu, 2006; Sun et al., 2015), *Kocuria flava* S43 was isolated from a coastal sponge *Siphonochalina* sp. (Sun et al., 2015), which had 99.7% similarity in 16S rRNA gene sequence with *K. flava* HO-9041 from the air of Xinjiang, an inland province in China (Zhou et al., 2008). Evidently, the inland airborne *K. flava* HO-9041 represents a terrestrial microorganism whereas the sponge-derived *K. flava* S43 is a marine representative. These two sister organisms can serve as a model for gaining insights into marine adaptation in *K. flava*. The complete genome sequence of *K. flava* HO-9041 is currently available (Zhou et al., 2016). Furthermore, a few complete or draft genome sequences of marine *Kocuria* isolates are publicly available, which provided an opportunity to make comparison with *K. flava* S43 at the genomic level. In this study, *de novo* genome sequencing for *K. flava* S43 was performed by TGS, and the genetic basis for marine adaptation in *K. flava* S43 was revealed by comparative genomic analysis of several *Kocuria* isolates from various marine and terrestrial environments.

MATERIALS AND METHODS

Strain and 16S rRNA Gene Phylogeny

K. flava S43 was previously isolated from the tissue of the marine sponge *Siphonochalina* sp., which was collected from Xincun Harbor (18.42°N, 109.97°E), the South China Sea. The 16S rRNA gene sequence of *K. flava* S43 was deposited to GenBank under the accession number: JX007971 (Sun et al., 2015).

The type strains of the genus *Kocuria* were retrieved from RDP database (Release 11.5) by using the hierarchy browser[1]. Furthermore, literature search for the novel *Kocuria* species validly published in recent years was performed to update the type strain pool. The 16S rRNA gene sequences of the currently described 26 *Kocuria* species and *K. flava* S43 were manually aligned using ClustalX 1.81 (Thompson et al., 1997). Phylogenetic analysis was conducted using the maximum likelihood method (Felsenstein, 1981) provided by the software package MEGA 6.06 (Tamura et al., 2013). The consistency of the tree was verified by bootstrapping (1,000 replicates) for parsimony (Felsenstein, 1985).

[1]http://rdp.cme.msu.edu/

Genomic DNA Extraction and Genome Sequencing

A single colony of *K. flava* S43 was inoculated to a 50-ml centrifuge tube with 10 ml of GYM4 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, and 1 L distilled water) medium (10 g glucose, 4 g yeast extract, 4 g malt extract, and 1 L artificial seawater) (Schneemann et al., 2010). After incubation at 28°C and 180 rpm for 2 days, the culture was collected by centrifugation and its genomic DNA was extracted using a DNeasy® Blood and Tissue Kit (Qiagen, United States). The integrity, concentration and purity of genomic DNA were detected by Agarose Gel Electrophoresis, Nanodrop and Qubit Fluorimeter. The genome was sequenced by the third generation sequencing (TGS), i.e., the Pacific Biosciences (PacBio) Single Molecule Real-Time (SMRT) sequencing technology. A whole-genome sequencing library was constructed using the SMRTcell Template Prep Kit (PacBio, United States) according to the manufacturer's protocol, and the library was sequenced by using g-Tube on the PacBio RS II platform (PacBio).

Genome Annotation and Comparative Genomic Analysis

De novo assembly of the sequences was performed using hierarchical genome assembly process (HGAP) version 3.0 (Chin et al., 2013). This whole-genome shotgun project was deposited at DDBJ/EMBL/GenBank under accession number LOMZ00000000. The version described in this paper was the first version, LOMZ01000000. The draft genome sequence of *K. flava* S43 was uploaded to NCBI and aligned with the deposited genomes using Basic Local Alignment Search Tool[2].

The genome sequences of *K. flava* HO-9041 from inland air, *Kocuria* sp. SM24M-10 from the mucus of the coral *Mussismilia hispida* (Palermo et al., 2016), *K. indica* DSM 25126 from a marine sediment sample (Dastager et al., 2014), *K. rhizophila* P7-4 from the intestine of the fish *Siganus doliatus* (Kim et al., 2011) and *K. rhizophila* DC2201 from a soil sample (Takarada et al., 2008) were downloaded from NCBI[3] and served as the reference genomes for subsequent analysis. Genome alignment of *K. flava* S43 and *K. flava* HO-9041 was carried out using MAUVE software (Darling et al., 2010). For rapid function-based comparison by the comparative tool in the SEED Viewer (Overbeek et al., 2014), all the six *Kocuria* genome sequences were annotated by using Rapid Annotation using Subsystem Technology (RAST) version 2.0[4] (Aziz et al., 2008). Genome Browser was used to visualize gene arrangement and annotation.

The subsystem category distribution pattern was compared between the *K. flava* S43 and HO-9041 genomes. The subsystems with significant difference in feature counts were marked and further compared at a finer scale. The common genes with marked increase in number in the *K. flava* S43 genome were screened. Meanwhile, the function-based comparison was performed among the 6 *Kocuria* genomes, the genes common in at least three marine *Kocuria* isolates but absent in two terrestrial *Kocuria* representatives were also screened. All the screened genes were listed as putative MAGs and uploaded to NCBI for protein match using BLASTp. The original RAST annotations were manually checked according to the BLAST hits using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) (Angiuoli et al., 2008). The genes incorrectly annotated by RAST were excluded.

Phylogenetic Analysis of Genes Potentially Relevant to Environmental Adaptation

The screened candidate genes were subjected to phylogenetic analysis to test for a shared evolutionary history with other bacteria or actinomycetes derived from marine and hyperosmotic environments. The top 15–20 BLASTp hits of each gene were downloaded from the NCBI protein database. Maximum likelihood phylogenies were constructed for each representative gene using the online tool MABL with default settings[5] (Dereeper et al., 2008). The genes that were clustered in the clades totally or largely comprising the homologs from the isolates of marine or hyperosmotic sources were kept in the final MAG pool.

RESULTS

Phylogeny and Genome Feature of *K. flava* S43

Based on 16S rRNA gene sequence analysis, the phylogenetic position of *K. flava* S43 with respect to currently described *Kocuria* species was shown in **Figure 1**. *K. flava* S43 was clearly positioned within the genus *Kocuria* and formed a subgroup together with *K. flava* HO-9041.

A total of 99,440 reads of *K. flava* S43 were obtained, with a mean read length of 16,484 bp, providing 361.84× average reference coverage. The final assembly yielded six contigs, with the largest contig of 3,548,480 bp. The remaining five contigs were from 2,899 to 152,580 bp in length. BLAST matches indicated that the largest contig represented chromosome genome, four contigs were plasmid genomes and the smallest contig could not match any known genome (**Table 1**).

The genome sequence of *K. flava* S43 was aligned with that of *K. flava* HO-9041. This alignment showed a high homology between *K. flava* S43 and *K. flava* HO-9041 (**Figure 2A**), supporting their close phylogenetic relationship as suggested by 16S rRNA gene phylogeny. Both genomes shared a large number of genomic traits, however, various translocations and inversions occurred in the genome of *K. flava* S43 relative to the reference genome. In addition, it appeared that gene transfer from plasmids to chromosomes happened in both genomes (**Figure 2B**). In specific, a few genes located in the *K. flava* S43 plasmid 1 and 2 showed a high homology with those in the *K. flava* HO-9041 genome and a few genes occurring in the *K. flava* HO-9041

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

[3]ftp://ftp.ncbi.nih.gov/genomes/genbank/bacteria/Kocuria

[4]http://rast.nmpdr.org/

[5]http://phylogeny.lirmm.fr/phylo_cgi/simple_phylogeny.cgi

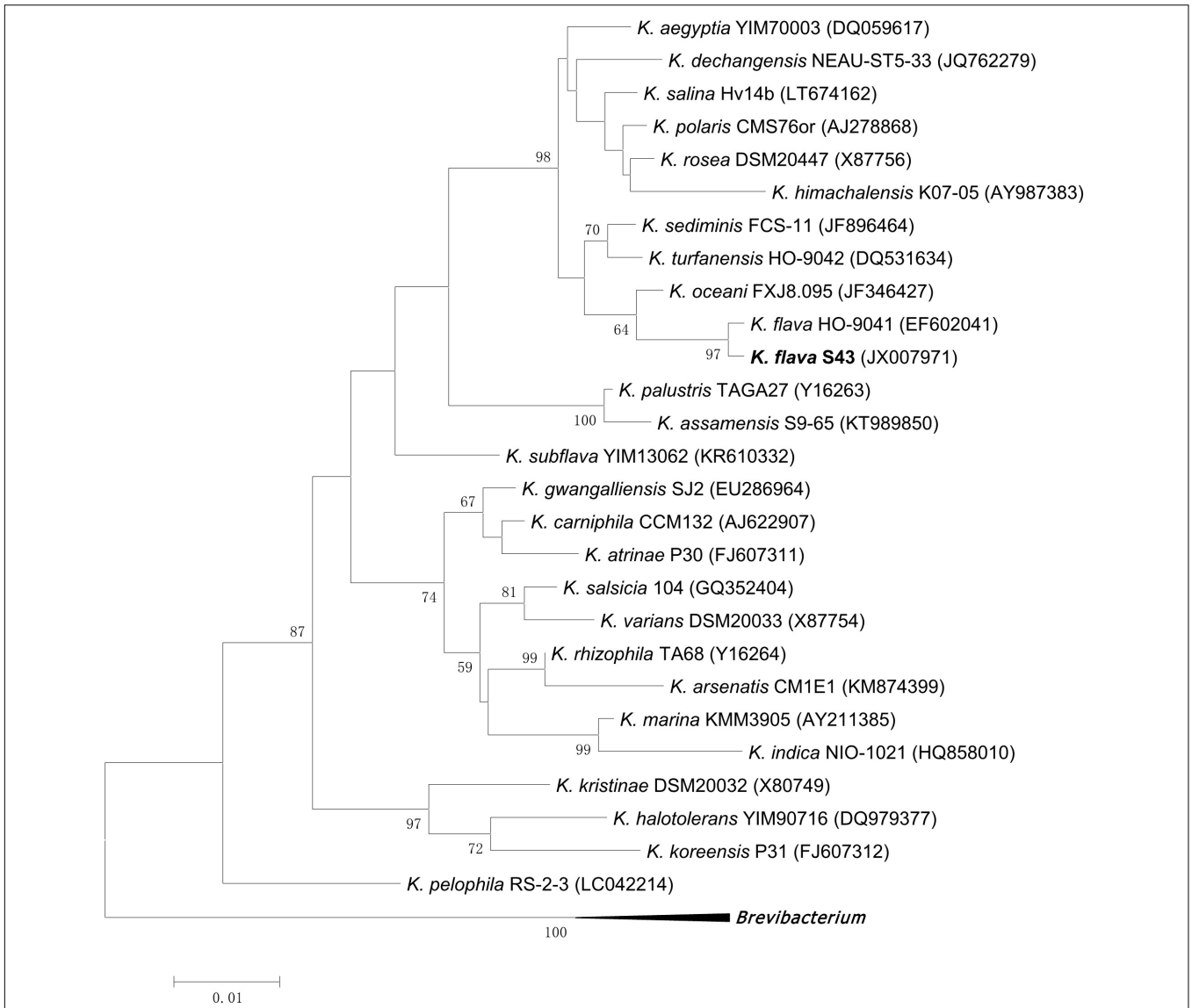

FIGURE 1 | Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences of *K. flava* S43 and all currently described *Kocuria* species. *Brevibacterium iodinum* DSM 20626 (X83813), *Brevibacterium linens* DSM 20425 (X7745), and *Brevibacterium casei* DSM 20657 (AJ251418) were used as the out-group. Only bootstrap values of being greater than 50% were shown on the tree.

TABLE 1 | BLAST match of the *K. flava* S43 genome against the deposited bacterial genomes.

Contig no.	Contig size (bp)	Top BLAST hit	Protein-coding sequences	Coverage (%)	Identity (%)
Contig 1	3,548,480	*K. flava* HO-9041 genome	3,194	87	98
Contig 2	152,580	*K. flava* HO-9041 plasmid 1	136	23	91
Contig 3	140,250	*K. flava* HO-9041 plasmid 1	116	41	99
Contig 4	41,752	*K. turfanensis* HO-9042 plasmid 2	34	24	91
Contig 5	11,314	*K. flava* HO-9041 plasmid 1	10	49	95
Contig 6	2,899	No significant similarity	4		

plasmid 1 showed a high homology with those in the *K. flava* S43 genome, suggesting potential horizontal gene transfer.

Gene annotation of the draft genome sequence yielded 3,494 protein-coding sequences (CDSs), 49 tRNA and 11 rRNA (three 5S rRNA, four 23S rRNA, and four 16S rRNA) genes. In total, 3,194 CDSs, 48 tRNA, and 11 rRNA genes were located in the chromosome genome. The rest of the CDSs and one tRNA gene were distributed in the plasmid genomes.

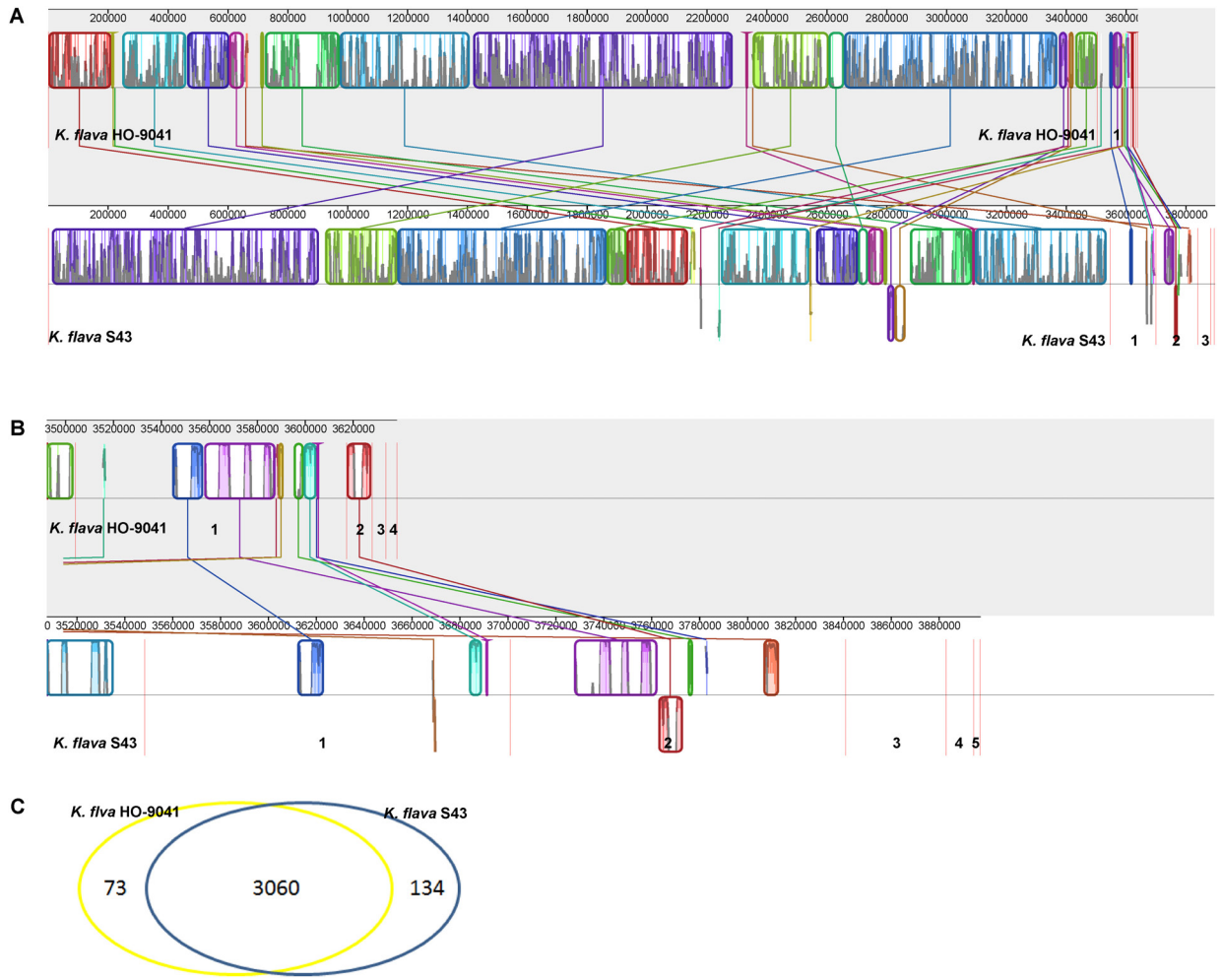

FIGURE 2 | MAUVE alignment of the chromosome and plasmid genome sequences of *K. flava* S43 and *K. flava* HO-9041. The contigs were separated by red lines. When boxes had the same color, this indicated syntenic regions. Boxes below the horizontal line indicated inverted regions. Rearrangements were shown by colored lines. Scale was in nucleotides. **(A)** Chromosome with the boundaries marked by strain name. **(B)** Plasmid with the figures referring to different contigs. **(C)** Venn diagram showing the unique and common ORFs in 2 *K. flava* genomes.

Genes Potentially Relevant to Marine Adaptation Based on Comparative Genomic Analysis

The comparison of the genomic characteristics between *K. flava* S43 and *K. flava* HO-9041 was presented in **Table 2**. The chromosome genome of *K. flava* S43 was slightly larger in size than that of *K. flava* HO-9041, suggesting the draft genome sequence was nearly complete. The same number of tRNA genes occurred in two genomes. Notably, although one copy was incomplete (5S rRNA missing and 23S rRNA partial) due to the draft sequence, the *K. flava* S43 genome owned one more copy of rRNA operon than the *K. flava* HO-9041 genome.

A Venn diagram showed the unique and common ORFs in two *K. flava* genomes (**Figure 2C**). The functional annotation of the unique ORFs in the *K. flava* S43 genome was shown in **Supplementary Table S1**. The comparison of the subsystem distribution between *K. flava* S43 and *K. flava* HO-9041 was demonstrated in **Table 3**. Significant difference was observed in the count of the genes categorized in six subsystems: respiration, potassium metabolism, virulence, disease and defense, phosphorus metabolism, cell wall and capsule, and nitrogen metabolism. The count of the genes related to respiration markedly increased in the *K. flava* S43 genome, which was due to the occurrence of the genes encoding respiratory

TABLE 2 | Genomic characteristics of *K. flava* HO-9041 and *K. flava* S43.

Feature	*K. flava* HO-9041	*K. flava* S43
Genome size (bp)	3,504,335	3,548,480
GC content	74.2	74.2
Protein-coding genes (CDSs)	3,113	3,194
rRNA operons	3	4*
tRNA genes	48	48

**One operon is incomplete.*

TABLE 3 | Subsystem distribution in the *K. flava* HO-9041 and *K. flava* S43 genomes.

Subsystem	*K. flava* HO-9041	*K. flava* S43
Cofactors, vitamins, prosthetic groups, pigments	221	221
Cell wall and capsule	67	40*
Virulence, disease and defense	54	62**
Potassium metabolism	16	21**
Photosynthesis	0	0
Miscellaneous	53	58
Phages, prophages, transposable elements, plasmids	1	1
Membrane transport	77	79
Iron acquisition and metabolism	7	7
RNA metabolism	98	101
Nucleosides and nucleotides	98	104
Protein metabolism	224	229
Cell division and cell cycle	24	22
Motility and chemotaxis	3	3
Regulation and cell signaling	26	26
Secondary metabolism	10	10
DNA metabolism	66	67
Fatty acids, lipids, and isoprenoids	145	148
Nitrogen metabolism	38	31*
Dormancy and sporulation	2	3
Respiration	49	78**
Stress response	102	99
Metabolism of aromatic compounds	51	51
Amino acids and derivatives	320	321
Sulfur metabolism	33	32
Phosphorus metabolism	40	47**
Carbohydrates	327	353

**The subsystems with significant decrease in feature counts of K. flava S43 were in light gray background rows.*
***The subsystems with significant increase in feature counts of K. flava S43 were in gray background rows.*

complex I, terminal cytochrome d ubiquinol oxidase and the genes associated with the biogenesis of c-type cytochromes. Additional 4 *kdpD* (osmosensitive K^+ channel histidine kinase) genes led to the higher number of genes related to potassium homeostasis in *K. flava* S43. Similarly, the counts of *czcD* (cobalt–zinc–cadmium resistance protein) and *copD* (copper resistance protein D) genes increased in *K. flava* S43, which were grouped into the virulence, disease, and defense subsystem. In the phosphorus metabolism subsystem, six *phoB* (phosphate regulon transcriptional regulatory protein) genes occurred in *K. flava* S43 whereas only three were present in *K. flava* HO-9041. In addition, the count of the genes categorized in cell wall/capsule and nitrogen metabolism markedly decreased in the *K. flava* S43 genome. In specific, more than 20 genes related to peptidoglycan biosynthesis were absent in *K. flava* S43. The number of the genes involved in nitrate and nitrite ammonification and encoding denitrifying reductase were less in *K. flava* S43 than in *K. flava* HO-9041.

Phylogenetic analyses of the *kdpD*, *czcD*, *copD*, and *phoB* genes in *K. flava* S43 were performed (**Figures 3A–D**). Apparently, the number increase of these genes was not due to gene duplication but gene gain of *K. flava* S43 or gene loss of *K. flava* HO-9041. Notably, each of the phylogenetic trees contained at least one clade totally comprising the homologs from the isolates of marine or hyperosmotic sources, such as *Kocuria* sp. SM24M-10 from a coral and *K. polaris* CD08-4 from a celiac disease patient (Chander et al., 2017), suggesting that a total of eight genes (two *kdpD*, one *czcD*, three *copD,* and two *phoB* genes) in such clades may be MAGs.

A total of 13 genes (*nuoA*, *nuoH*, *nuoJ*, *nuoK*, *nuoL*, *nuoM*, *nuoN*, *cydA*, *cydB*, *cydCD*, *aqpZ,* and two *dsbA* genes) common in at least three marine isolates but absent in two terrestrial representatives were screened as putative MAGs. Seven *nuo* genes constituted a partial NDH-1 operon, which encoded a part of a proton-pumping NADH dehydrogenase. The *nuo* genes were previously identified as MAGs in *Salinispora* (Penn and Jensen, 2012). The organization of the operon in *K. flava S43* was consistent with that in *S. arenicola* CNS-205 (**Figure 4A**). Three *cyd* genes in a cluster were responsible for encoding cytochrome d ubiquinol oxidase subunit I, II and an ATP-binding cassette-type transporter, respectively. As one type of terminal respiratory oxidases, cytochrome d ubiquinol oxidase acted as the terminal acceptor of electron transport chains. The arrangement of the genes in the cluster was similar to that in the marine actinobacterium PHSC20C1 genome (**Figure 4B**). The *aqpZ* gene was located next to *cydCD*, which encoded an aquaporin, a specific water channel that facilitated the rapid influx/efflux of water, and the *dsbA* gene was responsible for encoding a periplasmic thiol/disulfide interchange protein, which was involved in the biogenesis of cytochrome C.

The phylogenies of *nuoM* (**Figure 5A**), *cydB* (**Figure 5B**), *aqpZ* (**Figure 5C**), and *dsbA* (**Figure 5D**) indicated the presence of several clades totally or largely comprising the orthologs from the isolates of marine or hyperosmotic sources, suggesting that they shared evolutionary history with certain isolates derived from marine or other hyperosmotic environments.

The distribution and abundance of the MAGs in six *Kocuria* representatives were shown in **Table 4**. Besides four marine isolates, two strains from hyperosmotic environments, i.e., *K. polaris* CD08-4 from a celiac disease patient and *K. marina* SO9-6 from a copper iron sulfide mineral (Castro et al., 2015), were also included. Apparently, the MAGs were distributed in all or the majority of the analyzed *Kocuria* isolates though their abundance sometimes varied as the species, indicating that the *Kocuria* isolates possessed common genetic basis for marine adaptation. The result based on RAST annotation and comparison was consistent with the phylogeny test based on NCBI BLASTp.

DISCUSSION

Actinomycetes are widely distributed in marine and terrestrial environments. Besides a few obligate marine lineages

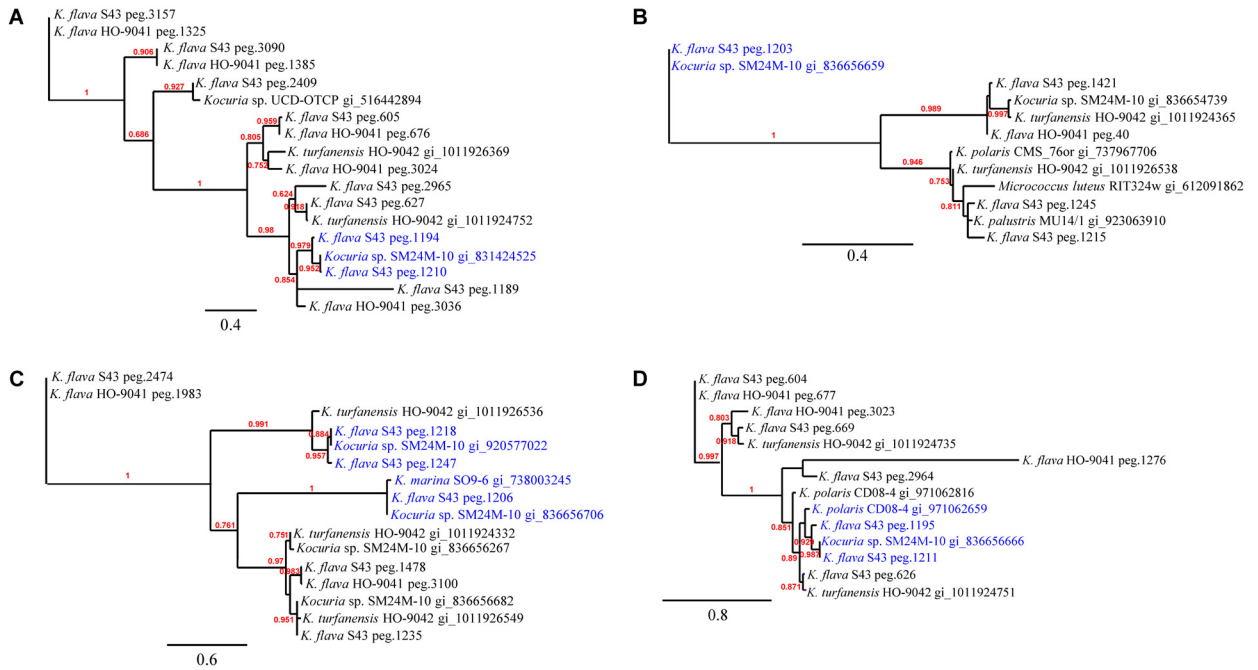

FIGURE 3 | The phylogenies of the genes occurring in higher number in the *K. flava* S43 genome potentially relevant to marine adaptation. **(A)** *kdpD*, **(B)** *czcD*, **(C)** *copD*, **(D)** *phoB*. The clades totally comprising the homologs from the isolates of marine or hyperosmotic sources were colored blue. Midpoint rooting was used and likelihood values shown for each node. Only bootstrap values of being greater than 0.5 were shown on the tree. Scale bar represented changes per site.

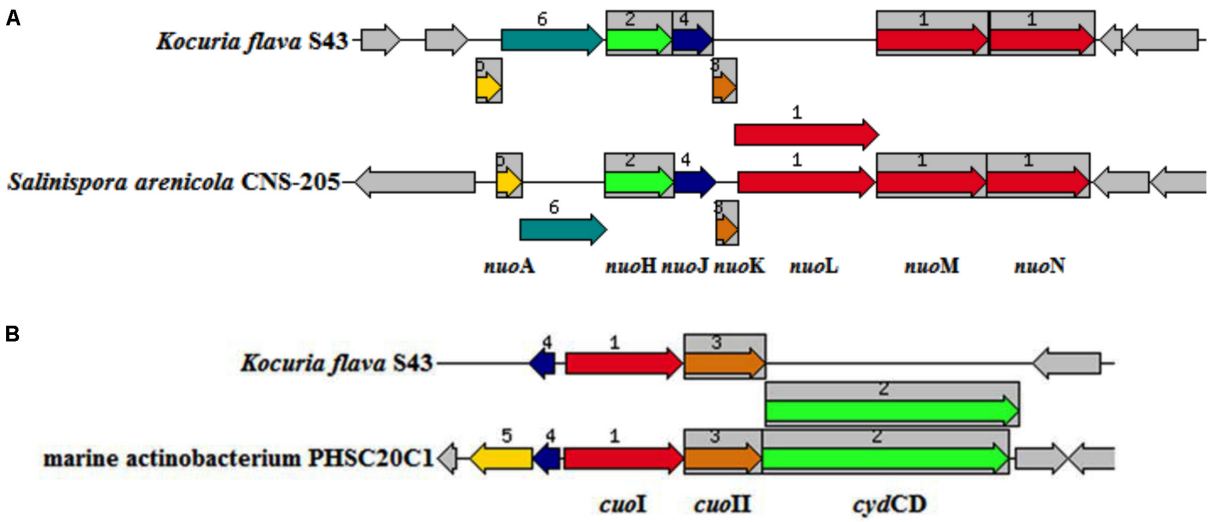

FIGURE 4 | The arrangement of the operons potentially relevant to environmental adaptation in *K. flava* S43 and obligate marine actinomycetes. **(A)** nuo (NADH-ubiquinone oxidoreductase) operon, **(B)** cuo (cytochrome ubiquinol oxidase) operon. The chromosomal region of the focus gene (top) was compared with similar organisms. The graphic was centered on the focus gene, which was red and numbered 1. Sets of genes with similar sequence were grouped with the same number and color. Genes whose relative position was conserved were functionally coupled and shared gray background boxes.

such as *Salinispora* and *Marinispora*, the majority of the actinomycetes can survive in both marine and terrestrial habitats. Undoubtedly, marine actinomycetes acquire marine adaptive traits relative to their terrestrial counterparts. The genes relevant to marine adaptation can be identified by comparing the genomes of closely related marine and terrestrial microorganisms. Previous comparative genomic analyses of marine actinomycetes were performed mainly at the species or genus level (Penn and Jensen, 2012; Tian et al., 2016). In this study, comparative genomic analysis of *Kocuria* spp. was carried out at both strain and species level. Several MAGs were finally identified, indicating that

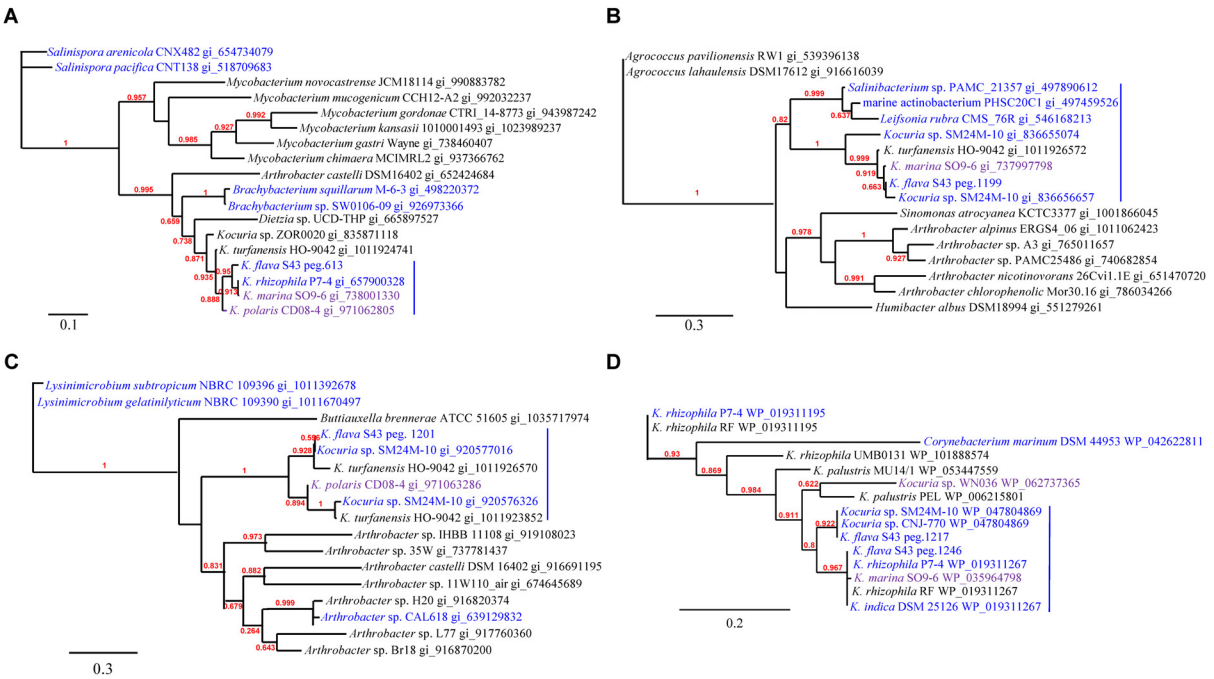

FIGURE 5 | The phylogenies of the genes newly acquired in the *K. flava* S43 genome potentially relevant to environmental adaptation. **(A)** *nuoM*, **(B)** *cydB*, **(C)** *aqpZ*, **(D)** *dsbA*. Strains of marine, hyperosmotic, and terrestrial sources were colored blue, purple, and black, respectively. Midpoint rooting was used and likelihood values shown for each node. Only bootstrap values of being greater than 0.5 were shown on the tree. Scale bar represented changes per site.

TABLE 4 | The abundances of the MAGs in the investigated *Kocuria* spp. genomes.

Strain Source gene	*K. flava* S43 (HO-9041) sponge tissue	*Kocuria* sp. SM24M-10 coral mucus	*K. rhizophila* P7-4(DC2201) fish intestine	*K. indica* DSM 25126 marine sediment	*K. polaris* CD08-4 patient	*K. marina* SO9-6 copper miner
kdpD	9 (5)	6	2 (1)	2	6	1
czcD	4 (1)	3	2 (1)	3	1	3
copD	6 (2)	5	2 (1)	2	1	3
phoB	6 (3)	6	3 (1)	6	5	6
nuo	1 (0)	0	1 (0)	1	1	1
cyd	1 (0)	2	1 (0)	1	0	1
aqpZ	1 (0)	2	1 (0)	2	1	2
dsbA	2 (0)	1	2 (0)	1	0	1

K. flava S43, Kocuria sp. SM24M-10, K. rhizophila P7-4 and K. indica DSM 25126 were representatives of various marine sources. K. polaris CD08-4 and K. marina SO9-6 were isolates from hyperosmotic environments. The numbers in brackets represented the gene number in its closely related terrestrial relative. kdpD, osmosensitive K^+ channel histidine kinase; czcD, cobalt–zinc–cadmium resistance protein; copD, copper resistance protein; phoB, phosphate regulon transcriptional regulatory protein; nuo, NADH-ubiquinone oxidoreductase; cyd, cytochrome d ubiquinol oxidase; aqpZ, aquaporin; dsbA, periplasmic thiol/disulfide interchange protein.

comparative genomic analysis at the strain level was efficient and informative.

Gene gain is a major force driving bacterial evolution. The presence of the MAGs may be attributed to gene gain in marine *Kocuria* or gene loss in terrestrial *Kocuria*. In this study, the quantification of gene gain and loss was not performed. However, the gene arrangement in the *K. flava* S43 genome can provide a clue on gene gain or loss. The identified 21 MAGs are not scattered in the genome but concentrate in two genomic regions. One region consists of the seven *nuo* genes, and the other centers on the *cyd* genes. Particularly, a total of 12 genes (*kdpD*, *phoB*, *cycA*, *cycB*, *cycCD*, *aqpZ*, *czcD*, *copD*, *kdpD*, *phoB*, *dsbA*, *copD*) arrange in a cluster. On the upstream and downstream of the cluster, respectively, locates one mobile element, suggesting the gene cluster largely comprising the MAGs is acquired by horizontal gene transfer. Thus, gene acquisition potentially represents a primary mechanism of marine adaptation in *K. flava* S43.

The MAGs identified in the *K. flava* S43 genome potentially function in various ways. The respiratory complex I encoded by the nuo operon can create a proton-motive force for the generation of ATP and help maintain a proton gradient in seawater (Tokuda and Unemoto, 1982). The terminal respiratory oxidase encoded by *cydA* and *cydB* can act as the terminal

acceptor of an electron transport chain and is therefore the key enzyme of respiration, and the ABC transporter encoded by *cydCD* can export cysteine, which is crucial for redox homeostasis in the periplasm (Pittman et al., 2002). The thiol/disulfide interchange protein encoded by *dsbA* can oxidize cysteine thiols of apocytochromes c and play a role in the biogenesis of c-type cytochromes (Peek and Taylor, 1992). Considering the high osmolarity in marine environments, the aquaporin AqpZ can provide the osmoregulation by mediating influx/efflux of water (Calamita, 2000), and the sensor kinase KdpD can osmotically regulate the potassium uptake (Jung et al., 1997). Surface seawater is known to contain trace amounts of heavy metals such as cadmium, copper, and zinc, the intracellular accumulation of which is harmful for the microorganisms. The copper resistance protein CopD can maintain the balance of copper by exporting copper and the cobalt–zinc–cadmium resistance protein CzcD can detoxify the periplasm by the export of toxic metal cations (cobalt, zinc, and cadmium) (Nies and Silver, 1995). Given the two-component response regulator PhoB can regulate phosphate assimilation in a sophisticated manner (Marzan and Shimizu, 2011), it probably facilitates the survival of *K. flava* S43 under phosphate-limiting niche. Taken together, the MAGs can help the marine *Kocuria* spp. adapt to oligotrophy and hyperosmolarity and resist to heavy metal ions.

The present study reveals that the *Kocuria* isolates from various marine and hyperosmotic environments have some common genomic features related to environmental adaptation. *K. flava* S43, *Kocuria* sp. SM24M-10, *K. indica* DSM 25126, *K. rhizophila* P7-4, *K. polaris* CD08-4, and *K. marina* SO9-6 were isolated from distinct habitats. The presence of some common MAGs suggests that the *Kocuria* isolates may adapt to respective niche in the same ways. The abundance of the MAGs in the *Kocuria* spp. varies as species, which can be attributed to the difference in geographic and environmental factors. It warrants attention that the *nuo* genes are missing in *Kocuria* sp. SM24M-10, and both *cyd* and *dsbA* are absent in *K. polaris* CD08-4. This phenomenon can be explained by their close associations with the hosts (coral mucus and patient), which provide nutrient-rich niches for the *Kocuria* isolates so they may reduce the energy requirement. Furthermore, *Kocuria* sp. SM24M-10, *K. indica* DSM 25126 and *K. marina* SO9-6 have more *aqpZ* genes than other *Kocuria* spp., implying the requirement of more aquaporins to regulate osmolarity. Taken together, these results indicate that the MAGs are not essential for the survival in marine environments. However, the presence of the genes potentially improves the environmental adaptability of the *Kocuria* isolates.

It was proposed that no common genetic basis for marine adaptation existed among different actinobacterial genera (Penn and Jensen, 2012). Our study shows that the MAG pool in marine *Kocuria* isolates markedly differs from those in *Salinispora* or marine *Streptomyces*, further supporting this suggestion. Specifically, over half the MAGs in *Kocuria* are grouped into electron transport systems, however, in *Salinispora* channels and pores account for more than half of the MAGs (Penn and Jensen, 2012). K^+ transporters (Trk) and betaine/carnitine/choline transporters are highly rich in marine *Streptomyces* group (Tian et al., 2016). It is noteworthy that although the MAG pool varies significantly in gene content and abundance among different actinomycete genera, the type and function of the MAGs are almost consistent, i.e., respiration/electron transport system, cation and ABC transporters and channels/pores. The results suggest that the functional adaptation of diverse actinomycetes to marine environments is convergent. Previous analyses revealed that the mechanisms of marine adaptation in *Salinispora* spp. were fundamentally different from those reported for Gram-negative bacteria (Penn and Jensen, 2012). Our finding that the gene content in the MAG pool of *Kocuria* significantly differs from that of Gram-negative bacteria also supports this conclusion. The role of Nqr in Gram-negative marine bacteria seems to be replaced by that of a proton-pumping NADH dehydrogenase in marine actinomycetes. In contrast to the well-studied Gram-negative bacteria, much fewer researches have focused on Gram-positive bacteria. With increasing Gram-positive bacteria investigated from a genomic view, a clear picture of marine adaptation in Gram-positive bacteria will be obtained.

CONCLUSION

This study reveals that *K. flava* S43 might adapt to the marine environment by increasing the number of the genes related to potassium homeostasis, phosphate metabolism and resistance to heavy metals, and acquiring the genes associated with electron transport and the genes encoding ABC transporter, aquaporin, and thiol/disulfide interchange protein. Gene acquisition is potentially a primary mechanism of environmental adaptation in *K. flava* S43. Furthermore, this study indicates that the *Kocuria* isolates from various marine and hyperosmotic environments have common genomic characteristics for environmental adaptation. The findings expand our knowledge of the genetic basis for marine adaptation in *Kocuria*.

AUTHOR CONTRIBUTIONS

ZL and WS conceived and designed the study. MZ was responsible for sequencing and assembly. WS, CL, and FZ performed data analyses. WS and ZL drafted the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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TABLE S1 | The functional annotation of the unique ORFs in the *K. flava* S43 genome.

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REVIEW
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Diversity and Applications of Endophytic Actinobacteria of Plants in Special and Other Ecological Niches

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Actinobacteria are wide spread in nature and represent the largest taxonomic group within the domain Bacteria. They are abundant in soil and have been extensively explored for their therapeutic applications. This versatile group of bacteria has adapted to diverse ecological habitats, which has drawn considerable attention of the scientific community in recent times as it has opened up new possibilities for novel metabolites that may help in solving some of the most challenging problems of the day, for example, novel drugs for drug-resistant human pathogens, affordable means to maintain ecological balance in various habitats, and alternative practices for sustainable agriculture. Traditionally, free dwelling soil actinobacteria have been the subject of intensive research. Of late, symbiotic actinobacteria residing as endophytes within the plant tissues have generated immense interest as potential source of novel compounds, which may find applications in medicine, agriculture, and environment. In the light of these possibilities, this review focuses on the diversity of endophytic actinobacteria isolated from the plants of extreme habitats and specific ecological niches. Furthermore, an attempt has been made to assign chemical class to the compounds obtained from endophytic actinobacteria. Potential therapeutic applications of these compounds and the utility of endophytic actinobacteria in agriculture and environment are discussed.

Keywords: endophytic actinobacterial divsersity, special habitats, chemical diversity, secondary metabolites, therapeutics, plant growth promotion, phytopathogens

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INTRODUCTION

Actinobacteria are Gram-positive bacteria with high G+C content, which are filamentous with substrate and aerial mycelia. They rose to prominence during the hunt for drugs from microbes following the discovery of Penicillin from *Penicillium notatum* by Alexander Fleming. They are now well established as prolific producers of a wide range of bioactive secondary metabolites such as antibiotics, enzymes, enzyme inhibitors, antioxidants, and others having therapeutic significance (Barka et al., 2016). Approximately, 22,000 bioactive secondary metabolites of microbial origin have been reported, of which fifty percent are from actinobacteria only. Approximately 160 antibiotics are being currently used in human therapy and in agriculture (Berdy, 2012). However, this is a small number to meet the ever-growing requirement for such compounds. Therefore, there is an urgent need for further exploration of actinobacteria to expand the repertoire of bioactive molecules. Since only a small part of the bioactive molecules isolated from actinobacteria and their biosynthetic gene clusters are studied, bioprospecting of actinobacteria for bioactive molecules holds a great promise.

In view of the emerging threat to deal with drug resistant pathogens, there is an increasing sense of urgency for discovery and development of new drugs. In order to address this challenge, novel approaches need to be devised for searching new molecules. One of which is "renaissance in antibacterial discovery from actinomycetes" (Baltz, 2008). Search of special ecological niches along with new methods of isolation of novel genera/species of actinobacteria may lead to the identification of new gene clusters, and hence, new products (Xu et al., 2010). Researchers are now focusing on the isolation of actinobacteria from diverse habitats like oceans (Subramani and Aalbersberg, 2013), extreme environments (Tang et al., 2003; Hamedi et al., 2013), inner tissues of plants (Chankhamhaengdecha et al., 2013), animals excreta (Cao et al., 2012), algae, and lichens (González et al., 2005; Yamamura et al., 2011). Hence, it is highly encouraging to explore the actinobacteria inhabiting special niches like extremophilic plants for discovering hitherto unexploited strains.[1,2,3]

For centuries plants have been extensively used as the sources of bioactive compounds for therapeutic purposes. In the recent times, plant associated microorganisms have been shown to produce compounds of high therapeutic value (Subbulakshmi et al., 2012). The microorganisms residing inside plant tissues, mostly in symbiotic relationship, may include different groups, for example, fungi and bacteria including actinobacteria (Pimentel et al., 2011; Singh and Dubey, 2015). The endophytes complete their life cycle within the host plants, normally without subjecting them to any disadvantage. Endophytes are ubiquitous in nature and remain in specific association with the host plants, for example, mutualism or antagonism but not parasitism (Nair and Padmavathy, 2014). They play significant roles in enhancing growth of the host plants by producing phytohormones and other growth promoting factors. In turn, they are benefited with nutrients and shelter within the host plant. They also improve the host's tolerance for abiotic and biotic stresses, while offering resistance against insects, pests, and pathogens.

Studies revealed that nearly all the plants harbor endophytes (Strobel and Daisy, 2003). These microbes attain a very special niche in plants by colonizing themselves in stem, root, petioles, leaf segments, inflorescence of weeds, fruit, buds, seeds along with dead, and hollow hyaline cells (Hata and Sone, 2008; Stepniewska and Kuzniar, 2013). However, the colonization of endophytes in different plants is highly variable depending on factors like host specificity, developmental stage of the host, geographical conditions, and the extant microbial diversity (Dudeja and Giri, 2014). Extensive and intensive studies are, however, required to interpret the relationship between the host and the endophytes, and effect of such interactions on the production of bioactive metabolites.

Considering the immense diversity of the flora and widespread plant-microbe associations, the structural and functional diversity among endophytic actinobacteria is expected to be immense. Such diverse group of endophytic actinobacteria could be source of novel biomolecules with myriad of applications. This review highlights the occurrence and diversity of endophytic actinobacteria in the plants from special ecological niches: arid zones, mangrove, and saline ecosystems, and aquatic habitats. Furthermore, chemical and structural diversity of the metabolites reported from endophytic actinobacteria and their potential applications in medicine, agriculture and environment are discussed.

ORIGIN AND EMERGING CONCEPTS OF ENDOPHYTES

Majority of the microbes colonizing the plants internally play key roles in plant's fitness and growth. A minor fraction of them may also cause diseases (Andreote et al., 2014). The overall interaction, however, is mutually beneficial (Mendes et al., 2013; Philippot et al., 2013). Thousands of microorganisms can be the inhabitants of a single plant either in the form of epiphytes in the phyllospheric region or as endophytes within tissues of leaves, roots, or stems. Endophytes display extensive diversity (Turner et al., 2013; Andreote et al., 2014).

The presence of microbial cells in the plant tissue was first observed by De Bary (1866), who coined the term endophytes. The study and exploration of these microbes remained ignored for long. De Bary defined endophytes as "any organism that grows within plant tissue." Subsequently, the definition had been modified as the studies on endophytes progressed. The most appropriate and comprehensive of the definitions states that endophytes include a suite of microorganisms that grow intra or inter-cellularly in the tissues of the plants without causing any harmful effect to them.

The endophytic communities have been categorized into different subgroups, such as "obligate" or "facultative" (Rosenblueth and Martínez-Romero, 2006). Obligate endophytes are the microbes that depend solely on the metabolism of plants for survival, and whose transmission amongst plants take place by the action of various vectors or by vertical transmission (Hardoim et al., 2008). On the other hand, the facultative endophytes spend certain stages of their life cycle independent of the host plant. They are indirectly associated with plants through neighboring soil environment and atmosphere (Abreu-Tarazi et al., 2010). As the studies advanced, more hypotheses appeared about the origin of endophytic organisms. Extensive studies were undertaken to find out the origin of endophytic organisms in different species of plants (Hallmann et al., 1997; Mitter et al., 2013). Microorganisms associated with the rhizosphere and with the seeds were considered to be the major source of endophytes. Studies based on genome organization revealed the specific endophytes-plant interactions in terms of plant specificity, abundance, and mode of transmission (Andreote et al., 2014). However, in order to understand the relationship of endophytes and the host-plants, scientists have thoroughly studied the

[1]Available online at: http://www.chemicalbook.com/ChemicalProductProperty_EN_CB6965824.htm

[2]Available online at: http://www.chemspider.com/Chemical-Structure.4450703.html

[3]Available online at: https://www.molinstincts.com/chemical-structure/7-Methoxy-3-6-3-4-tetrahydroxyflavone-cstr-CT1105232405.html

genome sizes and origins of endophytes and compared with those of bacteria and their lifestyles (Dini-Andreote et al., 2012). Studies revealed that the genome size of endophytic microbes were smaller than those of free leaving microbes. Presence of less number of mobile genetic elements accounted for the smaller size but increased genomes stability. These observations suggested that microbes with smaller and stable genome are more likely to establish endophytic association (Mitter et al., 2013). It has been observed that the association between the endophyte and its host-plant begins at very early stage of the plant development (Hasegawa et al., 2006). With advancement in the research on plant-microbe interactions, the definitions were modified to offer a clearer and comprehensive description of the endophytes.

ENDOPHYTIC ACTINOBACTERIAL DIVERSITY IN VARIOUS ECOSYSTEMS

Actinobacteria are predominantly free-living microorganisms found in diverse environments. Soil is the most dominant reservoir for actinobacteria and also represnts the zone of most active interaction between the actinobacteria and the root system of plants. On account of such interactions, the roots of plants can be considered to be the richest source of the endophytic actinobacteria. Studies have demonstrated that plant roots influence the soil region in their vicinity through exudates, which significantly impact plant-microbe interactions in the rhizosphere (Schenk et al., 2012).

Diversity of endophytic actinobacteria had been reviewed by several groups in recent times. Dinesh et al. (2017) had discussed endophytic actinobacteria from terrestrial plants, which included mainly medicinal plants. Nalini and Prakash (2017), Golinska et al. (2015) and Masand et al. (2015) had also reviewed the diversity of endophytic actinobacteria in medicinal plants. Qin et al. (2011) discussed biodiversity of endophytic actinobacteria from crop plants, medicinal plants, Chinese cabbage, and plants from tropical rain forests of China. It had been reported that medicinal plants of tropical rain forests were the richest source of novel endophytic actinobacteria (Qin et al., 2009). Evidently, endophytic actinobacteria from medicinal plants and tropical rain forests have drawn considerable attention of the scientific community till date. But such actinobacteria from the plants of some of the fascinating ecological niches, for example, arid zones, high salt zones (mangroves and halophytes), and aquatic ecosystems have not yet been specifically reviewed. An attempt has, therefore, been made to address this gap in the present review, wherein endophytic actinobacteria from the plants of special niches have been considered. The plants growing in the environments that are under abiotic stress have evolved to cope up with such factors like temperature, humidity, drought, high rainfall, soil salinity, nutrition limitations, and others. These plants are evolutionarily adapted to thrive in the environments characterized by a high degree of abiotic stresses. Physiological features like modification in the root and shoot systems, structure of leaves and anatomical changes like structure of cortex, xylem and phloem aid such plants to survive in conditions like drought, water logging, marshy and salinity. These plants are also referred to as extremophiles due to their ability to thrive in these extreme environmental conditions. Altered environmental conditions are likely to induce changes in plant physiology. Also, there is difference in the chemo-attractants or signal compounds as well as a change in nutrient availability (Kandeler et al., 2006; Haase et al., 2007). These alterations cause changes in the activity and the diversity of microbial communities associated with the plants (Drigo et al., 2009).

Ubiquitous occurrence of the endophytic actinobacteria is evident from their discovery from the plants of diverse climates: arid zones, saline habitats, aquatic ecosystems, and other ecological niches. They have been reported to colonize any tissue or organ of the host plant (Dinesh et al., 2017). It has been noted that different tissues and organs of the plant are colonized by different actinobacteria, which might be determined by the host-microbe interaction. Such processes, which seem to confer a level of selectivity, may impact the endophytic actinobacterial diversity (Nimnoi et al., 2010). From the reports available till date, it is evident that endophytic actinobacteria are abundant in roots, occur moderately in the stems, and have been found in least numbers in the leaves (Gangwar et al., 2014). Such a distribution pattern for the endophytic actinobacteria seems rational since the roots have maximum exposure to and interactions with the microbial population in the rhizosphere. The endophytic actinobacteria are broadly considered as *Streptomyces* spp. and non-*Streptomyces* spp. The non-*Streptomyces* spp. are uncommon and are classified as rare taxa.

Similarly, the variation in climatic conditions contributes to greater diversity in their flora and the resident microbial communities. For example, the microbial communities of tropical and temperate regions possess more diverse range of endophytes (Strobel and Daisy, 2003). Studies have revealed that physiological diversity is linked to the geographical diversity of the host plants (Du et al., 2013). Despite the relationship between abundance and diversity of endophytic actinobacteria and plants, no direct correlation among the host plants and their endophytic communities could be established. *Actinosynnema*, isolated from a grass blade, was the first probable actinobacterium of plant origin (Hasegawa et al., 1978). Afterwards, there have been numerous reports on the isolation of endophytic actinobacteria from various plant sources as discussed in the ensuing sections.

Diversity of Endophytic Actinobacteria in Arid Ecosystems

The flora of arid ecosystem remains under continuous abiotic stress like draft and salt accumulation, which induces the development of physiological and molecular stress responses in them. This allows the plants to grow normally under such harsh environment. Aridification is a worldwide problem in agriculture and crop production. It has been found that there is a huge built-up of microorganisms that support the plants to cope up with such environments by developing adaptation strategies (Asaf et al., 2017). It is evident from literature that plant-associated extremophilic and extremotolerant actinobacteria comprised the group of less investigated microbes. It has been reported that actinobacteria residing in deserts display immense capability to survive under adverse conditions of pH or salinity and possess noteworthy gene clusters to produce bioactive compounds

(Mohammadipanah and Wink, 2016). A very few reports are, however, available on the endophytic actinobacteria from arid plants, thus there are good opportunities to study and explore diversity of new microbial species in the plants from arid zones (Thumar et al., 2010).

Huang et al. (2012) reported actinobacteria from arid plants, which included several genera like *Streptomyces, Micromonospora, Nocardia, Nonomuraea,* and *Amycolatopsis*. Drought tolerant endophytic actinobacteria, *Streptomyces coelicolor* DE07, *Streptomyces olivaceus* DE10 and *Streptomyces geysiriensis* DE27 are some of the endophytic actinobacteria reported in the plants of arid regions (Yandigeri et al., 2012). Goudjal et al. (2014) reported 22 *Streptomyces* spp. and five non-*Streptomyces* spp. on the basis of morphology and chemotaxonomic analyses, isolated randomly from five plants well adapted to the poor sandy soil and arid climatic conditions of the Algerian Sahara. *Streptomyces mutabilis* strain IA1, isolated from Saharan soil, was another endophyte as it colonized inside the caryopsis up to the endocarp layer in the wheat plant (Toumatia et al., 2016). Wang et al. (2015a,b) had reported isolation of novel endophytic actinobacteria like *Frigoribacterium endophyticum* and *Labedella endophyticum* from the roots of plant *Anabis eliator* from Urumqi (cold arid region), China. Novel endophyte, *Streptomyces zhaozhouensis* was isolated from *Candelabra aloe* which is a succulent collected from Zhaozhou, China (He et al., 2014). A novel species, *Streptomyces ginkgonis* has been isolated from the seeds of *Ginkgo biloba* (Yan et al., 2017). *Glycomyces anabasis,* a novel endophytic actinobacterium had been reported from the roots of *Anabasis aphylla* L of arid region in China (Zhang et al., 2018). In a study on endophytic actinobacteria from the plant *Ferula sinkiangensis* K. M. Shen, 125 endophytic strains were isolated from the roots. These actinobacteria belonged to 3 phyla, 13 orders, 23 families and 29 genera which contained potential novel species (Liu Y. et al., 2017). Plant growth promoting actinobacteria belonging to genus *Amycolatopsis* were also identified during this work. Metagenomic study of arid soil has shown the presence of the genera *Streptomyces, Micromonospora, Saccharothrix, Streptosporangium, Cellulomonas, Amycolatopsis, Geodermatophilus, Lechevalieria, Nocardia*, and *Actinomadura*, but no such reports are available on endophytes from the arid plants (Mohammadipanah and Wink, 2016). *Streptomyces, Actinoallomurus, Amycolatopsis, Kribbella,* and *Microbispora* were isolated from healthy roots of wattle tree, *Acacia auriculiformis* (Thamchaipenet et al., 2010). Despite water and nutrient deprived conditions, the flora of arid region was inhabited by many endophytic actinobacteria that included majorly *Streptomyces*, followed by other rare genera and novel species (**Table 1**).

Diversity of Endophytic Actinobacteria in Mangrove and Non-mangrove Saline Ecosystems

The intertidal zone of estuaries, backwaters, deltas, creeks, lagoons, marshes, and mud-lands are the main habitats of mangrove forests. They are also known as coastal wetland forests which cover around 25% of the overall world's coastline (Spalding et al., 1997; Alongi, 2002). Environment of the mangroves in terms of geographical location, pH, temperature, salinity, moisture and nutrients is highly diverse and different (Amrita et al., 2012; Xu et al., 2014). In tropical mangroves, 91% of the total microbial biomass is bacteria and fungi, another 7% is algae, and 2% is protozoa (Alongi, 1988). They are known for their productivity but very few investigations have been carried on the microbial diversity, specifically for endophytic actinobacteria.

Soil and sediments of mangrove ecosystems have been found to be excellent sources of novel actinobacterial strains (Ara et al., 2007; Han et al., 2007; Eccleston et al., 2008; Huang et al., 2008; Azman et al., 2015). While similar extensive reports are not awailable for endophytic actinobacteria but the novelity in endophytic community could be expected since soil and sedinments serve as reservoir for them. Gupta et al. (2009) reported 105 isolates of endophytic actinobacteria from 19 mangrove plants of Bhitarkanika region, India. Characterization of these isolates led to identification of 20 *Strptomyces* spp., wherein *S. exfoliates* and *S. auranticus* were reported from all the plants. But *S. halstedii*, *S. longisproflavus* and *S. albidoflavus* were reported only from *Kandelia candel*. Upto 118 actinobacterial strains were isolated by Wei et al. (2010) from the plant tissue sampls of Shankou Mangrove Nature Reserve, China. Majority of these isolates were *Streptomyces* spp. (37%), followed by *Micromonospora* spp. (21%). The genera *Saccharothrix* and *Nocardia* contained three isolates each, while both *Nocardiopsis* and *Lentzea* had one isolate each. Higher numbers of endophytic actinobacteria were reported from the mangrove plant *Bruguiera gymnorrhiza* of the Andaman Islands; details are yet to be investigated (Baskaran et al., 2012). Another endophytic halotolerant actinobacteria, *Saccharopolyspora dendranthemae*, was isolated from a coastal salt marsh plant at Jiangsu, China (Zhang et al., 2013). Isolation of several actinobacterial strains belonging to the genera *Streptomyces*, *Nocardiopsis*, *Pseudonocardia*, *Agrococcus,* and *Isoptericola* have been reported from the mangrove plants in Beilun River, Beilum Estuary National Nature Reserve, China (Yang et al., 2015). These isolates were found to produce bioactive compounds which inhibited pathogens like *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus*, *Proteus vulgaris*, hemolytic *Streptococcus* sp., and *Klebsiella pneumoniae*. A novel endophyte, *Phycicoccus endophyticus*, was reported from the *Bruguiera gymnorhiza*, a plant that belonged to Zhanjiang Mangrove Forest National Nature Reserve, Guangdong, China (Liu et al., 2016). Isolation of 11 actinobacterial strains was reported from the mangrove plants, *Rhizophora mucronata* and *Sonneratia caseolaris*. These isolates were found to be good source of antimicrobial and antioxidant compounds (Mesta et al., 2017). A large number of mangrove forests still remain untapped and could prove to be novel sources for isolation of rare and environmentally important endophytic actinobacteria.

Mangrove endophytes are rich in diversity as more than 10 isotales belonging to new species have been discovered from this source in the last few years (Jiang et al., 2017; Liu S. W. et al., 2017; Sun et al., 2017). A new species of endophytic actinobacteria belonging to the genus *Marmoricola* was reported

TABLE 1 | Endophytic actinobacterial diversity in plants of special habitats.

Habitat	Host plant	Endophytic actinobacteria	References
CROPS PLANTS			
Saline	*Limonium sinense*	*Kineococcus endophytica* KLBMP 1274^T, *Streptomyces* sp. KLBMP 5084, *Glutamicibacter halophytocola* sp. nov. KLBMP 5180	Bian et al., 2012a; Feng et al., 2017
Arid	*Lupinus termis*	*Actinoplane missouriensis* CPWT, CPNWT, CNPM	El-Tarabily, 2003
Arid	*Triticum aestivum*	*Streptomyces* sp., *Microbispora* sp., *Micromonospora* sp., *Nocardioides* sp.	Coombs and Franco, 2003
Arid	*Curcuma phaeocaulis*	*Streptomyces phytohabitans* KLBMP 4601^T	Bian et al., 2012b
Arid (Cold)	*Solanum melongena*	*Nonomuraea solani* NEAU-Z6^T	Wang et al., 2013
Arid	*Glycine max*	*Actinoplanes hulinensis* NEAU-M9^T, *Streptomyces harbinensi* NEAU-Da3^T, *Wangella harbinensis* NEAU-J3^T	Jia et al., 2013; Liu et al., 2013; Shen et al., 2013
Aquatic	*Oryza sativa* (Thai jasmine rice plant)	*Actinoallomurus oryzae* sp. nov. GMKU 370^T	Indananda et al., 2011
WOODY PLANTS			
Arid	*Acacia auriculiformis*	*Actinoallomurus acaciae* GMKU 931^T, *Streptomyces* sp. GMKU 937, GMKU 940, *Actinoallomurus coprocola* GMKU 943, *Amycolatopsis tolypomycina* GMKU 932, *Kribbella* sp. GMKU 938, *Microbispora mesophila* GMKU 941 and GMKU 942,	Bunyoo et al., 2009; Thamchaipenet et al., 2010
Arid	*Eucalyptus microcarpa*	*Promicromonospora endophytica* EUM 273^T	Kaewkla and Franco, 2013
Arid	*Camptotheca acuminate*	*Blastococcus endophyticus* YIM 68236^T, *Plantactinospora endophytica* YIM 68255^T	Zhu et al., 2012, 2013
Semi- Arid	*Dracaena cochinchinensis*	*Streptomyces* sp. (HUST 001, HUST 011, HUST 014, 015, 018) *Nocardiopsis* sp. HUST 017, *Pseudonocardia* sp. HUST 013	Salam et al., 2017
Mangrove (Lowlands)	*Aquilaria crassna*	*Streptomyces javensis* GQ179657, *Nonomuraea rubra* GQ179656, *Actinomadura glauciflava* GQ179654, *Pseudonocardia halophobica* GQ179660, *Nocardia alba* GQ179653	Nimnoi et al., 2010
Mangrove	*Xylocarpus granatum*	*Jishengella endophytica* 202201^T	Xie et al., 2011
Mangrove	*Avicennia marina, Aegiceras corniculatum, Kandelia obovota, Bruguiera gymnorrhiza, and Thespesia populnea*	*Streptomyces* sp., *Curtobacterium* sp., *Mycobacterium* sp., *Micrococcus* sp., *Brevibacterium* sp., *Kocuria* sp., *Nocardioides* sp., *Kineococcus* sp., *Kytococcus* sp., *Marmoricola* sp., *Microbacterium* sp. *Micromonospora,* sp., *Actinoplanes* sp., *Agrococcus* sp., *Amnibacterium* sp., *Brachybacterium* sp., *Citricoccus* sp., *Dermacoccus* sp., *Glutamicibacter* sp., *Gordonia* sp., *Isoptericola* sp., *Janibacter* sp., *Leucobacter* sp., *Nocardia* sp., *Nocardiopsis* sp., *Pseudokineococcus,* sp., *Sanguibacter* sp., *Verrucosispora* sp.,	Jiang et al., 2018
Mangrove	*Thespesia populnea*	*Marmoricola endophyticus* 8BXZ-J1^T	Jiang et al., 2017
MEDICINAL PLANTS			
Arid	*Lobelia clavatum*	*Pseudonocardia endophytica* YIM 56035^T	Chen et al., 2009
Arid	*Elaeagnus angustifolia*	*Micromonospora* sp. D30401, D30202, D30511 C10401 and D30407, *Nonomureae* sp. D10204, *Pseudonocardia* sp. C20201 *planotetraspora* sp. C10404	Chen et al., 2011
Arid	*Aloe arborescens*	*Micrococcus aloeverae* AE-6^T, *Streptomyces zhaozhouensis* NEAU-LZS-5^T	He et al., 2014; Prakash et al., 2014
Arid (Cold)	*Psammosilene tunicoides*	*Allostreptomyces psammosilenae* YIM DR4008^T	Huang et al., 2017
Mangrove	*Centella asiatica*	*Streptomyces* sp., *wenchangensis* 234402, *Actinoplanes brasiliensis* IFO13938, *Couchioplanes caeruleus* SCC 1014, *Gordonia otitidis* IFM 10032, *Micromonospora schwarzwaldensis* HKI0641	Ernawati et al., 2016
Mangrove	*Terminalia mucronata*	*Micromonospora terminaliae* CAP94^T	Kaewkla et al., 2017
Mangrove	Mangrove medicinal Plants of Macao	*Friedmanniella* sp. 4Q3S-3 and *Nakamurellas* sp. 2Q3S-4-2	Li et al., 2017
OTHER PLANTS			
Mangroves	*Jatropha curcas*	*Jatrophihabitans endophyticus* S9-650^T, *Nocardioides panzhihuaensis* KLBMP 1050^T, *Nocardia endophytica* KLBMP 1256^T, *Kibdelosporangium phytohabitans* KLBMP 1111^T	Qin et al., 2011, 2012b, 2015; Xing et al., 2011; Madhaiyan et al., 2013
Mangrove	*Sonneratia apetala*	*Micromonospora sonneratiae* 274745^T	Li et al., 2013
Mangrove	*Bruguiera sexangula*	*Mangrovihabitans endophyticus* S3Cf-2^T	Liu S. W. et al., 2017
Saline	*Salicornia europaea*	*Modestobacter roseus* KLBMP 1279^T	Qin et al., 2013a

(Continued)

TABLE 1 | Continued

Habitat	Host plant	Endophytic actinobacteria	References
Saline	*Tamarix chinensis*	*Streptomyces halophytocola* KLBMP 1284^T	Qin et al., 2013b
Saline	*Dendranthema indicum*	*Glycomyces phytohabitans* KLBMP 1483^T, *Amycolatopsis jiangsuensis* KLBMP 1262^T	Xing et al., 2013, 2014
Saline	*Viola philippica*	*Micromonospora violae* NEAU-zh8^T	Zhang et al., 2014b
Saline	*Costus speciosus*	*Micromonospora costi* CS1-12^T	Thawai, 2015
Saline	*Glycyrrhiza uralensis*	*Phytoactinopolyspora endophytica*	Li et al., 2015
Saline	*Salsola affinis*	*Okibacterium endophyticum* EGI 650022^T, *Arthrobacter endophyticus* EGI 6500322^T,	Wang et al., 2015c, Wang et al., 2015d
Arid	*Dysophylla stellata*	*Rothia endophytica* YIM 67072^T	Xiong et al., 2013
Arid	*Anabasis elatior*	*Frigoribacterium endophyticum* EGI 6500707^T, *Labedella endophytica* EGI 6500705^T	Wang et al., 2015a, Wang et al., 2015b
Arid	*Anabasis aphylla* L.	*Glycomyces anabasis* EGI 6500139^T	Zhang et al., 2018
Arid	*Ferula sinkiangensis*	*Amycolatopsis* sp. SX2R71	Liu Y. et al., 2017
Aquatic	Seaweed	*Streptomyces sp.*	Hemalatha and Rasool, 2017
Aquatic	*Thalassia hemprichii*	*Micromonospora* sp. (HCZ27 HCZ42, M8Z28, G2Z37), *Saccharomonospora* sp. (M8Z39, G2Z41, G2Z21), *Mycobacterium* sp. G2Z43, *Actinomycetospora lutea* G2Z35, *Nonomuraea maheshkhaliensis* M1Z44, *Verrucosispora sediminis* M1Z33, *Nocardiopsis composta* M1Z45, *Microbacterium esteraromaticum* HCZ21, *Glycomyces arizonensis* HCZ4, *Streptomyces* sp.	Wu et al., 2012

for the first time from the mangrove plant (Jiang et al., 2017). Jiang et al. (2018) has recently reported endophytic actinobacteria from five different mangrove plants from Beilun Estuary National Nature Reserve, China. Prolific diversity of culturable endophytic actinobacteria had been reported, which included 7 orders, 15 families and 28 genera with significant novelty as there were potentially 7 new species of different genera. Furthermore, the genera, *Sanguibacter,* and *Citricoccus*, were reported for the first time in this study. Mangrove plants of Macao were also reported to be diverse in terms of novel genera and species of endophytic actinobacteria (Li et al., 2017). Two new species of rare genera *Friedmanniella* and *Nakamurella* were found among 192 endophytic actinobactrial strains isolated from 12 plants, which included 30 genera, 17 families, and 8 orders (Li et al., 2017). *Mangrovihabitans endophyticus*, a novel genus and novel species was reported from the mangrove plant *Bruguiera sexangula* (Liu S. W. et al., 2017). A large number of mangrove forests still remain untapped and could prove to be sources for isolation of rare and ecologically important endophytic actinobacteria.

Beside mangroves, halophytic plants from other saline environments have also been explored for endophytic actinobacteria. Saline conditions present a harsh environment for plant species as only <0.2% of the species are evolved to reproduce when exposed to seawater (Flowers and Colmer, 2015). Endophytes residing within such plants are reported to be one of the critical factors that contribute to the survival of these plants in saline environment (Sgroy et al., 2009). A few reports are available on the isolation and characterization of endophytic actinobacteria. A novel isolate, *Modestobacter roseus* KLBMP 1279^T, was obtained from the roots of *Salicornia europaea*, a halophyte of coastal region of Jiangsu Province, China (Qin et al., 2013a). A novel endophytic actinobacterium, *Okibacterium endophyticum,* capable of growth at high salt concentration (upto 7% NaCl) and at high pH was reported to be inhabitant of the roots of the halophyte *Salsola affinis* (Wang et al., 2015c). *Amycolatopsis jiangsuensis* sp. nov., was isolated from a coastal plant *Dendranthema indicum* (Linn.) Des Moul collected from the coastal region of Nantong, China. From this data and some other reports summarized in **Table 1**, it is evident that novel actinobacterial species had been reported from the halophytic plants of various regions. A scan through literature appeared to suggest that halophytic plants presented an attractive source for novel endophytic actinobacteria, most of which are yet to be explored.

Diversity of Endophytic Actinobacteria in Aquatic Ecosystems

The aquatic ecosystem is the largest contributor of the earth's biodiversity. Two fifths of earth's surface is freshwater ecosystem while it's 20th part of the total water (Alexander and Fairbridge, 1999). Lentic (include pools, ponds, and lakes), lotic (streams and rivers) and wetlands are the basic freshwater ecosystems. Rooted plants mainly occur in shallow water. Roots in aquatic plants are poorly developed (Sculthorpe, 1985). Association of diverse actinobacterial communities with marine sponges and soft corals has generated keen interest among researchers (Govindasamy et al., 2014). In view of these findings, it can be expected that significant diversity and novelty in endophytic actinobacterial communities of aquatic plants is waiting to be revealed. The wetlands, known for their fertility and nutrient richness, are among the most studied of the aquatic ecosystems. Rice plants from the wetlands had been explored for endophytic microbial communities as they might be associated with crop yield and also for their role in anaerobic methanogenesis causing global climate change (Bernstein et al., 2007).

A report on seagrass root-associated microorganisms suggested actinobacteria to be the first root colonizers (Jensen et al., 2007). In a study of 110 actinobacteria isolated from seagrass, ten genera of actinobacteria: *Streptomyces, Micromonospora, Saccharomonospora, Mycobacterium, Actinomycetospora, Nonomuraea, Verrucosispora, Nocardiopsis, Microbacterium,* and *Glycomyces* were reported along with four novel species (Wu et al., 2012). Twenty-one endophytic actinobacteria isolated from marine seaweed, Kovalam Beach, India, were used in generating copper nanoparticles that were active against *Klebsiella pneumoniae, Proteus mirabilis, Escherichia coli, Salmonella typhimurium, and* methicillin resistant *Staphylococcus aureus* (MRSA). The 16S rRNA analysis showed that 2 of the 21 isolates belong to the *Streptomyces* sp. (Hemalatha and Rasool, 2017). In another study of endophytic actinobacteria isolated from marine green algae *Cauler pataxifolia*, it was found that DSM3 had potential in producing antibiotic against multidrug resistant Gram-negative bacteria (Rajivgandhi et al., 2018). He also isolated *Nocardiopsis* sp. GRG1 (KT235640), an endophytic actinobacterium from brown algae. It was also active against multidrug resistant Gram-negative bacteria (Rajivgandhi et al., 2016). There are very few reports from the marine non-mangrove environments. Due to water logged condition and high salinity of oceans, the aquatic flora is under immense stress and is of immense importance due to its capacity to withstand such conditions. There is probability that the associating microorganisms could have played significant role in survival of this aquatic flora. Therefore, an extensive research on endophytic actinobacteria of marine plants is called for establishing their significance in growth and survival of the plants under such conditions.

16S rRNA Gene Sequence-Based Phylogenetic Analysis of Endophytic Actinobacterial Diversity in Special Niches

Nucleotide sequences of 16S rRNA gene of endophytic actinobacteria (**Table 1**) were retrieved from NCBI GenBank. Phylogenetic analyses of these sequences were performed using the software, Phylogeny.fr (Dereeper et al., 2008, 2010). Sequences were saved in FASTA format and then copied and pasted at the space provided in the online tool. The phylogenetic analyses were carried out using maximum likelihood. We have used one click mode which is an automated programme that performs step by step analysis starting from the alignment of the sequences (MUSCLE 3.8.31; Edgar, 2004), alignment refinement (Gblocks 0.91b), phylogeny (PhyML 3.1/3.0 aLRT) (Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006) to the tree rendering (TreeDyn 198.3; Chevenet et al., 2006). Further, the Inkscape 0.92 was used to make any highlighting and editing in the tree for clarity of facts (www.inkscape.org). *Escherichia coli* K-12 strain DH10B was used as outgroup for this phylogenetic study.

In order to consider evolutionary relatedness among the isolates falling in the same clade, bootstrap value of $\geq$85% and $\geq$90% were considered for determining closeness among different genera and different species of the same genus respectively. Some general observations that could be made based on this criterion from the data presented in **Figure 1**, included: (1) Different species of the same genus either from different or the same habitats showed high bootstrap values ($\geq$90%) indicating high degree of relatedness among them; (2) different genus from the same or different niche displayed bootstrap values of $\geq$85%, suggesting closer evolutionary relationship among them. In addition, different species of a genus from the same habitat were found to be placed within the same clade but the bootstrap values were <65%, indicating low confidence level in respect of their evolutionary closeness. However, for a deeper insight into evolutionary aspects of these microbes, further investigations involving other evolutionary parameters appear necessary.

METAGENOMICS BASED DIVERSITY OF ENDOPHYTIC ACTINOBACTERIA

Culturable approach to study diversity is often limited by access to only a minor fraction of the microbial community. The metagenomic approach, which employs PCR-based culture-independent method, offers a more comprehensive view of the diversity as it includes both culturable and non-culturable part of the population. Some of the recent metagenomic studies that highlighted the importance of the uncultured endophytic actinobacteria in the diversity of endophytic actinobacterial population are being discussed.

In the study of community structure of endophytic actinobacteria of the *Pseudowintera colorata* (Horopito), a native medicinal plant of New Zealand, it was seen that the microbial communities in leaves were more diverse as compared to the roots and stems on the basis of DGGE pattern. The abundance of actinobacterial taxa was highest in stems (39%), followed by leaves (34%), and roots (27%). However, three clones among them were identified as uncultured bacteria (Purushotham et al., 2018). Similar study of the endophytic actinobacterial population from medicinal plant *Centella asiatica* produced 16 major clones pertaining to the various communities of actinobacteria in the sample. Community of actinobacteria in the plant tissues was slightly more diverse than those of rhizosphere in this case (Ernawati et al., 2016). However, *Streptomyces* sp. was the abundant followed by members of the family *Micromonosporaceae* and *Gordoniaceae*. Furthrmore, the uncultured bacterial abundance was also significantly high. It shows that there is a need for optimization of isolation procedures in order to enhance the chances (Ernawati et al., 2016). According to a report on endophytic actinobacterial species diversity in the stem of *Gynura cusimbua* by 16S rRNA, it was observed that out of 63 positive clones, 16S rRNA sequence of 59 strains had higher similarity to the closest type strain and belonged to the genera *Microbacterium, Arthrobacter, Micrococcus, Curtobacterium, Okibacterium, Quadrisphaera, and Kineococcus* respectively (Zhang et al., 2016). On the other hand, the rest of clones were showing little similarity and belonged to unclassified families: *Micrococcineae*, *Intrasporangiaceae,* and *Microbacteriaceae* that are supposed to be uncultured endopohytic actinobacteria (Zhang et al., 2016). Culture independent methods were also used for the community study

FIGURE 1 | Phylogenetic diversity of some endophytic actinobacteria from extreme ecological niches based on 16S rRNA gene sequences. The tree was constructed using "one click" mode in Phylogeny.fr. *E. coli* was used as outgroup for this study.

of the endophytes related to the sponges, *Hymeniacidon perleve* and *Sponge* sp. An uncultured actinobacterium of the genus *Acidimicrobium* accounted for 33% and 24%, respectively in the above two sponges (Xin et al., 2008). Some other reports are also available on the metagenomic studies of stem and roots of rice (Tian et al., 2007), roots of wheat (Conn and Franco, 2004), roots and shoots of *Aquilaria crassna* (Nimnoi et al., 2010), leaves of grapevine (Bulgari et al., 2009), and *Maytenus austroyunnanensis* (Qin et al., 2012a). These studies revealed that the diversity of uncultured endophytic actinobacteria is comparable to those of the cultured endophytic actinobacteria. In order to get complete information on the diversity of endophytic actinobacteria and their functional importance, it is important to study the uncultured endophytic actinobacteria by metagenomic approach along with the culturable ones.

CHEMICAL DIVERSITY AND THERAPEUTIC SIGNIFICANCE OF SECONDARY METABOLITES PRODUCED BY ENDOPHYTIC ACTINOBACTERIA

Phytochemical profiling to identify and discover therapeutic compounds from plant extracts has been a common practice since long (Tiwari et al., 2011; Ahmad et al., 2013). However, comparable studies on microbial extracts are scanty. In view of immense potential of the secondary metabolites of actinobacteria for diverse applications, it is highly desirable that vigourous efforts are made to explore the chemical diversity of these metabolites. It is in the light of this observation that the relevant information from literature is being presented here.

Actinobacteria are known producers of a broad spectrum of bioactive molecules. They employ non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) pathways to achieve the structural and functional diversity of their secondary metabolites. They are reported to produce immunosuppressants (e.g., rapamycin), antibiotics (e.g., erythromycin), anticholesterol drugs (e.g., lovastatin), and anticancer drugs (e.g., epothilone B) which underlined their indispensibility for the pharmaceutical industries (Miller et al., 2012; Weber et al., 2015). Actinobacteria in general and *Streptomyces* spp. in particular have been explored extensively for drugs and therapeutics. The secondary metabolites produced by the endophytic actinobacteria were reported to fall majorly under pharmaceutically important classes like alkaloids, flavonoids, steroids, terpenoids, phenolics, quinones, and peptides (Yu et al., 2010). For instance, in a study of isolation and characterization of compounds from mangrove endophytic actinobacteria, 73 novel compounds and 49 known compounds were characterized as alkaloids, benzene derivatives, cyclopentenone derivatives, dilactones, macrolides, 2-pyranones and sesquiterpenes (Xu et al., 2014). Chemical diversity of therapeutically important metabolites from endophytic actinobacteria have been analyzed in the present work. Chemical classes have been defined for several of such compounds, as presented in **Table 2** and discussed briefly in the following sections. Strucutre of some of these compounds has been given in **Table 3**.

Alkaloids

Alkaloids are characterized by the presence of basic nitrogen (usually in a heterocycle). In recent years, the number of new alkaloids isolated, identified, and characterized for their medicinal potential from various sources including from the endophytic actinobacteria has increased. Paclitaxel (taxol), a well known anti-cancer drug, was originally isolated from the plant, *Taxus baccata*. Subsequently, it was also isolated from the endophytic actinobacteria of *Taxus baccata*: *Kitasatospora* sp. P and U 22869 (Caruso et al., 2000). Recently, various alkaloids, namely,1-acetyl-β-carboline, indole-3-carbaldehyde, tryptophol, 3-(hydroxyacetyl)-indole, brevianamide F, cyclo-(L-Pro-L-Phe), cyclo-(L-Pro-L-Tyr), cyclo-(L-Pro-L-Leu), and cyclo-(L-Val-L-Phe) were reported from the endophyte, *Aeromicrobium ponti* LGMB491 isolated from *Vochysia divergens* (Gos et al., 2017). These compounds displayed very good antibacterial activities against various *Staphylococcus* spp. Other alkaloids, 3-acetonylidene-7-prenylindolin-2-one, and 7-isoprenyl indole-3-carboxylic acid, isolated from endophytic *Streptomyces* sp. neau- D50, possessed anti-cancer activity (Zhang et al., 2014a). β-Carbolines and 3-Indole compounds were obtained from the endophyte *Microbiospora* sp. LGMB259, which showed inhibitory activities against Gram-positive bacterial strains: *Micrococcus luteus* NRRL B-2618 and *Kocuria rosea* B-1106. They aslo possess cytotoxic activities against two human cancer cell lines: prostate cancer cell line PC3 and the non-small-cell lung carcinoma cell line (Savi et al., 2015a). Diketopiperazine also belongs to this family with very good medicinal property. In this context the antifungal and anticancer compounds, Lansai A-D, were reported from the endophytic *Streptomyces* sp. SUC1 isolated from aerial roots of *Ficus benjamina* (Tuntiwachwuttikul et al., 2008). Diketopiperazine gancidin W (GW) was isolated from the *Streptomyces* sp. SUK10, an endophyte from the bark of *Shorea ovalis* tree, and it was tested *in vivo* against *Plasmodium berghei* PZZ1/100 (Zin et al., 2017). Kakadumycin A, a quinoxaline antibiotic with broad spectrum activity against Gram-positive bacteria and malaria parasites was reported from endophytic *Streptomyces* sp. NRRL 30566, isolated from the fern, *Grevillea pteridifolia*. It was structurally similar to echinomycin, but had shown better antibiotic activity (Castillo et al., 2003). Antifungal compound, 6-Prenylindole was isolated from *Streptomyces* sp. TP-A0595, an endophyte of liverwort (Sasaki et al., 2002). Igarashi (2004) isolated pure compound anicemycin, a cytotoxic compound potentially active against tumor cell line at very low concentration.

Polyketides

This group of actinobacterial secondary metabolites includes compounds with diverse chemical structures having significant activities of therapeutic importance, like, anticancer, antifungal, anticholesteremic etc. Many reports have shown that endophytic actinobacteria are proven to be good source for the discovery of polyketide compounds. Endophyte *S. hygroscopicus* TP-A0623, isolated from the root of *Clethra barbinervis,* produced a polyene polyketide, clethramycin, known to inhibit both spheroplast regeneration and germ tube formation in *Candida albicans*, (Furumai et al., 2003). Ansamitocin, a polyketide macrolide, was isolated from endophytic actinobacteria *Nocardia* sp. no. C-15003 (Higashide et al., 1977). It had potent antibacterial and antitumor activities, and was structurally similar to maytansine, a known antibacterial drug. Another drug, pterocidin, derived from endophytic isolate, *S. hygroscopicus* TP-A0451 was cytotoxic against some human cancer cell lines (Igarashi et al., 2006). The compound, linfuranone isolated from Thai medicinal plant has shown antidiabetic and antiatherogenic activities in mouse ST-13 pre-adiopocytes (Indananda et al., 2013). Several polyketides compounds of endophytic actinobacterial origin were reported as summarized in **Table 2**.

Terpenes and Terpenoids

Terpenes and terpenoids are known to be the primary constituents of the essential oils of many types of medicinal plants and flowers. They have been widely used for fragrances in perfumery, and in medicines. Besides plants, microbes have also been proved to be good sources for such compounds (Tholl, 2015). Biologically active terpenes and terpenoids have been isolated from several endophytic actinobacteria (**Table 2**). Cedarmycin A and B, isolated from an endophyte, *Streptomyces* sp. TPA0456, were found to possess broad spectrum anti-bacterial, and anti-fungal activities (Sasaki et al., 2001). It was seen to be specifically more potent against *Candida glabrata*. Only a few compounds with anti-viral activity had been reported from endophytic actinobacteria. One among them being xiamycin, produced by *Streptomyces* sp. GT2002/1503, isolated from *Bruguiera gymnorrhiza* that specifically blocked R5 tropic HIV infection (Ding et al., 2010).

TABLE 2 | Chemical diversity of the metabolites of endophytic actinobacteria and their therapeutic significance.

Bioactive Compounds	Habitat	Plant source	Therapeutic application	Actinobacteria species	References
ALKALOIDS					
6-Prenylindole	Temperate	*Allium tuberosum*	Antifungal	*Streptomyces* sp. TP-A0595	Sasaki et al., 2002
Anicemycin	Temperate	*Aucuba japonica*	Antitumor (anchorage-independent growth inhibitor)	*Streptomyces thermoviolaceus* TP-A0648	Igarashi, 2004
Kakadumycin A	Arid	*Gravillea pteridifolia*	Antibacterial, Antimalaria	*Streptomyces* sp. NRRL 30566	Castillo et al., 2003
Diketopiperazine, Gancidin W	Tropical	*Shorea ovalis*	Low toxic, Antimalarial agent	*Streptomyces* sp. SUK10	Zin et al., 2017
Cyclo-(L-Val-L-Pro), Cyclo-(L-Leu-L-Pro), Cyclo-(L-Phe-L-Pro), Cyclo-(L-Val-L-Phe), and N-(7-hydroxy-6-methyl-octyl)-acetamide. (Diketopiperazines)	Tropical	*Zingiber spectabile*	Antibacterial	*Streptomyces* sp.SUK 25	Alshaibani et al., 2017
1-Acetyl-β-carboline, Indole-3-carbaldehyde, 3-(Hydroxyacetyl)-Indole, Brevianamide F, and Cyclo-(L-Pro-L-Phe)	Aquatic (wetland)	*Vochysia divergens*	Antibacterial	*Aeromicrobium ponti* LGMB491	Gos et al., 2017
1-Vinyl-b-carboline-3-Carboxylic acid, Indole-3- carbaldehyde, Indole-3-acetic acid and Indole-3- carboxylic acid	Aquatic (wetland)	*Vochysia divergens*	Antibacterial activity	*Microbispora* sp. LGMB259	Savi et al., 2015b
Lansai A-D	Tropical	*Ficus benzamina*	Antifungal and anticancer	*Streptomyces* sp. SUC1	Tuntiwachwuttikul et al., 2008
3-Acetonylidene-7-Prenylindolin-2-one and 7-Isoprenylindole-3-carboxylic acid	Arid (Cold)	*Glycine max*	Antifungal	*Streptomyces* sp. neau-D50	Zhang et al., 2014a
2-(furan-2-yl)-6-(2S,3S,4-trihydroxybutyl)pyrazine	Mangrove	*Xylocarpus granatum*	Antiviral	*Jishengella endophytica* 161111	Wang et al., 2014
POLYKETIDES					
Pterocidin	Subtropical	*Pteridium aquilinum*	Anticancer	*Streptomyces hygroscopicus* TP-A0451	Igarashi et al., 2006
Linfuranone	Tropical	*Clinacanthus siamensis*	Antimicrobial, non-cytotoxic	*Microbispora* sp. GMKU 363	Indananda et al., 2013
Clethramycin	Tropical	*Clethra barbinervis*	Antifungal	*Streptomyces hygroscopicus* TP-A0623	Furumai et al., 2003
Ansamitocin	Tropical	*Maytenus serrata*	Antibacterial and antitumor	*Actinosynnema pretiosum*	Higashide et al., 1977; Siyu-Mao et al., 2013
TERPENES AND TERPENOIDS					
Demethylnovobiocins	Temperate	*Aucuba japonica*	Antimicrobial	*Streptomyces* sp. TPA0556	Igarashi, 2004
Cedarmycin A and B	Temperate	*Aucuba japonica*	Antibacterial, anti-Candida	*Streptomyces* sp. TP-A0456	Sasaki et al., 2001
Xiamycin	Mangrove	*Bruguiera gymnorrhiza*	Antiviral	*Streptomyces* sp.GT2002/1503	Ding et al., 2010
COUMARINS (ALPHA BENZOPYRONES)					
5,7-Dimethoxy-4-pmethoxylphenylcoumarin; 5,7-Dimethoxy-4-phenylcoumarin	Tropical	*Zingiber officinale*	Antifungal agent, Antioxidants, Antitumor	*Streptomyces aureofaciens* CMUAc130	Taechowisan et al., 2007
Saadamycin	Marine	*Aplysina fistularis*	Antifungal agent	*Streptomyces* sp. Hedaya 48	El-Gendy and El-Bondkly, 2010
FLAVONOIDS (GAMMA BENZOPYRONES)					
7-Methoxy-3, 3′,4′,6-tetrahydroxyflavone and 2′,7-Dihydroxy-4′,5′-Dimethoxyisoflavone, Fisetin, Naringenin, 3′-Hydroxydaidzein, Xenognosin	Tropical	*Boesenbergia rotunda (L.)*	Antibacterial	*Streptomyces* sp. BT01	Taechowisan et al., 2014
Kaempferol, Isoscutellarin,Umbelliferone and Cichoriin	Temperate	*Alpinia galanga*	Antioxidants	*Streptomyces* sp. Tc052	Taechowisan et al., 2009
QUINONES					
Alnumycin	Temperate	*Alnus glutinosa*	Antibacterial	*Streptomyces* sp.DSM 1175	Bieber et al., 1998
Celastramycins A and B	Tropical	*Celestracae* family plants	Antimycobacterial, Antibacterial	*Streptomyces setonii,* sp. Q21, *Streptomyces sampsonii,* QuH- 8	Pullen et al., 2002

(Continued)

TABLE 2 | Continued

Bioactive Compounds	Habitat	Plant source	Therapeutic application	Actinobacteria species	References
Lupinacidin C	Arid	*Lupinus angustifolius*	Antitumor	*Micromonospora lupini* Lupac 08	Trujillo et al., 2007; Igarashi et al., 2011
Naphtomycin A	Tropical	*Maytenus hookeri*	Antitumor	*Streptomyces* sp. CS	Lu and Shen, 2007
TANNINS					
Streptol	Arid (Cold)	*Cucubalus* sp.	Anti-fungal	*Dactylosporangium*sp. *strain* SANK 61299	Okazaki, 2003
PEPTIDES AND THEIR DERIVATIVES					
Actinomycin X2	Temperate	*Rhododendron* sp.	Antimicrobial	*Streptomyces* sp. R-5	Shimizu et al., 2004
Munumbicins A, B, C and D	Tropical	*Kennedia nigricans*	Antimicrobial, Antimalarial, Antitumor	*Streptomyces* sp. NRRL 30562	Castillo et al., 2002
Coronamycin	Tropical	*Monstera* sp.	Antifungal, Antimalarial	*Streptomyces.* sp. MSU-2110	Ezra et al., 2004
Munumbicins E-4 and E-5	Tropical	*Kennedia nigricans*	Antimalarial, antibacterial	*Streptomyces* sp. NRRL 30562	Castillo et al., 2006
S-adenosyl-Nacetylhomocysteine	Tropical	*Puereria candollei*	Antioxidant, Neuroprotection	*Micromonospora* sp. PC1052.	Boonsnongcheep et al., 2017
Proximicin	Arid	*Sonchus oleraceus*	Antibacterial, Antitumor	*Verrucosispora maris* AB-18-032.	Fiedler et al., 2008; Roh et al., 2011; Ma et al., 2016
FATTY ACID DERIVATIVE					
6-alkalysalicilic acids, salaceyins A and B	Arid	*Ageratum conyzoides*	Anticancer	*Streptomyces laceyi* MS53	Kim et al., 2006
7-Octadecenamide	Arid	*Sonchus oleraceus*	Antimicrobial	*Nocardia caishijiensis*	Tanvir et al., 2016
9, 12- Octadecadienamide (Linoleamide)	Arid	*Sonchus oleraceus*	Antimicrobial	*Pseudonocardia carboxydivorans* SORS 64b	Tanvir et al., 2016

Benzopyrones

The compounds from this class consist of a benzene ring joined to a pyrone ring. The benzopyrones can be subdivided into the benzo-alpha pyrones, which include mainly coumarins and the benzo-gamma pyrones, of which the flavonoids are principal members (Jain and Joshi, 2012).

Coumarins

(Benzo-alpha pyrones): Several novel compounds isolated from the endophytic actinobacteria belong to this class. *Streptomyces* sp. TPA0556 isolated from the plant *Aucuba japonica* produced two novel antibiotics: 7′- demethylnovobiocin and 5″-demethylnovobiocin with broad spectrum antibiotic activity against both Gram-positive and Gram-negative bacterial pathogens (Sasaki et al., 2001). A novel coumarin, saadamycin was reported from *Streptomyces* sp. Hedaya48 with significant antimycotic activity specifically against dermatophytes (El-Gendy and El-Bondkly, 2010). 5, 7-Dimethoxy-4-phenylcoumarin extracted from endophytic *S. aureofaciens* was effective in preventing or delaying formation of metastases (Taechowisan et al., 2007).

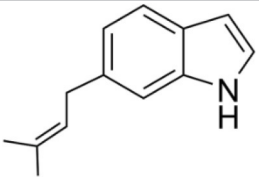
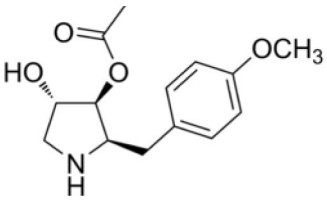
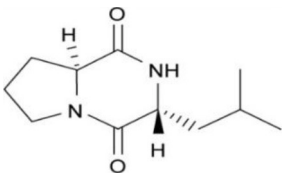
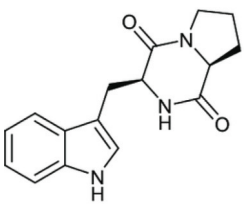
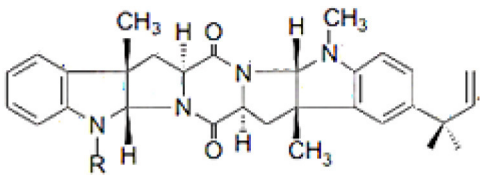
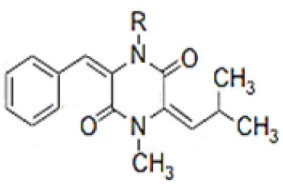
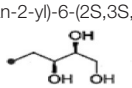
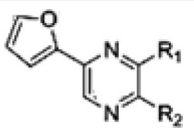
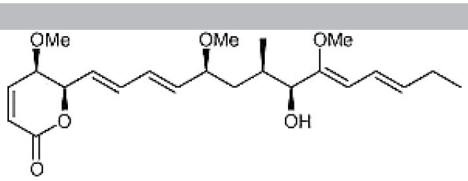
Flavonoids

(Gamma-benzopyrones): Flavonoids are polyphenolic structures, widely found in fruits, vegetables and certain beverages. They are known for their biochemical and antioxidant effects associated with various diseases such as cancer, Alzheimer's disease (AD), atherosclerosis etc. (Panche et al., 2016). Some of the flavonoids from endophytic actinobacteria include: 7-methoxy-3,3′,4′,6-tetrahydroxyflavone; 2′,7-dihydroxy-4′,5′-dimethoxyisoflavone, fisetin, naringenin, 3′-hydroxydaidzein and xenognosin B. They were isolated from endophytic *Streptomyces* sp. BT01. It has shown good activity against Gram-positive bacteria; *Staphylococcus aureus* ATCC25932, *Bacillus cereus* ATCC 7064, and *Bacillus subtilis* ATCC 6633 (Taechowisan et al., 2014). Kaempferol, isoscutellarin, umbelliferone, and cichoriin are some other flavonoids obtained from the endophytic *Streptomyces* sp. Tc052, which showed anti-oxidative and inhibitory activities on nitric oxide production (Taechowisan et al., 2009).

Tannins

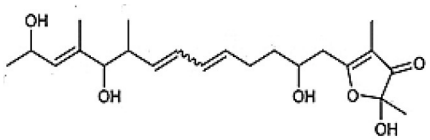
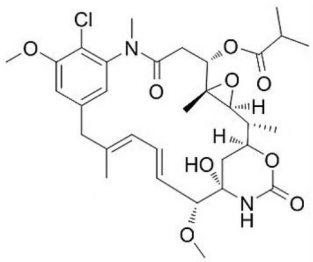
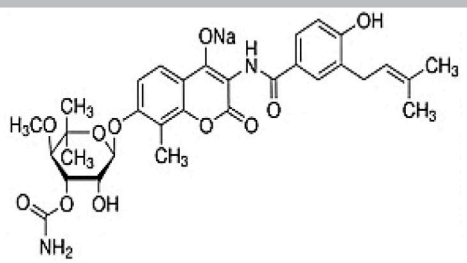
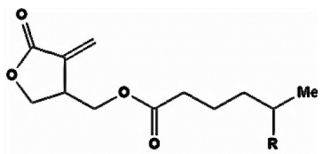
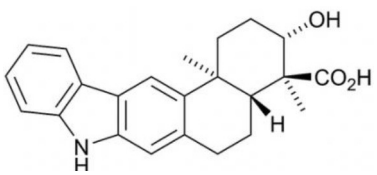
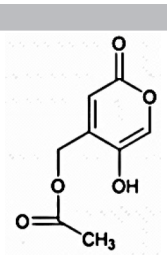
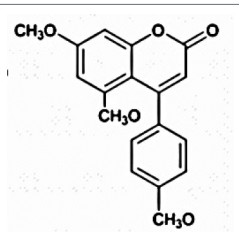
They are water soluble polyphenols that are majorly found in plant foods. They are reported to be involved in improving human health by providing anticancer and antibacterial activities. However, in many cases they can be inhibitory as they decrease the nutritional value of foods by forming complexes with proteins, starch, and digestive enzymes (Chung et al., 1998). Not many compounds of tannins family are reported from endophytic actinobacteria. Streptol, a tannin isolated from endophytic strain SANK 61299 of the actinobacterium

TABLE 3 | Chemical Structures of some important therapeutic compounds of different chemical classes isolated from endophytic actinobacteria.

Bioactive compounds	Chemical structure	References
ALKALOIDS		
6 Prenylindole		Sasaki et al., 2002
Anicemycin		Igarashi, 2004
Gancidin W		Zin et al., 2017
Brevinamide F		Amar et al., 2012
Lansai A; R = H, Lansai B; R = CH_3		Tuntiwachwuttikul et al., 2008
Lansai C; R = OH, D; R = H		Tuntiwachwuttikul et al., 2008
2-(furan-2-yl)-6-(2S,3S,4-trihydroxybutyl)pyrazine R1 =  R2 = H		Wang et al., 2014
POLYKETIDES		
Pterocidin		Igarashi et al., 2006

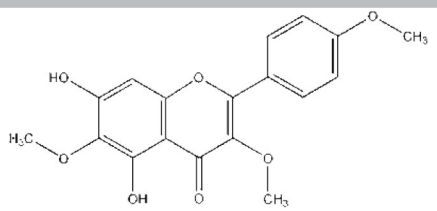
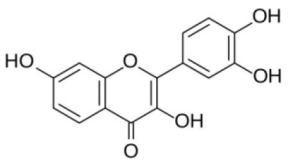
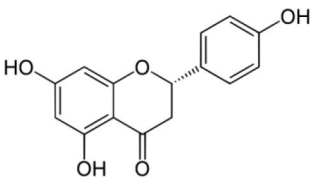
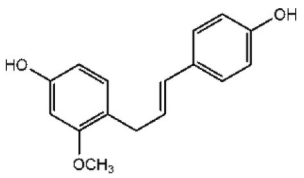
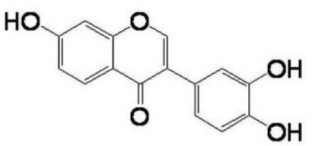
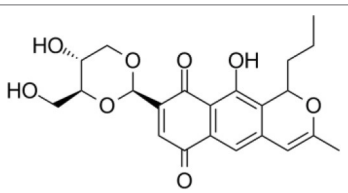
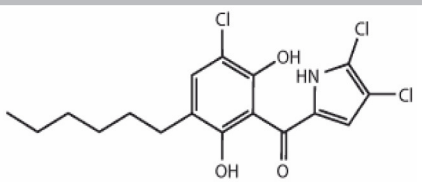
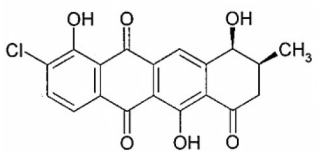
(Continued)

TABLE 3 | Continued

Bioactive compounds	Chemical Structure	References
Linfuranone		Indananda et al., 2013
Ansamitocin		Siyu-Mao et al., 2013
TERPENES AND TERPENOIDS		
Demethylnovobiocins		Kominek, 1972
Cedarmycin		Sasaki et al., 2001
Xiamycin		Li et al., 2012
COUMARINS (ALPHA BENZOPYRONES)		
5,7-Dimethoxy-4- pmethoxylphenylcoumarin		Indraningrat et al., 2016
Saadamycin		Indraningrat et al., 2016

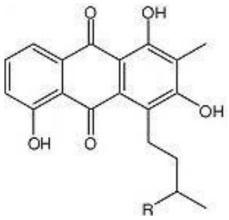
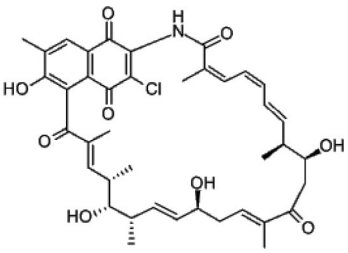
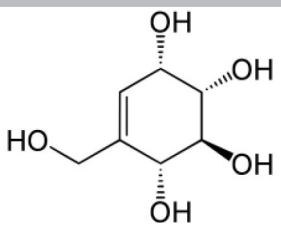
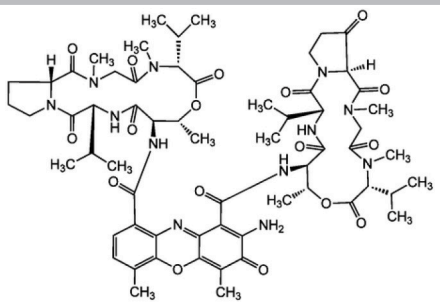
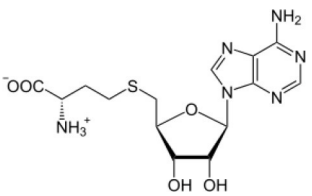
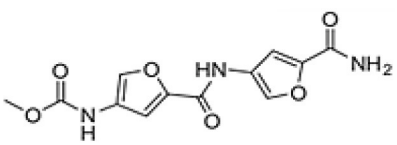
(Continued)

TABLE 3 | Continued

Bioactive compounds	Chemical Structure	References
FLAVONOIDS (GAMMA BENZOPYRONES)		
7-Methoxy-3, 3′,4′,6-tetrahydroxyflavone		Molinstincts chemical structures ID CT1105232405
Fisetin		Khan et al., 2013
Naringenin		Taechowisan et al., 2014
Xenognosin		Taechowisan et al., 2014
Hydroxydaidzein		Taechowisan et al., 2014
Alnumycin		Bieber et al., 1998
QUINONES		
Celestamycin A		Pullen et al., 2002
Celestamycin B		Pullen et al., 2002

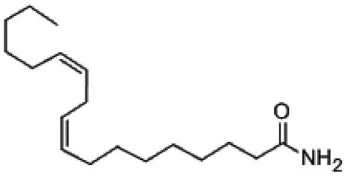
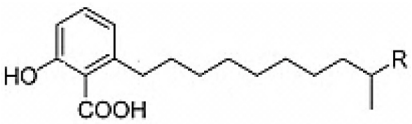
(Continued)

TABLE 3 | Continued

Bioactive compounds	Chemical Structure	References
Lupinacidin (R = CH3 or H)		Igarashi et al., 2011
Naphthomycin A		Lu and Shen, 2007
TANNINS		
Streptol		Chemspider ID 4450703
PEPTIDES AND THEIR DERIVATIVES		
Actinomycin X2		Shimizu et al., 2004
S-adenosyl-N acetylhomocysteine		Boonsnongcheep et al., 2017
Proximicin A		Fiedler et al., 2008

(Continued)

TABLE 3 | Continued

Bioactive compounds	Chemical Structure	References
FATTY ACID DERIVATIVE		
Linoleamide		CAS:3072-13-7
Salaceyins A(R = CH_3) ; B (R = CH_2CH_3)		Kim et al., 2006

Dactylosporangium sp. from the plant, *Cucubalus* sp. exibited broad spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria (Furumai et al., 2003).

Quinones

Quinones are a group of compounds produced by plants, fungi, and bacteria. They have an aromatic di-one or di-ketone system, generally derived from the oxidation of hydroquinones. They show potential therapeutic activities like neurological, antibacterial, antiplasmodial, antioxidantal, trypanocidal, antitumor, and anti-HIV that are shown to be related to the redox properties of their carbonyl functions (Villamil et al., 2004).

Streptomyces sp. CS, isolated from *Maytenus hookeri*, came into focus for the production of naphthomycin K, together with two known naphthomycins A and E which had shown cytotoxicity against P388 and A-549 cell lines (Lu and Shen, 2007). Another naphthoquinone, alnumycin was isolated from *Streptomyces* sp. DSM 11575, an endophyte inhabiting the root nodules of *Alnus glutinosa*. It has displayed narrow spectrum antibacterial activity against *Bacillus subtilis* tested by well diffusion assay. Endophytic actinobacterium *Micromonospora lupine* had been reported to produce anticancer compound lupinacidin (anthraquinone) A-C. Lupinacidin C was shown to inhibit invasion of murine colon carcinoma cells into the reconstituted basement membrane (Igarashi et al., 2011). Endophytic *Streptomyces* sp. MaBQuH-8, isolated from woods of the trees of family *Celastraceae*, was found to produce antibiotics: celastramycins A and B having antimicrobial activity against Gram positive bacteria and mycobacteria.

Fatty Acid Derivatives

Some bioactive compounds reported from endophytic actinobacteria fall into the category of fatty acid derivatives. The compounds: 7-coctadecenamide and 9,12-octadecadienamide (Linoleamide), were derived from two rare actinobacteria, *Nocardia caishijiensis* and *Pseudonocardia carboxydivorans,* isolated from the plants *Sonchus oleraceus* and *Ageratum conyzoides* respectively. These compounds had shown very good activity against various human pathogens including *Candida* spp. strains, Gram-positive and Gram-negative bacteria (Tanvir et al., 2016). Kim et al. (2006) studied cytotoxic activity of 6-alkalysalicilic acids, salaceyins A and B from *S. laceyi* MS53 against human breast cancer cell line.

Peptides and Their Derivatives

Microorganisms play a significant role in the production of peptide antibiotics. Endophytic actinobacteria being unique in their habitat can be a source of some novel therapeutic peptides.

Streptomyces sp. NRRL 30562, an endophyte isolated from *Kennedia nigriscans* was reported to produce actinomycin X2, a broad-spectrum polypeptide antibiotic having potential activity against human and plant pathogenic bacteria and fungi (Castillo et al., 2006). Another endophyte, *Streptomyces* sp. TC022, isolated from the roots of *Alpinia galangal*, is capable of producing actinomycin D, highly potential as antifungal, and antitumor polypeptide. It was also active against plant and human fungal pathogens, for example, *Colletotrichum musae* and *Candida albicans* (Taechowisan et al., 2006). Coronamycin, identified as a complex of novel peptides antibiotics, was obtained from verticillate *Streptomyces* sp. MSU-2110 isolated from *Monstera* sp. It has potential activity against pythiaceous fungi and the human fungal pathogen *Cryptococcus neoformans.* It was also reported to be active against *Plasmodium falciparum* with IC_{50} value of 9.0 ng/ml. In another study on the secondary metabolites of endophytic actinobacteria isolated from roots and root nodules of *Pueraria candollei* Graham ex Benth, isolation of S-adenosyl-N-acetylhomocysteine (an antibacterial and antioxidant compound) was isolated from *Micromonospora* sp. for the first time (Boonsnongcheep et al., 2017).

Therapeutic Spectrum of Uncharacterized Metabolites

There are several reports demonstrating wide range of therapeutic properties for the metabolite preparations from endophytic actinobacteria. But isolation and characterization of the active compounds have not been undertaken for many of them. Endophytic *Streptomyces* sp. SUK 06, isolated

from the plant *Thottea grandiflora,* was reported to produce metabolites with strong activity against bacterial pathogens including MRSA as well as phytopathogens (Ghadin et al., 2008). In a study on the endophytes associated with medicinal plants, many actinobacteria producing compounds with antibacterial activity against a panel of Gram positive and Gram negative bacterial pathogens were reported (Li et al., 2008). Like-wise endophytic actinobacteria isolated from the roots of *Alpinia galangal*, *Streptomyces* sp. Tc052, was shown to possess antimicrobial activity with MIC values lying between 64-128 μg/ml (Taechowisan et al., 2008). *Streptomyces* sp. TQR12-4, an endophyte of *Citrus nobilis* Lour was reported to produce thermostable antifungal compound with MIC values of 100 μg/ml and 400 μg/ml (Thao et al., 2016). Crude extracts of the metabolites from *Microbispora* sp. LGMB259, LGMB250, LGMB255 and LGMB256 showed more than 98% antitumor activity against Glioblastoma multiforme cells (Savi et al., 2015b). Furtermore, the metabolites of actinobacteria strains: SORS64b, SORS124, AGRS16, AGLS2, and AGRS19 isolated from the plants of *Asteraceae* family showed antioxidant property using DPPH assay (Tanvir et al., 2014). In another study on five endophytic isolates, it was reported that their metabolites displayed excellent antioxidant properties and were found to increase the survival of plants under oxidative stress (Babu et al., 2014). Ethyl acetate extract of cell-free broth of actinobacteria isolated from *Catharantus roseus* showed marked α-glucosidase inhibitory activity (Yokose et al., 1983). Some *in vivo* studies also showed that the partially purified metabolite preparations of endophytic actinobacterium, *Streptomyces* sp. worked effectively against diabetes by reducing systemic glucose levels in the experimental animals (Kulkarni-Almeida et al., 2011). Remarkable α-glucosidase inhibitory activity was reported by the metabolites produced by several strains of *Streptomyces* sp. isolated from the roots of Indonesian medicinal plants, *Caesalpinia sappan* (Pujiyanto et al., 2012). Antidiabetic activity was studied in the extract of *Streptomyces* sp. JQ92617, isolated from *Rauwofia densiflora*, while crude extract of *S. longisporoflavus* JX96594 was found to be a potential α-amylase and α-glucosidase inhibitor (Akshatha et al., 2014).

The foregoing discussion thus presents a strong evidence for the endophytic actinobacteria to be an excellent source for the production of a wide range of natural products with significant potential for therapeutic applications. The diversity of the compounds produced could be comparable to that of plants. The use of endophytic actinobacteria for production of therapeutic compounds may offer some distinct advantage over plants in terms of production under controlled environmental conditions for strict adherence to quality, shorter production period and sustainability.

BIOSYNTHETIC GENE CLUSTERS IN ENDOPHYTIC ACTINOBACTERIA

It is evident from the literature that the genes involved in the synthesis of bioactive secondary metabolites are present in the actinobacterial genome in the form of gene clusters (Doroghazi and Metcalf, 2013). Genome mining tools have made it more convenient to look for innovations in natural product discovery (Corre and Challis, 2007). Majority of the bioactive compounds are encoded by large gene clusters such as polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) with highly repetitive modules (Jackson et al., 2018).

The biosynthetic pathway of polyketides is governed by a complex enzyme system, called polyketide synthase encoded by PKS gene cluster. Aromatic polyketide synthases are encoded by PKS II. On the other hand, the polyketides formed as a result of condensation of acetate, propionate and butyrate acyl units are synthesized by modular polyketide synthase that is encoded by PKS I gene (Shinwari et al., 2013). Other than polyketide synthases, there is another class of enzyme known as Non-ribosomal peptide synthetases (NRPS). NRPS gene clusters are involved in the biosynthesis of wide range of peptide antibiotics, toxins, siderophore, anti-inflammatory compounds, immunosuupressants etc. (Cane and Walsh, 1999; Crosa and Walsh, 2002; Mansson et al., 2011). Another gene cluster ANSA (ansamicin) is involved in the production of ansamycins; while OxyB codes for glycopeptide antibiotics (Ayuso-Sacido and Genilloud, 2005). In a study on taxonomic diversity and metabolic activity of endophytic actinobacteria of some medicinal plants, it was reported that all the 80 bioactive isolates possessed atleast one test gene. The frequency of occurrence biosynthetic gene clustures in the isolates was reported to be 55.0, 58.8, 90.0, 18.8, and 8.8% for the PKS-I, PKS-II, NRPS, ANSA, and OxyB genes respectively. In this study, it was also found that ANSA gene was more active in the rare actinobacteria, while PKSII genes were frequent in *Streptomyces* sp. However, the NRPS gene was found at almost same frequency in *Streptomyces* and non-*Streptomyces* spp. Approximatley, 46.3% of the isolates were found to be positive for all the four gene clusteres together (Qiu et al., 2015). This implied that gene clusteres are not species specific. In another study of 81 endophytic actinobacteria isolated from *Rhynchotoechum ellipticum* showing antimicrobial and antioxidant activities, presence of gene clusures were analyzed. The results showed that PKS I, PKS II, and NRPS gene clusteres occurred in 19, 51, and 30% of isolates respectively. However, there were 17 isolates possessing all the three genes clusteres. This study also reported that these isolates synthesized several antibiotics: erythromycin, ketoconazole, fluconazole, chloramphenicol, rifampicin and miconazole, phenolic compounds; catechin, kaempferol, chebulagic acid, chlorogenic acid, asiatic acid, ferulic acid, arjunic acid, gallic acid and boswellic acid, and paclitaxel. It is thus evident that these gene clusteres were reposnsible for the production of secondary metabolites (Passari et al., 2018). Frequencies for the presence of biosynthetic genes in the endophytic actinobacteria of the plant *Dracaena cochinchinensis* Lour was 29.4, 70.6, and 23.5%, for PKS-I, PKS-II, and NRPS respectively (Salam et al., 2017). Gene cluster encoding hybrid trans-acyltransferase (AT) polyketide was identified during the genome analysis of endophytic actinobacterium, *Actinoplanes* isolated from the plant *Amphipterygium adstringens* for the first time (Centeno-Leija et al., 2016). In genome analysis of the endophyte, *Streptomyces kebangsaanensis,* it was established

that 24 gene clusters were involved in the production of a novel phenanzine. It was also predicted to be involved in the biosynthesis of polyketide, nonribosomal peptide, terpene, bacteriocin, and siderophore (Remali et al., 2017). Presence of PKS and NPRS genes were also repoted in the endophytic actinobacteria of aquatic ecosystem. In a study on endophytic actinobacteria isolated from sea grass *Thalassiahemprichii*, PKS-II, and NRPS genes were detected in almost all of the isolates while PKS-I could be amplified in only half of them (Wu et al., 2012). In another study involving 23 endophytic actinobacteria from tropical plants of Papua New Guinea and Mborokua Island, Solomon Islands, presence of all the three gene clusters PKS I, PKS II and NPRS were reported but no *in vitro* activity was detected. This observation led the authors to interpret that there was no correlation between the presence of gene cluster and the activity (Janso and Carter, 2010). But the inability to detect the bioactive metabolite might be due to non-expression of the genes under the experimental conditions for production.

Available whole genome draft of endophytic actinobacteria also revealed the presence of PKS and NPRS genes suggesting that these microbes are the possible source for many novel bioactive compounds. Genome Sequence of *Microbispora* sp. GMKU 363 harbor three type I PKS gene clusters associated with the synthesis of linfuranone (Komaki et al., 2015). *Streptomyces scabrisporus* NF3, an endophyte isolated from *Amphipterygium adstringens*, carries at least 50 gene clusters for the synthasis of polyketides and nonribosomal peptides (Vazquez-Hernandez et al., 2017). Genome sequence of *Streptomyces* sp. PRh5, a novel endophytic actinobacterium isolated from wild rice root, has been predicted to have putative nigericin and nocardamine biosynthetic gene clusters (Yang et al., 2014). Similarly, *Streptomyces* sp. XY006, an endophyte isolated from Tea (*Camellia sinensis*) had 27 possible secondary metabolite biosynthetic gene clusters encoding for antimycin, melanin, informatipeptin, albaflavenone, and roseoflavin (Vazquez-Hernandez et al., 2017). Genome mining of *Streptomyces wadayamensis* A23, an endophytic strain showed the presence of biosynthetic gene clusteres for antimycins and candicidin (Angolini et al., 2016).

It has been reported that these gene clusters are involved not only in the biosynthesis of bioactive secondary metabolites, but also in the production plant growth promotion factors. Endophytic actinobacteria isolated from *Artemisia annua* possessed herbicidal activity against *Echinochloa crusgalli* and carried PKSI, PKSII, and NRPS gene clusters (Li et al., 2012). *Kibdelosporangium phytohabitans* KLBMP 1111^{T}, an endophyte of *Jatropha curcas*, possessed the gene clusters responsible for polyketide and nonribosomal peptide synthesis and also the genes related to the plant growth promoting factors, such as zeatin, 1-aminocyclopropane-1-carboxylate deaminase (ACCD) and siderophore (Qin et al., 2015).

Streptomyces sp. XY006 genome sequence revealed the presence of genes associated with synthesis of indole-3-acetic acid, 1-aminocyclopropane-1-carboxylate deaminase, mineral phosphate solubilization, transport and assimilation, and also genes involved in fungal cell-wall degradation, such as a family 19 chitinases (Vazquez-Hernandez et al., 2017). Genome sequence of *Streptomyces* sp. GKU 895 isolated from sugarcane root, showed gene cluster related to plant growth-promoting activity (Kruasuwan et al., 2017). Endophytic actinobacteria isolates of halophytic plant *Salsola imbricate* were found to be active against phytopathogens *Phytophthora capsici* and *Pythium ultimum* and also possessed the biosynthetic gene cluster PKS I, II and NRPSs (Bibi et al., 2018). The screening of the gene clusters in the endophytic actinobacteria can be used as a high throughput method to carry out screening for the presence of bioactive secondary metabolites.

OPPORTUNITIES FOR APPLICATION OF ENDOPHYTIC ACTINOBACTERIA IN AGRICULTURE

Use of chemical fertilizers, insecticides, pesticides, and other synthetic agents as nutrients or as biocontrol agents has led to increase in crop productivity and yield (Aktar et al., 2009). However, prolonged use of these substances had adverserly affected the physico-chemical properties of soil, leading to decline in soil fertility and microbial diversity. Also, use of synthetic insecticides and pesticides have resulted in resistant pests and insects along with bioaccumulation of harmful agricultural chemicals and their residues in the consumers invoking serious health concerns (Prashar and Shah, 2016). Evidently, such agricultural practices have come to be considered as non-viable and called for the development and implementation of sustainable alternatives.

Advances in the field of soil microbiome, plant microbiome and increasing understanding of plant-microbe interactions have opened up new vistas for sustainability in agriculture. In this section, we briefly discuss the potential applications of endophytic actinobacteria in protection of plants from diseases, plant nutrition, growth, and tolerence to biotic and abiotic stresses.

Endophytes in Protection Against Plant Diseases

It has been earlier reported in several studies that endophytic actinobacteria can effectively inhibit plant pathogens, thus protect the host-plants from diseases (Taechowisan et al., 2003; Cao et al., 2004). An endophytic actinobacterial strain CEN26 of *Centella asiatica* was inhibitory against the fungal pathogen *Alternaria brassicicola*. It acts by inhibiting germination of conidia and morphological development (Phuakjaiphaeo and Kunasakdakul, 2015). In a study involving 260 isolates of endophytic actinobacteria, majority of them were seen to be inhibiting plant pathogens, and thus had a protective role for protection of the hosts against diseases (Palaniyandi et al., 2013). The type of interaction among the actinobacteria and their host-plants seems to be important in suppression of diseases (Hasegawa et al., 2006; Maggini et al., 2017). Leaf blight disease of rice was cured to a greater extent by *S. platensis* (Wan et al., 2008). Mycolytic, parasitic, and antibiotic activities against *Fusarium* sp. was reported in *Nocardiopsis* sp. (Sabaou et al., 1983). Fifty-five percent of the total endophytes recovered from leaves of *Paeonia*

lactiflora and *Trifalium repens* were found to inhibit growth of the phytopathogen, *Rhizoctonia solani* (Gu et al., 2006). Fistupyrone, isolated from *Streptomyces* sp. TP-A0569 inhibited *in vivo* infection of the seedlings of Chinese cabbage by necrotrophic plant pathogen, *Alternaria brassicicola* TP-F0423 (Igarashi et al., 2000). In a recent study, *Streptomyces* sp. PRY2RB2, an endophyte of the medicinal plant *Pseudowintera colorata* (Horopito), a native of New Zealand, have shown inhibitory activity against various phytopathogens: *Neofusicoccum luteum* ICMP 16678, *N. parvum* MM562, *Ilyonectria liriodendri* WPA1C and *Neonectria ditissima* ICMP 14417 (Purushotham et al., 2018). Endophytic actinobacterium, strain AR3, isolated from *Emblica officinalis*, inhibited the growth of *Fusarium oxysporum* (Kamboj et al., 2017). Another endophyte, *Streptomyces* sp. EN27, has also been reported to display antifungal activity against *F. oxysporum* (Conn et al., 2008). He also concluded that resistance to *F. oxysporum* by this strain occurred via an NPR-dependent pathway alongwith salicylic acid but was independent of jasmonic acid/ethylene (JA/ET) pathway. Some other reports of plant protection against phytopathogens have been documented in **Table 4**.

Endophytic Actinobacteria in Plant Growth Promotion

Endophytic actinobacteria are known for their potential to affect plant growth and nutrient uptake (Rajkumar et al., 2006). They are reported as plant growth promoters in cereals and legumes (Mishra et al., 1987; Salam et al., 2016). In some studies, it was observed that endophytes are very good producers of plant growth promoting factors like phytohormones. Indole acetic acid (IAA) is among the highly reported growth regulator from endophytic actinobacteria (Khamna et al., 2010; Palaniyandi et al., 2013). Endophytic *Streptomyces* strains producing IAA significantly improved the growth of tomato plants (Verma et al., 2011). There are reports suggesting that the endophytic actinobacteria isolated from one plant could promote growth of the other plants. For instance, rhizospheric endophytic actinobacteria of Yam plant of Yeoju, South Korea, enhanced the growth of *Arabidopsis thaliana* by producing IAA, phosphate solubilization, and prominent 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity (Palaniyandi et al., 2013). However, some of the studies suggested that there is negative impact of IAA as it has been verified as one of the virulence factors in some phytpathogenic actinobacteria (Vandeputte et al., 2005). Endophytic actinobacteria of wheat crop, for example, *S. olivaceoviridis, S. rimosus* and *S. rochei* produced auxins, gibberellins, and cytokinin-like substances that positively influenced plant growth (Aldesuquy et al., 1998). It has been suggested that formulations of endophytic actinobacteria can be a potential step to promote sustainable agriculture (Hasegawa et al., 2006; Palaniyandi et al., 2013). Plant growth promoters, Pteridic acids A and B, isolated from endophytic *S. hygroscopicus* TP-A045 had auxin-like activity and helped in the improved formation of adventitious root in hypocotyls of kidney beans. They showed potency comparable to the auxins (Igarashi et al., 2002). Another *Streptomyces* sp. MBR-52 also augmented the emergence and elongation of plant adventitious roots (Hasegawa et al., 2006; Meguro et al., 2006). Ethylene a phytohormone is responsible for the physiological responses to both abiotic and biotic environmental stresses to plants (Sun et al., 2006). Extreme physiological conditions like temperature, drought and salinity can induce the production of ethylene. Endophytic actinobacteria residing in such plants might lessen the negative impact of stress by production of enzyme ACC deaminase. This enzyme hydrolyzes 1-aminocyclopropane-1-carboxylic acid (ACC) and hence decreases the ethylene production in plants. In some studies, it is also reported that this enzyme is capable of hydrolyzing ACC into a-ketobutyrate and ammonia, and provide nitrogen to the microorganisms for their growth (Viterbo et al., 2010; Xing et al., 2012).

Phosphorus is one of the essential elements for plants necessary for various biological processes like transport of glucose, stimulation of cell proliferation and promotion of organ development (Ahemad and Kibret, 2014). But the phosphorus available in the soil is not accessible to plants directly (Ezawa et al., 2002). Endophytic actinobacteria play important role in solubilization of phosphates and enhance its availability to plants by means of acidification, chelation, redox changes and mineralization of organic phosphorus (Nautiyal et al., 2000; Van der Hiejden et al., 2008). Phosphate solubilization activity along with the phytase secretion was reported from endophytic *Streptomyces* sp. isolated from *Triticum aestivum* plant. It has shown significant plant growth improvement (Jog et al., 2014).

Besides phosphorus, iron also plays an imperative role in the physiological processes and enzymatic activities in plants (Bothwell, 1995). For iron to be taken up by plants, it needs to be solubilised. The availability of iron to plant roots was enhanced by siderophores produced by microorganisms in the form of bacterial siderophore-iron complex, or phytosiderophore-iron complex (Rajkumar et al., 2009; Ma et al., 2011). *S. acidiscabies* E13 being an excellent producer of siderophore, promotes the growth of *Vigna unguiculata* under nickel stress conditions (Dimkpa et al., 2009; Sessitsch et al., 2013). In a recent study on the community of endophytic actinobacteria of New Zealand native medicinal plant *Pseudowintera colorata* (Horopito), *Streptomyces* sp. UKCW/B and *Nocardia* sp. TP1BA1B were found to solubilise phosphate and produce siderophores (Purushotham et al., 2018).

Some reports had shown direct benefits of endophyte on plants by tissue culture. For example, inoculation of halophytic plants with plant growth-promoting (PGP) actinobacteria was reported for the improvement of salt tolerance in plants (Qin et al., 2017). *Streptomyces coelicolor* (MAR1), an endophyte isolated from mangrove plants of Pichavaram, Tamilnadu (India) was reported to be active against various bacterial and fungal pathogens. It was also tolerant for high salt concentration and high pH (Gayathri and Muralikrishnan, 2013).

The endophytic actinobacteria also possess the ability to produce growth inhibitors in order to improve health of the host-plants. Some herbicidal compounds had been reported from the endophytic actinobacteria. Herbicidin H isolated from *Streptomyces* sp. SANK 63997, an endophyte of

TABLE 4 | Endophytic actinobacteria in protection of host-plants against phytopathogens.

Endophyte	Host-plant	Target pathogen	References
Streptomyces sp. S30, *Streptomyces* sp. R18(6)	*Lycopersicon esculentum*	*Rhizoctania solani*	Cao et al., 2004; De Oliveira et al., 2010
Actinoplanes campanulatus, Micromonospora chalcea and *Streptomyces spiralis*	*Cucumis* sp.	*Pythium aphanidermatum*	El-Tarabily et al., 2010
Microbispora sp. A004 and A011, *Streptomyces* sp. A018	*Brassica rapa*	*Plasmodiophora brassicae*	Lee et al., 2008
Streptomyces diastaticus, Streptomyces fradiae, Streptomyces olivochromogenes, Streptomyces collinus, Streptomyces ossamyceticus Streptomyces griseus (CC1, CC4, CC12, CC20, CC23, CC29, CC31, CC38, CC41, CC42, CC52 and CC53)	Medicinal plants	*Sclerotium rolfsii, Rhizoktonia solani, Fusarium oxysporum, Alternaria solani*	Singh and Gaur, 2016
Streptomyces sp., LBR02, AB131-1 and AB131-2	Host plant information not available	*Xanthomonas oryzae* pv. *oryzae* (Xoo)	Hastuti et al., 2012
Streptomyces sp. DBT204	*Solanum lycopersicum*	*Fusarium proliferatum*	Passari et al., 2016
Leifsonia xyli BPSAC24,*Streptomyces* sp. BPSAC34	*Eupatorium odoratum, Musa superb, Mirabilis jalapa, Curcuma longa, Clerodendrum colebrookianum, Alstonia scholaris, Centella asiatica,*	*Rhizoctonia solani, Fusarium graminearum, Fusarium oxysporum ciceri, Fusarium prolifratum, Fusarium oxysporum, Fusarium graminearum, Colletotrichum capsici*	Passari et al., 2015a
Microbispora sp. isolate 1and 5	*Solanum tuberosum* L.	*Streptomyces scabies*	Goodman, 2014
Streptomyces sp. R-5	*Rhododendron* sp.	*Phytophthora cinnamom, Pestalotiopsis sydowiana*	Shimizu et al., 2000
Streptomyces sp., MBPu-75 *Streptomyces* sp. MBCu-56	*Cucumis* sp.and *Cucurbita* sp.	*Colletotrichum orbiculare*	Shimizu et al., 2009
Streptomyces sp. RM 365	Leguminous plants	*Xanthomonas campestris* pv. *glycine*	Mingma et al., 2014
Streptomyces thermocarboxydus TF23, *Streptomyces* sp. TF2, TF21 and TF30	*Lycospersicum esculentum*	*Rhizoctonia solani*	Inderiati and Franco, 2008
Nocardiopsis sp. ac9, *Streptomyces Violaceorubidus* 6ca11, *Streptomyces* sp. ac19	*Elaeis guineensis* Jacq.	*Ganoderma boninense*	Ting et al., 2014
Streptomyces mutabilis CA- 2, *Streptomyces cyaneofuscatus* AA-2	*Aristida pungens* *Cleome arabica* *Solanum nigrum* *Panicum turgidum, Astragallus armatus* *Peganum harmala* *Hammada scoparia* *Euphorbia helioscopia*	*Rhizoctonia solani*	Goudjal et al., 2014
Streptomyces felleus YJ1	*Brassica napus*	*Sclerotinia sclerotiorum*	Cheng et al., 2014
Streptomyces sp.	*Garuga pinnata* *Gmelina arborea* *Stephania venosa* *Melastoma malabathricum* *Merremia vitifolia*	*Pectobacterium carotovorum*	Chankhamhaengdecha et al., 2013
S. griseorubiginosus-like strain	*Musa acuminata*	*Fusarium oxysporum*	Cao et al., 2005
Actinoplanes missouriensis CPWT	*Lupinus termis*	*Plectosporium tabacinum*	El-Tarabily et al., 2010
Streptomyces sp. EACK and *Streptomyces* sp. EAOP	*Streptomyces lycopersicum* Mill.	*Ralstonia solanacearum*	Sreeja and Gopal, 2013
Streptomyces sp. AzR-051, AzR- 049, AzR- 010	*Azadirachta indica* A. Juss.	*Alternaria alternata*	Verma et al., 2011
Streptomyces sp.AOK-30	*Kalmia latifolia* L.	*Pestalotiopsis sydowiana*	Nishimura et al., 2002
Streptomyces violaceusniger	Trees of Dehradun	*Phanerochaete chrysosporium*, *Coriolus versicolor, Gloephyllum trabeum*	Shekhar et al., 2006
Streptomyces sp. PRY2RB2	*Pseudowintera colorata*	*Neofusicoccum luteum* ICMP 16678, *Nocardia parvum* MM562, *Ilyonectria liriodendri* WPA1C, *Neonectria ditissima* ICMP 14417	Purushotham et al., 2018

Setaria viridis var. *pachystachys*, gamma-glutamylmethionine sulfoximine from *Microbispora* sp. residing in *Carex kobomugi* are some of the examples of herbicides of endophytic origin (Okazaki, 2003). A plant growth inhibitor was isolated from actinobacteria *Dactylosporangium* sp. of *Cucubalus* sp. found to inhibit germination of *Brassica rapa* (Furumai et al., 2003). Clethramycin, a pollen tube growth inhibitor extracted from the fermentation broth of *S. hygroscopicus*, was isolated from the roots of *Clethra barbinervis*. This metabolite has mode of action similar to inhibition of MAP kinase by staurosporine and herbimycin (Igarashi et al., 2003). Endophenazines A-D, produced by *S. anulatus* possessed herbicidal activity against *Lemna minor* (Gebhardt et al., 2002).

Besides these beneficial properties, a few phytotoxins have also been reported from the endophytic actinobacteria (Berdy, 2012), but majorly, they play beneficial roles. Thus, it can be safely summed up that there is enormous potential for application of endophytic actinobacteria in agriculture. Some more studies of plant growth promotion by endophytic actinobacteria are reported in **Table 5**.

ENVIRONMENTAL APPLICATIONS OF ENDOPHYTIC ACTINOBACTERIA

Some endophytic actinobacteria isolated from the plant, *Salix caprea,* were reported to release metal binding secondary

TABLE 5 | Plant growth promoting activities of endophytic actinobacteria.

Plant growth promotion			
Plant growth promoting factor	**Endphytic actinobacteria**	**Plant source**	**References**
Nitrogen Fixation	Actinomycetes strains DII, G2	*Casuarina equisetifolia*	Gauthier et al., 1981
IAA and siderophore	*Streptomyces* sp. CMU-PA101 and *Streptomyces* sp. CMU-SK126	*Curcuma mangga*	Khamna et al., 2009
Solubilization of phosphate, production of phytase, chitinase, IAA, siderophore and malate	*Streptomyces* sp. mhcr0816, mhce0811	*Triticum aestivum*	Jog et al., 2014
Production of IAA and ACC deaminase	*Actinoplanes campanulatus, Micromonospora chalcea, Streptomyces spiralis*	*Cucumis sativus*	El-Tarabily et al., 2010
Production of IAA	*Streptomyces* sp. PT2	Plants of Algerian Sahara	Goudjal et al., 2013
Production of chitinase, phosphatase and siderophore	*Streptomyces* sp. AB131-1, LBR02	Isolates of Microbiology Laboratory, Bogor Agricultural University	Hastuti et al., 2012
Production of rooting-promoting plant hormones	*Streptomyces* sp. MBR-52	*Rhododendron ferrugineum*	Meguro et al., 2006
Solubilization of phosphate, production of siderophores, HCN, ammonia, production of chitinase and IAA	*Streptomyces* sp. BPSAC34, *Leifsonia xyli* BPSAC24, *Microbacterium* sp. BPSAC 21, 27, 28 and 29	Medicinal plants	Passari et al., 2015b
IAA	*Streptomyces* sp., *Nocardia* sp., *Nocardiopsis* sp., *Spirillospora* sp., *Microbispora* sp. *and Micromonospora* sp.	*Citrus reticulata*	Shutsrirung et al., 2013
Siderophores	*Streptomyces* sp. GMKU 3100	*Oryza sativa* L. cv. KDML105	Rungin et al., 2012
Indole-3-acetic acid (IAA), hydroxamate and catechol type siderophore, protease	*Streptomyces* sp. S4202, *Nonomuraea* sp. S3304, *Actinomadura* sp. S4215, *Pseudonocardia* sp. S4201	*Aquilaria crassna*	Nimnoi et al., 2010
Glucoamylase	*Streptosporangium* sp. L21	*Zea mays*	De Araujo et al., 2000; Stamford et al., 2002
Cell wall degrading enzymes	*Streptomyces galbus* R-5	Rhododendron seedlings	Minamiyama et al., 2003
Endo- chitinase enzymes	*Streptomyces violaceusniger* XL- 2	Trees of Dehradun	Shekhar et al., 2006
IAA, siderophore production, Phosphate solubilisation	*Streptomyces sp.* R18(6)	*Lycopersicon esculentum*	De Oliveira et al., 2010
Homoserine lactone degrading enzymes	*Streptomyces* sp. LPC026, LPC029, PC005, PC052, and PC053	*Garuga pinnata*, *Gmelina arborea*, *Stephania venosa, Melastoma malabathricum*, *Merremia vitifolia*	Chankhamhaengdecha et al., 2013
Herbicidin H (herbicide)	*Streptomyces* sp. strain SANK 63997	*Setaria viridis* var. *pachystachys*	Okazaki, 2003
Cellulase, Xylanase, Lignolytic activity	*Nocardiopsis* sp. ac9, *Streptomyces, Violaceorubidus* 6ca11, *Streptomyces* sp. ac19	*Elaeis guineensis* Jacq.	Ting et al., 2014
Phosphate solubilisation and seiderophore production	*Streptomyces* sp. UKCW/B, *Nocardia* sp. TP1BA1B	*Pseudowintera colorata*	Purushotham et al., 2018

metabolites that mobilize zinc (Zn) and cadmium (Cd) from the soil, enhancing their accumulation in the leaves of this plant (Kuffner et al., 2010). In another study involving the endophyte *S. tendae* from sunflower plant, it was reported to mediate phytoremediation of Cd (Dimkpa et al., 2009). These studies have clearly established the potential of endophytic actinobacteria in removal of heavy metals from the soil and their increased uptake by plants growing in the metal-contaminated soil. A combinatorial study of plants and their endophytes can be explored for their application in remediation of heavy metals from contaminated soil sites and consequent improvement in soil health.

A few reports on the potential application of endophytic actinobacteria in pesticide bioremediation have also appeared. Chlordane, pesticide banned due to its adverse effect on human health and environment, was successfully removed by using a mixed culture of actinobacteria. It was shown that the mixed culture produced bioemulsifiers that facilitated bioavailability and uptake of the pesticide (Fuentes et al., 2017).

Endophytic *Streptomyces* sp. had been reported in improving phytoremediation efficiency of petroleum contaminated soil by their petroleum degradation ability (Baoune et al., 2017). Significant biosurfactant and biodegradation activities had been reported from the endophyte *Nocardia* sp. A9. Furthermore, it was able to degrade plastic and petroleum by 22 and 10% on weight loss basis respectively (Singh and Sedhuraman, 2015).

CONCLUSIONS AND FUTURE PERSPECTIVES

Microbes and plants are keys to sustenance of life on the planet earth. They are the drivers of natural processes, like biogeochemical cycles, maintenance of various ecological habitats (supporting specific flora and fauna under such special niches), production of oxygen, utilization of carbon dioxide, production of organic compounds used as food, feed, and medicine. These naturally thriving and beautifully maintained biosystems have come under serious threat due to deleterious consequences of rampant industrialization and unthoughtful use of myriad of chemicals for human, animal, and agriculture purposes. These problems have drawn attention of the researchers to find appropriate solutions. It is during such endeavors that we are learning more about various biotic and abiotic factors, which interact in a very rationale and scientific manner to balance and sustain each other. Endophytic actinobacteria and their host plants provide an exciting model to explore and to understand their biology and chemistry to develop suitable, non-deleterious applications for human health, agriculture and environment.

While the genus *Streptomyces* had been reported to be the most abundant, the non-*Streptomyces* category was also frequently reported in the endophyte of various categories of plants from extreme habitats (**Table 1**). The biosynthetic gene clusters (BGCs) of these isolates are largely unexplored. But significant diversity in chemical classes was evident from the reports summarized in **Tables 2**, **3**. The therapeutic properties of these compounds strongly suggested abudance of potentially novel drug molecules in the expansive chemical repertoire of endophytic actinobacteria. The data presented in **Tables 4**, **5** demonstrated important roles of endophytic actinobacteria in plant protection and growth, thereby their significance in agriculture.

From the studies analyzed in this review, it is evident that the plants from the extreme environments are potential source for novel and widely diverse endophytic actinobacteria. While these natural reservoirs remain largely untapped as of now, they seem to be drawing considerable attention of the scientific community in present times. Furthermore, progress in endophytic actinobacterial diversity will depend on the advancements in tools and technology for both culturable and non-culturable approaches. This would enhance the repertoire of culturable endophytic actinobacteria from the special habitats besides expanding the information on non-culturable forms. Advancement in the genomic science and technology can be expected to offer rapid means to establish the endophytic actinobacterial diversity in extreme habitats and to indentify potentially useful strains based on the detection of BGCs or other genetic determinants.

Due to multiple drug resistant (MDR) and extensive drug resistant (XDR) pathogens on the scene and the problems associated with the treatment and management of the diseases caused by "ESKAPE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pnumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species), the need for discovery and development of novel drugs is more urgent than ever before. The diverse and novel endophytic actinobacteria from the special habitats offer unparallel opportunities for mining drugs and therapeutics. Besides application in medicine, the novel bioactive molecules may be useful for agriculture purposes for their ability to promote plant growth; ward off phytopathogens, insects and pests; enhance nutrient uptake and help maintain crop productivity under severe abiotic stress of salinity, draft and water logging. Also, these endophytic actinobacteria may play crucial roles in maintenance and sustenance of specific natural habitats but these endeavors call upon extensive scientific investigation to develop the necessary knowledge base.

It can thus be summed up that the novelty and the diversity of the endophytic actinobacterial strains and their bioactive molecules offer tremendous opportunities to address the current and future needs in medicine, agriculture, and environment.

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Diversity, Novelty, and Antimicrobial Activity of Endophytic Actinobacteria From Mangrove Plants in Beilun Estuary National Nature Reserve of Guangxi, China

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Endophytic actinobacteria are one of the important pharmaceutical resources and well known for producing different types of bioactive substances. Nevertheless, detection of the novelty, diversity, and bioactivity on endophytic actinobacteria isolated from mangrove plants are scarce. In this study, five different mangrove plants, *Avicennia marina*, *Aegiceras corniculatum*, *Kandelia obovota*, *Bruguiera gymnorrhiza*, and *Thespesia populnea*, were collected from Beilun Estuary National Nature Reserve in Guangxi Zhuang Autonomous Region, China. A total of 101 endophytic actinobacteria strains were recovered by culture-based approaches. They distributed in 7 orders, 15 families, and 28 genera including *Streptomyces*, *Curtobacterium*, *Mycobacterium*, *Micrococcus*, *Brevibacterium*, *Kocuria*, *Nocardioides*, *Kineococcus*, *Kytococcus*, *Marmoricola*, *Microbacterium*, *Micromonospora*, *Actinoplanes*, *Agrococcus*, *Amnibacterium*, *Brachybacterium*, *Citricoccus*, *Dermacoccus*, *Glutamicibacter*, *Gordonia*, *Isoptericola*, *Janibacter*, *Leucobacter*, *Nocardia*, *Nocardiopsis*, *Pseudokineococcus*, *Sanguibacter*, and *Verrucosispora*. Among them, seven strains were potentially new species of genera *Nocardioides*, *Streptomyces*, *Amnibacterium*, *Marmoricola*, and *Mycobacterium*. Above all, strain 8BXZ-J1 has already been characterized as a new species of the genus *Marmoricola*. A total of 63 out of 101 strains were chosen to screen antibacterial activities by paper-disk diffusion method and inhibitors of ribosome and DNA biosynthesis by means of a double fluorescent protein reporter. A total of 31 strains exhibited positive results in at least one antibacterial assay. Notably, strain 8BXZ-J1 and three other potential novel species, 7BMP-1, 5BQP-J3, and 1BXZ-J1, all showed antibacterial bioactivity. In addition, 21 strains showed inhibitory activities against at least one "ESKAPE" resistant pathogens. We also found that *Streptomyces* strains 2BBP-J2 and 1BBP-1 produce bioactive compound with inhibitory activity on protein biosynthesis as result of translation stalling. Meanwhile, *Streptomyces* strain

3BQP-1 produces bioactive compound inducing SOS-response due to DNA damage. In conclusion, this study proved mangrove plants harbored a high diversity of cultivable endophytic actinobacteria, which can be a promising source for discovery of novel species and bioactive compounds.

Keywords: mangrove plants, endophytic actinobacteria, diversity, antimicrobial activity, novel species

INTRODUCTION

The increased prevalence of "ESKAPE" pathogens, along with the rapid development of multidrug resistances became the driving force in new antibiotics discovery (Spellberg et al., 2008; Bassetti et al., 2013; Pendleton et al., 2013; Singh et al., 2017). New types of antibacterial drugs are so extremely limited that clinicians are forced to the situation as "Bad Bugs, No Drugs," which made novel antibiotic discovery become a very important and urgent issue (Talbot et al., 2006; Boucher et al., 2009; Khan and Khan, 2016). It is well known that actinobacteria, especially the genus *Streptomyces*, are major producers of bioactive compounds, it account for nearly 45% of the total bioactive metabolites produced by microorganisms (Bérdy, 2012; Genilloud, 2017). However, after excavation for many decades, the discovery of new species and new antibiotics from common enviroments is becoming increasingly difficult. On the contrary, it is also becoming increasingly evident that un- and under-explored habitats are rich and new sources of actinobacteria for interesting novel bioactive metabolites, including antibiotics (Rateb et al., 2011; Manivasagana et al., 2014; Jiang et al., 2015; Mohammadipanah and Wink, 2015; Axenov-Gibanov et al., 2016; Hassan and Shaikh, 2017). Microbes have to adapt and evolve in metabolite and genetic level to resist the stress from their habitats, thus, have the capability to synthesis of novel chemicals to carry out special biofunctions and bioactivities (Wilson and Brimble, 2009). In fact, a large number of new bioactive compounds produced by actinobacterial strains from special enviroments have been discovered in recent years. (Wilson and Brimble, 2009; Rateb et al., 2011; Xu et al., 2014; Hassan and Shaikh, 2017).

Mangroves locate in the intertidal zone of tropical and subtropical coastlines and possess a unique environment with highly productive ecosystems, it harbors many kinds of microorganisms including actinobacteria (Jensen et al., 1991; Hong et al., 2009; Xu et al., 2014; Azman et al., 2015; Sun et al., 2017). Since 2007, 66 new species and 8 novel genera of actinobacteria have been isolated and identified from mangrove environments (Biswas et al., 2017; Hamada et al., 2017; Jiang et al., 2017; Law et al., 2017; Li F. et al., 2017; Liu et al., 2017; Sun et al., 2017; Qu et al., 2018). Furthermore, at least 84 new compounds including some "hot molecules," such as salinosporamides, xiamycins and novel indolocarbazoles, were discovered from mangrove actinobacteria. (Kyeremeh et al., 2014; Xu et al., 2014; Ding et al., 2015; Fu et al., 2016; Han et al., 2016; Mangamuri et al., 2016a,b; Chen et al., 2017; Ye et al., 2017).

Endophytic actinobacteria have become a hot spot with increasing actinobacteria prospecting from a range of plant types (Coombs and Franco, 2003; Tian et al., 2007; Qin et al., 2009; Gohain et al., 2015; Passari et al., 2015; Trujillo et al., 2015; Nalini and Prakash, 2017). Moreover, novel endophytic actinobacteria from various tissues of plants have been increasingly reported, and some produced bioactive metabolites with new chemical structures (Qin et al., 2009; Golinska et al., 2015; Matsumoto and Takahashi, 2017; Sun et al., 2017). Until the present, some studies have implemented to isolate endophytic actinobacteria and their secondary metabolites from mangrove plants (Lin et al., 2005; Hong et al., 2009; Wang et al., 2010; Xie et al., 2011; Ding et al., 2012, 2015; Li et al., 2013, 2016; Xu et al., 2014, 2016; Li F. et al., 2017; Li F.N. et al., 2017). According to Xu et al. (2014), 29 of 34 compounds isolated from mangrove endophytic actinobacteria had novel structures, suggesting that mangrove endophytic actinobacteria have the ability to produce new bioactive metabolites. Even though, more than 10 new endophytic actinobacteria species have been characterized from mangrove plants (Jiang et al., 2017; Liu et al., 2017; Sun et al., 2017), studies on mangrove plants endophytic actinobacteria are still rather scarce when compared to actinobacteria prospecting for mangrove soil or sediment. Our results presented in this article revealed that actinobacteria isolated from mangrove plants are important sources for new species and diverse bioactive compounds, and researches on this area should draw much more attention (Yan et al., 2010; Liu et al., 2016, 2017; Tuo et al., 2016a,b; Jiang et al., 2017).

Beilun River is the boundary river between China and Vietnam and the Chinese part is located in Guangxi Zhuang Autonomous Region. The Beilun Estuary National Nature Reserve was estabished to protect mangrove plants in the year of 2000. The reserve contains rich mangrove flora composed of 14 plant species and is mostly unexplored (Liang et al., 2004). To our knowledge, few studies have focused on the endophytic actinobacterial communities of Beilun Estuary mangrove plants. In this study, the diversity and novelty of cultivable actinobacteria from mangrove plants of the Beilun Estuary National Nature Reserve was investigated and their ability to produce antimicrobial activity against "ESKAPE" was evaluated. Meanwhile, a high-throughput screening model based on double fluorescent protein reporter was also implemented to find strains producing secondary metabolites as ribosome and DNA biosynthesis inhibitors.

MATERIALS AND METHODS

Sampling of Mangrove Plants

Mangrove plants were collected from Beilun Estuary National Nature Reserve (21°36'N, 108°12'E) in Guangxi Zhuang

Autonomous Region, China, in July, 2015. A total of 19 tissues including leaves, branches, barks, roots, also flowers, and fruits (if present) were collected from five plants: *Avicennia marina*, *Aegiceras corniculatum*, *Kandelia obovota*, *Bruguiera gymnorrhiza*, and *Thespesia populnea*. After flame sterilization, the cut ends of root and branch were sealed with parafilm, and then all samples were transported to the laboratory and processed within 48 h.

Isolation of Endophytic Actinobacteria

All plant samples were washed thoroughly with tap water for a few minutes to remove organic debris and soil. After air-drying, the samples were processed according to the five-step sterilization method described by Qin et al. (2009), and ground into powder by using micromill, and distributed on plates containing different isolation media. The plates were incubated at 28°C for 2–5 weeks. Colonies that displayed differentiable morphologies were transferred onto ISP2 agar plates and repeatedly isolated and incubated until pure isolates were obtained. The purified cultures were maintained on ISP2 medium slants at 4°C and stored in 20% (v/v) glycerol suspensions at −80°C. A total of 10 media were used for the isolation of mangrove endophytic actinobacteria (Supplementary Table S1), and actidione (40 mg L^{-1}), nystatin (40 mg L^{-1}), and nalidixic acid (25 mg L^{-1}) were added to the media to inhibit the growth of fungi and Gram-negative bacteria.

The efficacy of the sterilization process was confirmed using methods described by Qin et al. (2009). Briefly, the surface-sterilized tissues were washed three times in sterile distilled water, soaked in sterile water for 1 min with continuous stirring, and then, a 0.1 milliliter aliquot of the last washed water was inoculated onto ISP2 agar plates and incubated at 28°C. Meanwhile, the surface-sterilized tissues were imprinted onto ISP2 agar plates and incubated at 28°C. If no microbial growth was observed on the surface of the media, the sterilization was considered as effective.

Molecular Identification and Phylogenetic Analysis of Isolates

Genomic DNA was extracted from pure isolates as described by Zhou et al. (2010). Universal primers 27F and 1492R (Lane, 1991) were used for amplification of 16S rRNA gene fragments. Cycling conditions were as follows: initial denaturation at 95°C for 5 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, 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 PRISM 3730XL DNA Analyzer from Life Sciences Solutions Group, Thermo Fisher Scientific (Beijing). The genus-level affiliation of the sequences was validated using available 16S rRNA gene sequences from the EzTaxon-e server[1] (Kim et al., 2012). Sequence alignment and phylogenetic analysis were carried out using MEGA version 5 (Tamura et al., 2011). Phylogenetic trees were constructed by using the neighbor-joining method (Saitou and Nei, 1987) with bootstrap values based on 1000 replications (Felsenstein, 1985) using MEGA5 program. The 16S rRNA gene sequences of the potential novel isolates were deposited in GenBank under the accession numbers: MG563365-563372, and the partial 16S rRNA gene sequences of the remaining isolates were deposited in GenBank with the following accession numbers: MG563311-563364.

[1]http://eztaxon-e.ezbiocloud.net

Antibacterial Activity Screening

Based on phylogenetic and phenotypic characteristics analysis, 63 strains were selected for antimicrobial assay. Each strain was transferred to 500 mL Erlenmeyer flasks containing 100 mL of YIM 38 medium (Jiang et al., 2015) and cultivated for 7 days at 28°C with 180 rpm orbital shaking. The 600 mL fermentation broth obtained from each of the isolates was separated from the mycelium by centrifugation at 4500 rpm at 20°C for 20 min. The supernatants were extracted twice with ethyl acetate (1:1, v/v), and the whole organic layer and 50 mL of water layer were concentrated under vacuum and freeze-dried, respectively, to obtain dried samples. The mycelium were soaked in acetone for 12 h and then filtered, the filtration were concentrated under vacuum to obtain dried samples. Finally, each of three kinds of dried samples was dissolved in 3 mL HPLC grade methanol and used in the antibacterial assay by agar disk diffusion method. The methanol sample (60 μL) was dripped on paper disk (diameter, 5 mm). Meanwhile, 60 μL methanol without sample was used as the negative control, and levofloxacin (10 μL, 0.1 mg/mL) was used as the positive control. After being dried in a hood, the paper disks were transferred to agar plates containing pathogenic bacteria and incubated at 37°C for 24 h. The diameters of the inhibition zones were measured by vernier caliper. The indicator bacteria used for antimicrobial assay were: *Enterococcus faecalis* (*E. faecalis*) (ATCC 29212, ATCC 51299), *Staphylococcus aureus* (*S. aureus*) (ATCC 25923, 2641), *Klebsiella pneumoniae* (*K. pneumoniae*) (ATCC 10031, ATCC 700603), *Acinetobacter baumannii* (*A. baumannii*) (ATCC19606, 2799), *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC 27853, 2774), and *Escherichia coli* (*E. coli*) (ATCC 25922, 2800). Isolate 2774 is resistant to carbapenem and quinolone, isolates ATCC 51299, 2641, and 2799 are resistant to vancomycin, methicillin, and carbapenem, and strains 2800 and ATCC 700603 are both ESLB-producing species, respectively.

Mechanism of Action Determination

Reporter strain JW5503-pDualrep2 was used as previously described (Osterman et al., 2016). Briefly, 1 ml of the extracts from ethyl acetate layer was dried in hood and 100 μl DMSO was added to each sample, and a total of 63 samples were prepared. A total of 2 μl of each sample were applied to agar plate containing a lawn of the reporter strain. After overnight incubation at 37°C, the plate was scanned by ChemiDoc (Bio-Rad): "Cy3-blot" for RFP and "Cy5-blot" for Katushka2S. Erythromycin and levofloxacin were used as positive controls for ribosome and DNA biosynthesis inhibitors, respectively.

RESULTS

Isolation and Diversity of Actinobacteria From Mangrove Plants of Beilun Estuary

A total of 318 strains of endophytic microbes were isolated from 19 tissues collected from 5 mangrove plants. A total of 158 presumed endophytic actinobacteria were selected on the basis of colonial morphology and were further identified by their 16S rRNA gene sequences. A total of 101 isolates were confirmed as actinobacteria and phylogenetic analysis based on the partial 16S rRNA genes sequences (approximately 700 bp) revealed that the 101 endophytic actinobacterial strains were assigned to 28 genera in 7 orders of 15 families: *Streptomyces*, *Curtobacterium*, *Mycobacterium*, *Micrococcus*, *Brevibacterium*, *Kocuria*, *Nocardioides*, *Kineococcus*, *Kytococcus*, *Marmoricola*, *Microbacterium*, *Micromonospora*, *Actinoplanes*, *Agrococcus*, *Amnibacterium*, *Brachybacterium*, *Citricoccus*, *Dermacoccus*, *Glutamicibacter*, *Gordonia*, *Isoptericola*, *Janibacter*, *Leucobacter*, *Nocardia*, *Nocardiopsis*, *Pseudokineococcus*, *Sanguibacter*, and *Verrucosispora* (**Figure 1**). Relative abundance of endophytic actinobacteria at the genus level revealed that *Streptomyces* and *Curtobacterium* were most abundant with 33.0 and 14.0%, respectively (**Figure 2A**). All endophytic actinobacterial strains were isolated from 10 isolation media, which had a major influence on the number of isolates recovered. The ISP2-M medium was the most effective as regards the number and diversity of isolates obtained; the TWYE medium produced the second-highest numbers and diversities. On the contrary, the TP medium yielded the lowest numbers (**Figure 2B**). All tissues produced at least one isolate, which indicated that endophytic actinobacterial isolates can colonize different tissues throughout the plants (**Figure 2C**). Out of 101 isolates, the majority (n = 35, 34.65%) were isolated from barks, followed by stem (n = 24, 23.76%), leaf (n = 22, 21.78%), fruits (n = 10, 9.90%), roots (n = 9, 8.91%), and flower (n = 1, 0.99%).

Phylogenetic Novelty of Isolated Actinobacteria

A total of 7 strains exhibited low sequence similarities (< 98.65%) with validly described species based on a 16S rRNA gene sequence search via the EzTaxon server, suggesting that these strains could represent novel taxons within the phylum *Actinobacteria* (Kim et al., 2014). These putative novel isolates belong to genera *Marmoricola* and *Nocardioides* in family *Nocardioidaceae*, genus *Streptomyces* in family *Streptomycetaceae*, genus *Mycobacterium* in family *Mycobacteriaceae*, and genus *Amnibacterium* in family *Microbacteriaceae*. In addition, strain 8BXZ-J1 has been characterized as a new species of the genus *Marmoricola* (Jiang et al., 2017). The almost complete sequencing (> 1375bp) of the 16S rRNA gene was performed in 7 potential novel strains and the new species. The phylogenetic tree based on 16S rRNA gene sequences generated by using the neighbor-joining method indicated that strain 1BXZ-J1 was clustered within the genus *Marmoricola*, strains 10BTP-3 and 6BMS-J1 were clustered within the genus *Nocardioides*, strains 5BQP-J3 and 7BMP-1 were clustered within the genus *Streptomyces*, strain 3BMZ-J1 was clustered within the genus *Amnibacterium*, and strain 3BMS-J1 was clustered within the genus *Mycobacterium* (**Figure 3**). The 16S rRNA gene sequences of strain 1BXZ-J1, 10BTP-3, 6BMS-J1, 5BQP-J3, 7BMP-1, 3BMZ-J1, and 3BMS-J1 showed highest similarities with *Marmoricola aequoreus* NRRLB-24464^T (96.3%), *Nocardioides soli* mbc-2^T (97.0%), *Nocardioides aquiterrae* GW-9^T (97.3%), *Streptomyces yogyakartensis* NBRC 100779^T (97.9%), *Streptomyces phaeoluteichromatogenes* NRRL 5799^T (98.2%), *Amnibacterium kyonggiense* KSL51201-037^T (98.1%), and *Mycobacterium peregrinum* ATCC 14467^T (98.6%), respectively. These isolates will be further characterized in a polyphasic approach to determine their taxonomic positions. The phylogenetic analysis presented implied a considerable cultivable actinobacteria with novelty in mangrove plants from Beilun Estuary.

Antibacterial Activity of Actinobaterial Isolates

Antimicrobial activity was evaluated against a set of pathogenic bacteria. Out of 63 isolates, 31 (49.2%) exhibited antagonistic activity against at least one of the tested pathogens (Supplementary Table S2). They were affiliated to 15 different genera, i.e., *Streptomyces* (14), *Curtobacterium* (2), *Micromonospora* (2), *Marmoricola* (2), *Micrococcus* (1), *Nocardioides* (1), *Mycobacterium* (1), *Kocuria* (1), *Microbacterium* (1), *Leucobacter* (1), *Gordonia* (1), *Citricoccus* (1), *Kytococcus* (1), *Nocardia* (1), and *Nocardiopsis* (1). The antimicrobial profile of the actinobacteria against different pathogenic bacteria was shown in **Figure 4**. Regarding the sensitive pathogenic strains tested, activity against *P. aeruginosa* was clearly the most frequent (21 isolates, 33.3%), and activity against *E. coli* was the least frequent (7, 11.1%), while 27.0% (17), 19.0% (12), 14.3 (9), and 12.7% (8) of the isolates were active against *S. aureus*, *E. faecalis*, *K. pneumonia*, and *A. baumannii*, respectively. Concerning the resistant pathogenic strains tested, activity against *S. aureus* was the most frequent (12 isolates, 19.0%), and activity against *K. pneumonia* was the least frequent (2, 3.2%), while 12.7% (8), 9.5% (6), 7.9% (5), and 4.8% (3) of the isolates were active against *P. aeruginosa*, *E. faecalis*, *A. baumannii*, and *E. coli*, respectively. In all, 21 strains showed inhibitory activities against at least one "ESKAPE" resistant pathogens, suggesting that these strains might represent a valuable source of biologically active compounds with inhibitory activities against "ESKAPE" resistant pathogens.

Notably, three of the seven potential novel strains and new species (8BXZ-J1) showed activity against at least one of the tested pathogenic bacteria. Strain 7BMP-1 exhibited strong antimicrobial activities against *S. aureus* ATCC 25923 (19.1 mm), *S. aureus* 2641 (16.1 mm), *E. faecalis* ATCC 29212 (16.0 mm), *P. aeruginosa* ATCC 27853 (14.0 mm), *E. faecalis* ATCC 33186 (13.8 mm), and *A. baumannii* ATCC 19606 (9.7 mm). Strain 5BQP-J3 showed strong inhibitory activity against *P. aeruginosa* ATCC 27853 (19.1 mm), which is resistant to carbapenem and quinolone. Both strains 1BXZ-J1 and 8BXZ-J1 showed strong inhibitory activity against *P. aeruginosa* ATCC 27853, and the inhibition zones were 19.4 and 17.1 mm, respectively.

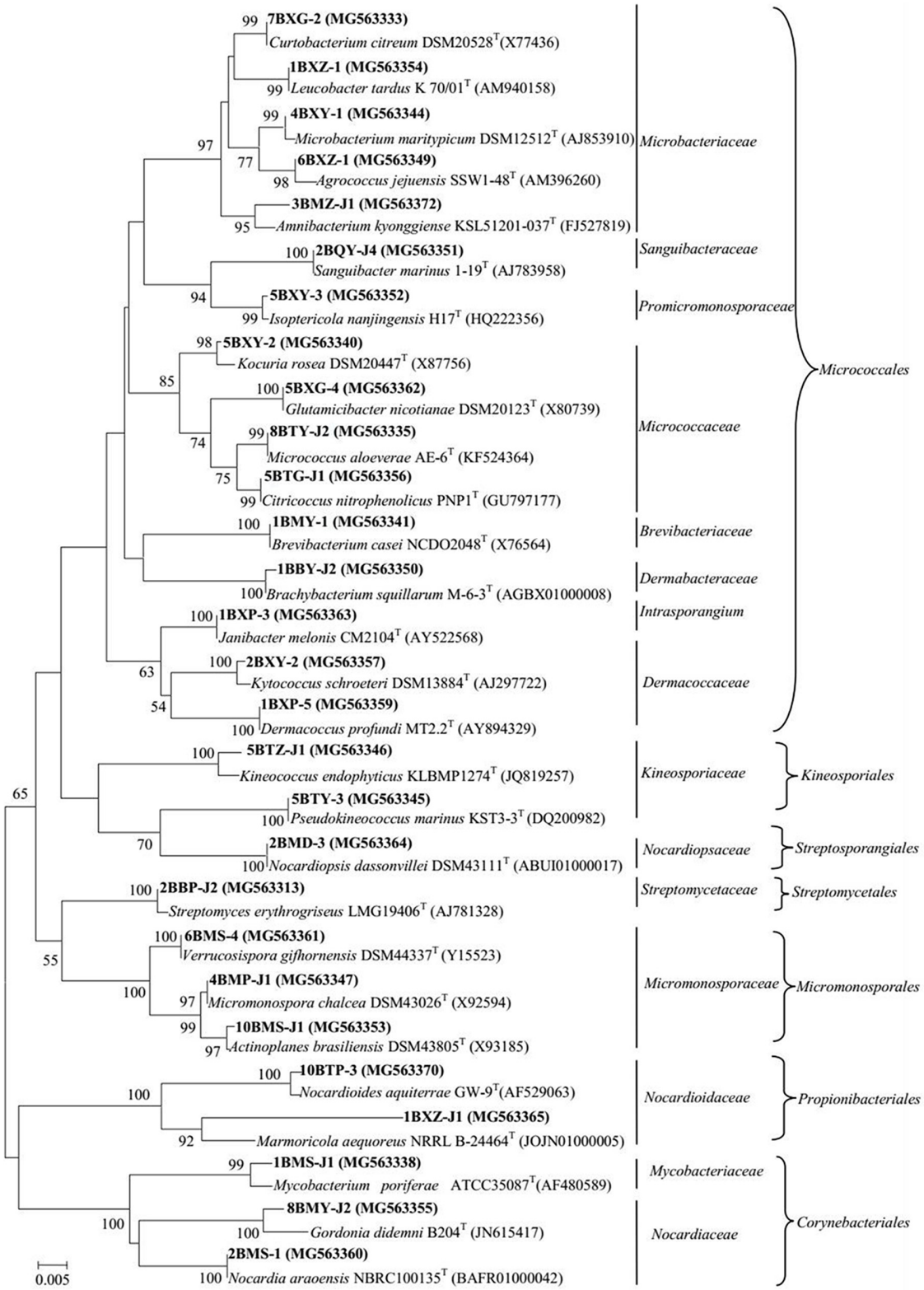

FIGURE 1 | Phylogenetic tree of actinobacterial isolates from mangrove plants of Beilun Estuary that belong to the orders *Micrococcales*, *Kineosporiales*, *Streptosporangiales*, *Streptomycetales*, *Micromonosporales*, *Propionibacteriales*, *Corynebacteriales*, and closely related representative species. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of the strains and closely related species of the genera. Numbers at nodes indicate the level of bootstrap support (> 50%) based on 1000 replications. Bar, 5 nt substitutions per 1000 nt.

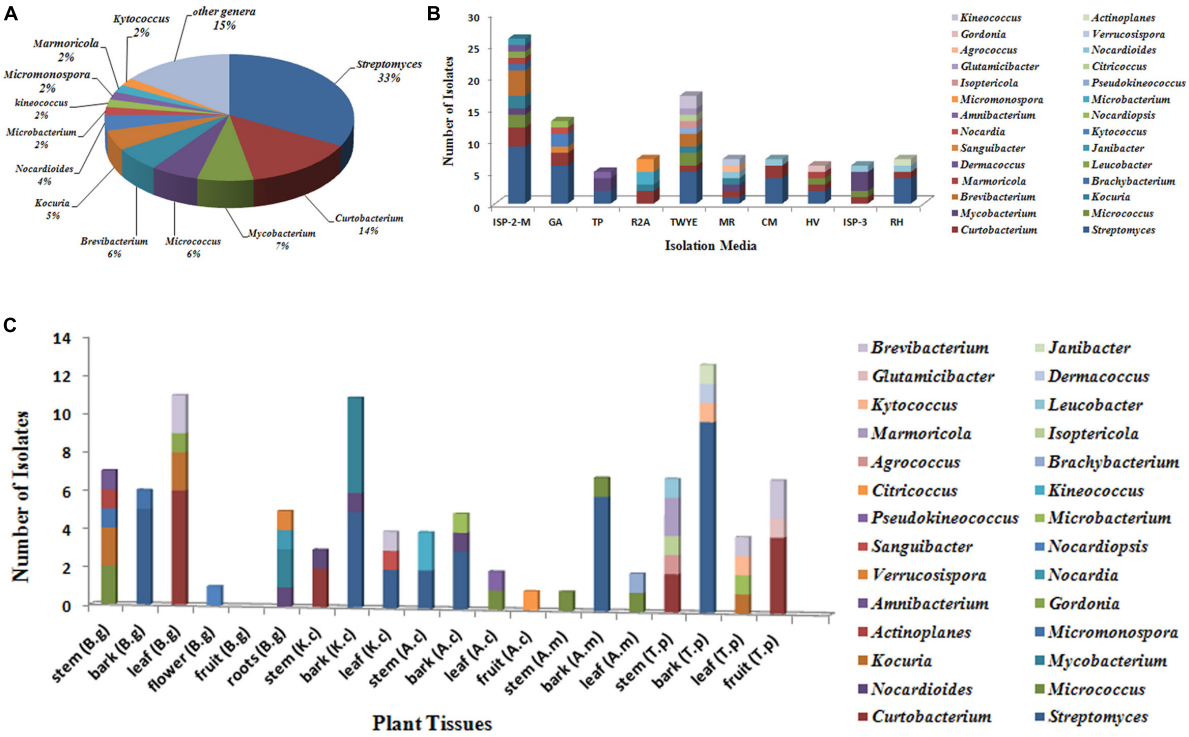

FIGURE 2 | Diversity of culturable actinobacteria from mangrove plants of Beilun Estuary. **(A)** Pie chart representation of the percentage frequency of actinobacterial genera within the total number of isolates. **(B)** Number of actinobacterial isolates recovered from the different culture media used. **(C)** Number of actinobacterial isolates from different tissues of mangrove plants (B.g: *Bruguiera gymnorrhiza*; K.c: *Kandelia candel*; A.c: *Aegiceras corniculatum*; A.m: *Avicennia marina*; T.p: *Thespesia populnea*).

The 63 ethyl acetate extracts were assayed by a double fluorescent protein reporter (pDualrep2 reporter system) screening model and the results are shown in **Figure 5**. Strains 2BBP-J2 and 1BBP-1 induced Katushka2S expression, which demonstrated strong translation inhibition activity as erythromycin did, meanwhile, strain 3BQP-1-induced RFP expression and SOS-response because of DNA-damage as levofloxacin did.

DISCUSSION

Mangroves plants harbor a great diversity of culturable actinobacteria, and are proven to be a valuable microorganism source for discovery of new bioactive metabolites (Hong et al., 2009; Xu et al., 2014, 2016; Li et al., 2016; Li F.N. et al., 2017). In this study, a considerable diversity of endophytic actinobacteria was obtained from the mangrove plants collected in Beilun Estuary National Nature Reserve of Guangxi Zhuang Autonomous Region, China. In addition, to our knowledge, this is the first time strains that were rarely recovered in genera *Sanguibacter* and *Citricoccus* have been isolated and cultured from the inner parts of plants. Recently, Li F.N. et al. (2017) reported that 198 endophytic actinobacterial strains isolated from mangrove plants of Macao, China, were distributed in 30 genera affiliated to 8 orders. Li et al. (2016) isolated 146 endophytic actinobacteria belonging to 8 orders (18 genera) from mangrove plants of Dongzhaigang of Hainan Province, China. The 159 isolates of 19 actinobacteria genera belonging to 8 orders obtained from mangrove plants of Zhanjiang in Guangdong Province were reported in 2016 (Xu et al., 2016). Hong et al. (2009) identified 237 actinobacterial isolates belonging to five orders (13 different genera) from soil and plant samples of 8 mangrove sites in China. In our study, we identified 28 genera from 101 isolated actinobacterial strains, the diversity of endophytic actinobacteria is apparently higher than the previous studies. Therefore, it is clear that endophytic actinobacteria in mangrove plants are diverse, and that the variation in the diversity and richness of endophytic actinobacteria recovered from mangrove plants are closely related with the isolation media and different tissues.

Some novel endophytic actinobacteria have been discovered from mangrove plants, suggesting that they have potential as excellent sources of novel species with actinobacterial reagents (Liu et al., 2017; Sun et al., 2017). In this study, isolates from the genus *Streptomyces* were the most abundant, it is consistent with previous studies on actinobacterial communities in mangrove plants (Hong et al., 2009; Xu et al., 2014, 2016; Li et al., 2016; Li F.N. et al., 2017). Two *Streptomyces* strains (5BQP-J3 and 7BMP-1) are probably new species due to their relatively low gene sequence similarities to their closest type strains. Meanwhile, the gene sequence similarity between

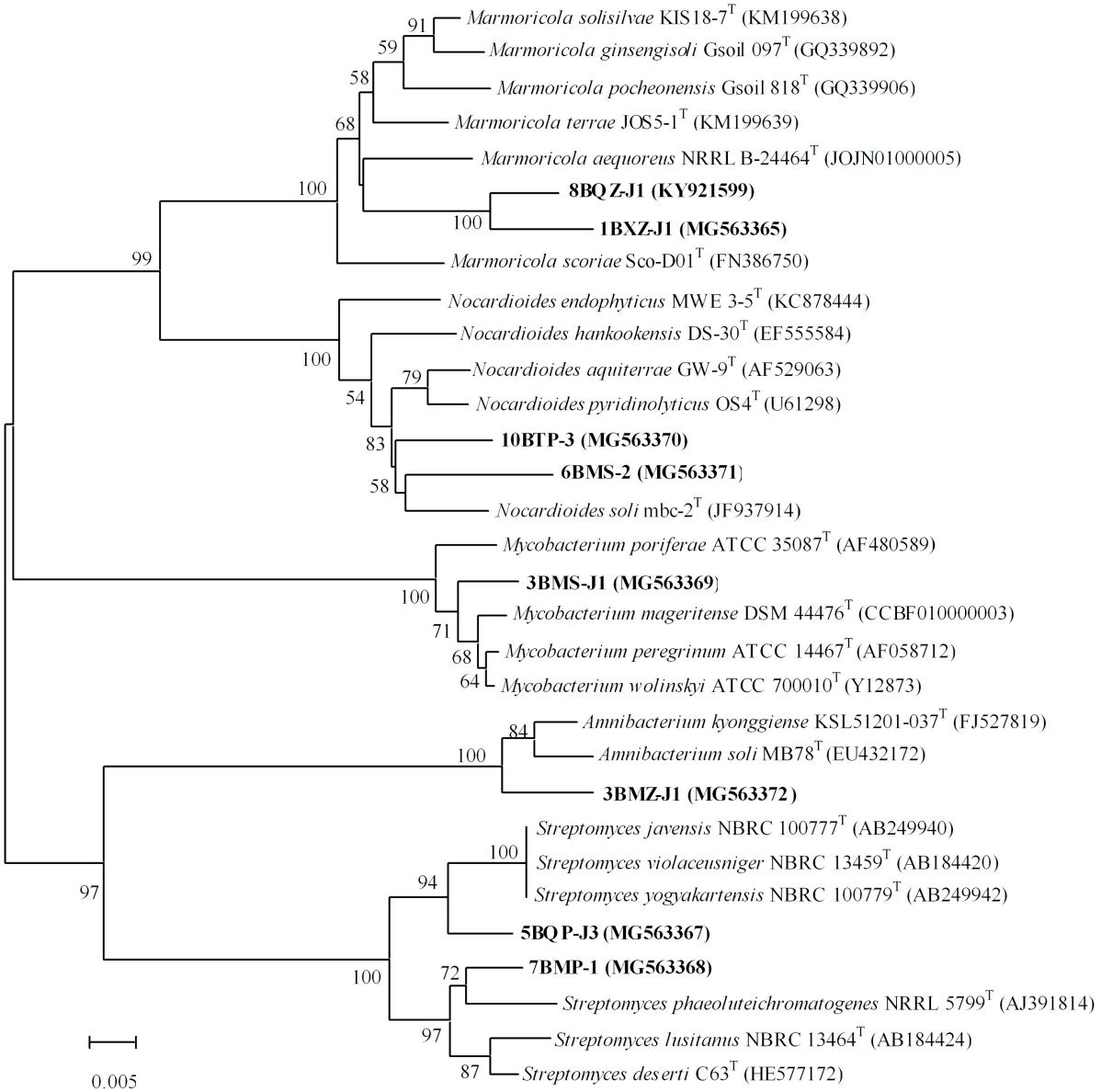

FIGURE 3 | Phylogenetic tree of 7 potential novel strains and 1 new species from mangrove plants of Beilun Estuary and closely related representative species. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of the strains and related species of the genera. Numbers at nodes indicate the level of bootstrap support (> 50%) based on 1000 replications. Bar, 5 nt substitutions per 1000 nt.

5BQP-J3 and 7BMP-1 was 96.5%. In addition, five putative new rare actinobacteria species are proposed: *Nocardioides* sp. 10BTP-3, *Nocardioides* sp. 6BMS-2, *Marmoricola* sp. 1BXZ-J1, *Mycobacterium* sp. 3BMS-J1, and *Amnibacterium* sp. 3BMZ-J1, based on numerical thresholds related to 16S rRNA gene sequences (Kim et al., 2014; Supplementary Table S2). Further phylogenetic analysis of the isolates is shown in **Figure 3**. Pairwise comparison of the 16S rRNA gene sequences from the two *Nocardioides* strains (10BTP-3 and 6BMS-2) showed relatively low similarities (97.4 and 97.0%) to the type strains of *Nocardioides aquiterrae* and *Nocardioides soli*, respectively. The sequence similarity between 10BTP-3 and 6BMS-2 was 97.5%. Furthermore, the phylogenetic analysis suggested that the two isolates were diversely distributed within genus *Nocardioides* and clustered singly in the phylogenetic tree (**Figure 3**), indicating that isolates 10BTP-3 and 6BMS-2 might belong to new species. The *Marmoricola* strains 1BXZ-J1 and 8BXZ-J1 had lineages that were distinct from each other and from other members of the genus; and they also formed distinct subclades in the tree supported by high bootstrap values (**Figure 3**). The 16S rRNA gene sequences of 1BXZ-J1 and 8BXZ-J1 showed 96.3 and 96.9% identities to the nearest neighbors, *Marmoricola aequoreus* and *Marmoricola solisilvae*, respectively. The sequence similarity between 1BXZ-J1 and 8BXZ-J1 was 97.8%. Strain 8BXZ-J1 has

been characterized as the first endophytic actinobacteria new species belonged to the genus *Marmoricola* (Jiang et al., 2017). *Mycobacterium* strains have been isolated from mangrove plants (Xu et al., 2016; Li F.N. et al., 2017) and other medicinal plants (Qin et al., 2009). However, novel species of the genus *Mycobacterium* from mangrove ecosystem have not yet been reported. 3BMS-J1 is probably a new *Mycobacterium* species because it presents relatively low 16S rRNA gene sequences similarity and forms a distinct phylogenetic branch when compared to strains from the genus *Mycobacterium* (**Figure 3**). Comparison of the 16S rRNA gene sequences of the isolate and its closest neighbors suggested that 3BMZ-J1 was closely related to the type strains of the genera *Amnibacterium*. To date, this genus comprises only two species that were isolated from Anyang stream and grass soil (Kim and Lee, 2011; Jin et al., 2013). Strain 8BXZ-J1 was isolated with HV medium, the potential novel strains 3BMS-J1 and 3BMZ-J1 were both isolated with TP medium, and the others potential novel strains 1BXZ-J1, 5BQP-J3, 6BMS-2, 7BMP-1, and 10BTP-3 were isolated with ISP-2-M medium, TWYE medium, MR medium, CM medium, and RH medium, respectively. This result demonstrated that it is still worthwhile to use traditional cultivating methods for isolating new actinobacterial species.

Analysis of composition in genera level of 31 positive strains from 63 tested strains indicated that *Streptomyces* strains predominated. In 63 tested strains, there are 15 *Streptomyces* strains, among them, 14 *Streptomyces* strains showed positive result. It is quite reasonable, since *Streptomyces* is the largest antibiotic-producing genus in the microbial world discovered, and it is well documented that mangrove *Streptomyces* are able to produce bioactive metabolites with a wide-range of activities, including antibacterial, antifungal, anti-HIV, and anticancer (Ding et al., 2010; Yuan et al., 2013; Khieu et al., 2015; Tan et al., 2015; Xu et al., 2015; Ser et al., 2017; Wang et al., 2017). *Curtobacterium* as the second most dominant genus in 63 tested strains, showed very low positive rate, only 2 of 11 displayed antibacterial activities against tested pathogens. *Curtobacterium* traditionally is viewed as a plant pathogen, and most studies focused on its role as an economically important plant pathogen (Huang et al., 2009; Buonaurio et al., 2015; Osdaghi et al., 2015). Recently, Undabarrena et al. (2016) reported *Curtobacterium* isolates showing potent antimicrobial bioactivity against more than one tested pathogens, and their antimicrobial activity was dependent on the culturing media.

It is noteworthy to highlight that the two rare actinobacterial strains, *Kytococcus* strain 2BXY-2 and *Leucobacter* strain 1BXZ-1, showed the strong inhibitory activity against *Pseudomonas aeruginosa* as shown in Supplementary Table S2. To our knowledge, antibacterial activities from these genera have been received scarce attention for potential development of novel antimicrobial reagents.

It is extensively accepted that novel microorganisms are a good source for the discovery of new secondary bioactive metabolites, as exemplified by salinosporamide A, a potent cytotoxic activity compound produced by *Salinispora* strain CNB-392 (Feling et al., 2003) and teixobactin, a new cell wall inhibitor with exceptional activity against *Clostridium difficile* and *Bacillus anthracis* produced by *Eleftheria terrae* (Ling et al., 2015). In the study, two potential new *Streptomyces* spieces (5BQP-J3 and 7BMP-1) displayed very strong antibacterial activities (Supplementary Table S2), thus it would be interesting to see if new antibiotics can be identified from these strains. In addition, antibacterial activities of *Marmoricola* isolates (1BXZ-J1 and 8BXZ-J1) against *P. aeruginosa* and *S. aureus* are highlighted by their novelty. Few have reported the antimicrobial activity potential of *Marmoricola* so far. *Marmoricola* isolates (1BXZ-J1 and 8BXZ-J1) may also be good candidates for production of bioactive compounds. Further experiments will be carried out

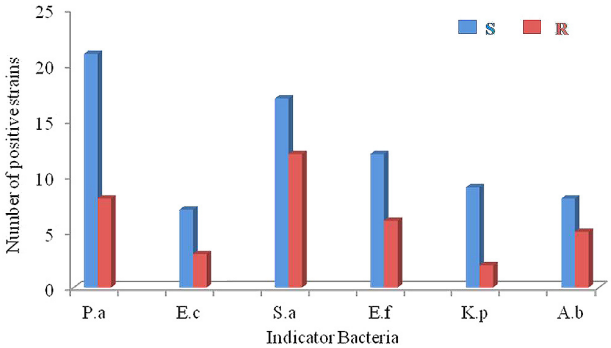

FIGURE 4 | The antimicrobial profiles of the actinobacteria against ESKAPE (P.a: *Pseudomonas aeruginosa*; E.c: *Escherichia coli*; S.a: *Staphyloccocus aureus*; E.f: *Enterococcus faecalis*; K.p: *Klebsiella peneumoniae*; A.b: *Acinetobacter baumannii*). S: sensitive pathogenic strains; R: drug-resistant pathogenic strains.

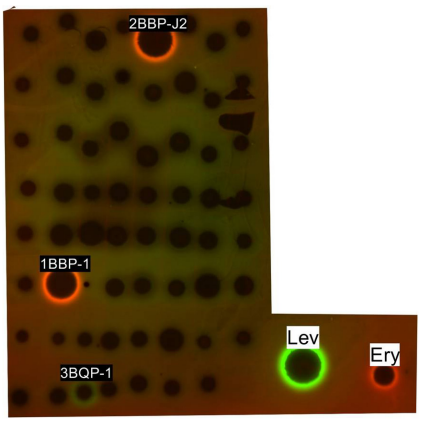

FIGURE 5 | Induction of a two-color dual reporter system sensitive to inhibitors of the ribosome progression or inhibitors of DNA replication, respectively. Spots of erythromycin (ERY), levofloxacin (LEV), and tested compounds were placed on the surface of an agar plate containing *E. coli tolC* cells transformed with the pDualrep2 reporter plasmid. Shown is the fluorescence of the lawn of *E. coli* cells scanned at 553/574 nm (green pseudocolor) for RFP fluorescence and 588/633 nm (red pseudocolor) for Katushka2S fluorescence. Induction of expression of Katushka2S is triggered by translation inhibitors, while RFP is upregulated by induction of DNA damage SOS response.

to deepen our knowledge on the antibacterial activities of these novel isolates.

In the antibacterial assay, pDualrep2 reporter system was implemented to indicate existence of antibiotics in the culture broth, the system can distinguish simultaneously between antibiotics that induce the SOS response, a major general stress response caused by inhibitors of DNA biosynthesis and those that cause ribosome stalling. The existence of ribosome inhibitor such as erythromycin will lead Katushka2S expression, and the existence of inhibitor of DNA biosynthesis such as levofloxacin will lead RFP expression. Both 2BBP-J2 and 1BBP-1 fermentation broth demonstrated ribosome inhibition activities in their ethyl acetate extractions, indicating that Katushka2S expression had been induced. Meanwhile, strain 3BQP-1 demonstrated inhibition activity against DNA biosynthesis in its fermentation broth extract as shown by the induced RFP expression.

The diversity, novelty, and antimicrobial activity of endophytic actinobacteria from mangrove plants of Beilun Estuary were first comprehensively investigated in this study. The 101 selected isolates were assigned to 7 orders, 15 families, and 28 genera. Notably, 31 isolates belonging to 15 different genera show inhibitory activities against at least one "ESKAPE" pathogens, and 7 isolates affiliated with 5 genera are below the 98.65% sequence identity threshold and therefore may be potential candidates of new taxons. In addition, a new species of the genus *Marmoricola* isolated from the mangrove plants has been published. These results indicated that mangrove plants are potentially unique sources of novel actinobacteria with promising potential to produce highly bioactive metabolites.

AUTHOR CONTRIBUTIONS

Z-kJ carried out the sampling, the selective isolation, the primary identification of the isolates, the screening of antimicrobial activities, analyzed the data, and prepared the manuscript. LT and D-lH carried out the sampling, the selective isolation, and edited the manuscript. S-wL and F-nL prepared the samples for screening. OD, AT, DL, PS, IO, and VK sorted out the antibiotics' mechanisms of action of the extracts by means of a double fluorescent protein reporter for high-throughput screening of ribosome and DNA biosynthesis inhibitors and edited the manuscript. C-hS conceived and designed the experiments, performed the sampling, and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00868/full#supplementary-material

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Microbial Flora Associated with the Halophyte–*Salsola imbricate* and Its Biotechnical Potential

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Halophytes are associated with the intertidal forest ecosystem of Saudi Arabia and seemingly have an immense potential for yielding useful and important natural products. In this study we have aimed to isolate and characterize the endophytic and rhizospheric bacterial communities from the halophyte, *Salsola imbricata,* In addition these bacterial strains were identified and selected strains were further studied for bioactive secondary metabolites. At least 168 rhizspheric and endophytic bacteria were isolated and of these 22 were active antagonists against the oomycetous fungal plant pathogens, *Phytophthora capsici* and *Pythium ultimum*. Active cultures were mainly identified with molecular techniques (16S r DNA) and this revealed 95.7–100% sequence similarities with relevant type strains. These microorgansims were grouped into four major classes: *Actinobacteria, Firmicutes*, β*-Proteobacteria,* and γ*-Proteobacteria*. Production of fungal cell wall lytic enzymes was detected mostly in members of *Actinobacteria* and *Firmicutes*. PCR screening for type I polyketide synthases (PKS-I), type II polyketide synthases (PKS-II) and nonribosomal peptide synthetases (NRPS) revealed 13 of the 22 strains (59%) were positive for at least one of these important biosynthetic genes that are known to be involved in the synthesis of important antibiotics. Four bacterial strains of *Actinobacteria* with potential antagonistic activity including two rhizobacteria, EA52 (*Nocardiopsis* sp.), EA58 (*Pseudonocardia* sp.) and *two* endophytic bacteria *Streptomyces sp.* (EA65) and *Streptomyces* sp. (EA67) were selected for secondary metabolite analyses using LC-MS. As a result, the presence of different bioactive compounds in the culture extracts was detected some of which are already reported for their diverse biological activities including antibiotics such as Sulfamethoxypyridazine, Sulfamerazine, and Dimetridazole. In conclusion, this study provides an insight into antagonistic bacterial population especially the *Actinobacteria* from *S. imbricata*, producing antifungal metabolites of medical significance and characterized taxonomically in future.

Keywords: halophyte, antifungal activity, enzyme production, 16S rRNA gene sequence, LC/MS analyses

INTRODUCTION

Coastal dune plants consist of shrubs that are associated with tropical and subtropical coastal areas. This ecosystem is considered as a low nutrient environment due to the sparseness of nitrogen and phosphorus (Frosini et al., 2012). Coastal dune areas of the world usually represent a unique flora as a result of variations in salinity, anaerobicity, and temperature fluctuations during different seasons. All of these conditions make this habitat unusual and thus may have been selective for the presence of truly unique microorganisms having a rich biodiversity. Under these conditions microorganisms associated with these costal dune plants play a role in their survival and perform diverse biological functions in plants (Del Vecchio et al., 2015).

Bacteria associated with Coastal dune plants are responsible for various activities including resistance against different pathogens by producing different bioactive metabolites and plant growth promotion compounds. Bacteria are associated with these plants either as rhizospheric or endophytic forms and use nutrients from plants for their growth (de Melo Pereira et al., 2012). Endophytic bacteria get access to internal parts of plants by producing enzymes to hydrolyze cell wall of plants (Cho et al., 2007). These hydrolytic enzymes are also involved in defense of plants against various bacterial and fungal pathogens (Luo et al., 2013).

Endophytes produce metabolites that possess antimicrobial activities (Strobel, 2003). Due to their beneficial effects and broad range, endophytes have been isolated from plants widely ranging from the poles of the earth to the steaming tropics. Plants provide these microorganisms, particularly endophytic bacteria and fungi, a nutrient-rich environment for their growth and development (Strobel et al., 2004; de Melo Pereira et al., 2012). Endophytic *Actinobacteria* associated with plant roots or leaves are a source of novel potential bioactive substances. *Actinobacteria* produce various secondary metabolites including antibiotics, antitumor, immunosuppressive, and enzymes widely used in industrial, agriculture, and pharmaceutical industry (Manivasagan et al., 2014). Recently, various antimicrobial compounds and enzymes have been isolated from marine *Actinobacteria* (Hong et al., 2009; He et al., 2012). Secondary metabolite salinosporamide A, isolated from a marine *Actinobacteria* is currently in clinical trials as a cancer treatment (Feling et al., 2003). Several other bioactive compounds have been isolated from different marine *Actinobacteria* (Fu et al., 2012).

The Genus *Salsola* is comprised of different halophyte species including *Salsola imbricata* that inhabit the coastal area of the Thuwal region in Jeddah, Saudi Arabia. Recent studies on *S. imbricata* reported the presence of triterpenesaponins and a new isorhamnetin from the methanolic extract of this plant (Osman et al., 2016). In a recent study from Saudi Arabia, phytochemical analysis of *S. imbricata* showed presence of bioactive biphenylpropanoids i.e., biphenylsalsonoid A and B. These two bioactive compounds exhibited antioxidant and antibacterial activity against human pathogenic bacteria (Oueslati et al., 2017). Although the coastal dune environment is an unexplored source of natural compounds with diverse activities there has been little or no work on the bacterial flora of this plant. Based on previous studies, it would appear that certain microbial flora components may be present in this plant that has biotechnical potential. Thus, this study was undertaken to explore potential antifungal microflora of halophyte that may produce products of medicinal, agricultural, and industrial significance.

MATERIALS AND METHODS

Collection of Plant Samples and Isolation of Rhizobacteria and Endophytic Bacteria

The halophyte, *S. imbricata* was collected from the coastal area of Jeddah, Saudi Arabia (22° 15′ 54″ North, 39° 6′ 44″ East). We used soil, roots and leaves of plant sample for the isolation of both rhizopheric and endophytic bacteria and the sample plant were placed in sterile plastic bag at the time of collection. For isolation of bacteria from soil associated with roots they were dipped in sterile distilled water and serial dilutions were made (10^{-3}, 10^{-4}, and 10^{-5}) in filtered autoclaved sea water (FAS) and spread in triplicate on four different media used for culturing of bacteria. We have used half strength R2A (½ R2A) [0.25 g yeast extract, 0.25 g proteose peptone No. 3 (Difco), 0.25 g casamino acid, 0.25 g dextrose, 0.25 g soluble starch, 0.15 g sodium pyruvate, 0.15 g K_2HPO_4, 0.03 g $MgSO_4$], half Tryptic soy agar (½ TSA) [Pancreatic Digest of Casein, 7.5g Papain Digest of Soybean, 2.5 g Sodium Chloride, 2.5 g Agar, 15.0 g], marine agar (MA) [Peptone, 5.0 g Yeast Extract, 1.0 g Ferric Citrate, 0.1 g Sodium Chloride, 19.45 g Magnesium Chloride, 8.8 g Sodium Sulfate, 3.24 g Calcium Chloride, 1.8 g Potassium Chloride, 0.55 g Sodium Bicarbonate, 0.16 g Potassium Bromide, 0.08 g Strontium Chloride, 34.0 mg Boric Acid, 22.0 mg Sodium Silicate, 4.0 mg Sodium Fluoride, 2.4 mg Ammonium Nitrate, 1.6 mg Disodium Phosphate, 8.0 mg Agar,15.0 g] and half nutrient agar (½ NA) [Beef Extract, 1.5 g Peptone, 2.5 g Agar, 15.0 g] (Difco Laboratories, Detroit, MI) in 1000 ml filtered Sea water for bacterial culturing. For isolation of endophytic bacteria, roots, and leaves were surface sterilized. Both roots and leaves were washed several times with tap water to remove adhering soil particles and then sterilized by washing with disinfectants as described previously (Bibi et al., 2012). After sterilization, small pieces of sterilized root and leaf segments were ground in FAS using a sterile mortar with pestle. Aliquots were further serially diluted and plated in triplicate on four different media mentioned above. To suppress fungal growth, 50 μg/ml cycloheximide was mixed to the medium before pouring. The plates were incubated at 28°C for 2 weeks for bacterial growth.

Isolation of Rhizopheric and Endophytic Actinobacteria

For the isolation of rhizosphere and endophytic *Actinobacteria*, the same dilutions from soil, roots, and leaves were inoculated onto starch-casein agar (Himedia) in sea water supplemented with cycloheximide and nystatin (50 μg/ml). Further, plates were incubated at 28°C for up to 10-12 days. By checking morphological characteristics, *Actinobacteria* were selected and

subcultured using the same isolation medium. Individual colonies were streaked to check purity of the strains and stored in 15% (v/v) glycerol. All bacterial strains were labeled according to acquisition numbers of the King Abdulaziz University special infectious agents unit and stored at −70 C.

Screening of Bacteria for Antifungal Activity

All isolated bacteria were screened for their antifungal activity. We used five different fungal pathogens i.e., *Phytophthora capsici* (*P. capsici*), *Pythium ultimum (Py. ultimum),* (obtained in this laboratory from *Capsicum annuum* and *Cucumis sativus* respectively), *Magnaporthe grisea* (KACC 40415), *Altenaria malli* (KCTC 6972) and *Fusarium moniliforme* (KCTC 6149) were obtained from Korean Agricultural Culture Collection (KACC) and Korean type culture collection center (KCTC). Antifungal activity was determined by using the cross streak method (Bibi et al., 2012). All isolates were streaked on PDA medium supplemented with ½ R2A in sea water. Each 6 mm mycelial disc of freshly grown fungal pathogen was placed in the center of plate perpendicular to streak of isolates at 4 cm distance from edges of plate and incubated for 4–6 days at 28°C. Antagonistic strains were checked in duplicate for antagonistic activity evaluated by measuring the inhibition zone of fungal pathogens around each bacterial streak.

Bacterial DNA Extraction and 16S rRNA Gene Sequencing

Genomic DNA was extracted from the selected antagonistic bacterial isolates using a DNA extraction kit (Thermo Scientific, Waltham, USA). To identify antagonistic bacteria, 16S rRNA gene sequencing was performed. Approximately 1500 bp fragment of the 16S rRNA gene was amplified using bacterial universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′- GGTTACCTTGTTACGACTT-3′), the 16S rRNA gene fragment was amplified under the following PCR conditions: one cycle of 95°C for 5 min followed by 28 cycles of 95°C for 1 min, and annealing at 58°C for 50 s with extension at 72°C for 50s, and a final extension step at 72°C for 10 min (Bibi et al., 2012). PCR products were purified using PCR purification kit (Thermo Scientific, Waltham, USA), and sequenced commercially (Macrogen, South Korea). Antagonistic bacteria were identified by blast searches of their 16S rRNA gene sequences obtained using the EzTaxon server (https://www.ezbiocloud.net) (Kim et al., 2012) to identify antagonistic bacteria. Phylogenetic positions of the antagonistic bacteria were confirmed using CLUSTALX (Thompson et al., 1997) multiple alignments of the bacterial sequences and BioEdit software (Hall, 1999) was used to edit the gaps. The neighbor-joining method in the MEGA6 Programme with bootstrap values based on 1,000 replications was used for construction of the phylogenetic tree based on the 16S rRNA gene sequences (Tamura et al., 2013).

Evaluation of Hydrolytic Enzymatic Activity

Antagonistic bacterial isolates were further evaluated for their lytic enzyme production. Protease activity was checked using skim milk ½ R2A agar plates. Bacteria exhibiting protease production made clear zone on skim milk agar plates. Amylase production was checked on starch medium. Starch hydrolysis was seen as a clear zone on agar plates by positive strains (Kumar et al., 2012). For lipase activity, tributyrin ½ R2A agar medium was used. After 48 h of incubation at 28°C a clear zone was detected around bacterial colonies after hydrolysis of tributyrin. To check cellulase activity, CMC agar (carboxy methyl cellulose agar) medium was used. Bacteria were streaked on plates with the respective enzyme medium and incubated at 28°C for 2 days. Congo red (0.1%) solution was added to plates and put on orbital shaker for 30 min and then rinsed with 1MNaCl (Hendricks et al., 1995). Cellulase activity was observed as a clear zone on CMC agar plates around bacterial colonies.

Detection of Antimicrobial Genes

It has been well established that many antimicrobial products contain polyketides. Thus, to determine if the antagonistic bacteria might contain such enzymes, a search of the bacterial genome was done to check for the presence of genes coding for these enzymes. To detect biosynthetic genes, polyketide synthetase I (PKS-I) and nonribosomal peptide synthetase (NRPS) genes, the following primer pairs K1F/M6R (5′-TSAAGTCSAACATCGGBCA-3′; 5′-CGCAGGTTSCSGTAC CAGTA-3′) and A3F/A7R (5′GCSTACSYSATSTACACSTCS GG-3′; 5′-SASGTCVCCSGTSCGGTAS-3′), respectively were used (Ayuso-Sacido and Genilloud, 2005). Amplification of polyketide synthetase II (PKS-II) gene was performed using primer pair KSα/Kβ (5′-TSGCSTGCTTGGAYGCSATC-3′; 5′-TGGAANCCGCCGAABCCTCT-3′) (Metsa-Ketela et al., 1999). PCR reaction mixture (25 μl) contained 12.5 μl of PCR mastermix (promega), 1 μl of each primer, 1 μl of template DNA, and 9.5 μl of Nuclease-Free Water. Amplifications were performed under following PCR conditions: one cycle of 95°C for 5 min followed by 28 cycles of 95°C for 1 min, and annealing at 58°C (K1F/M6R), 56°C (A3F/A7R) and 62°C (KSα/Kβ) for 50 s with extension at 72°C for 50 s, and a final extension step at 72°C for 10 min. PCR amplification products were then checked by using 0.8% agarose through gel electrophoresis, and bands of 1.2–1.4 kb, 600 bp, and 700–800 bp were detected as products of PKS-I, PKS-II, and NRPS genes, respectively.

Optimization of Bacterial Culture Conditions for Production Bioactive Compounds

To optimize culture conditions of selected bacterial strains four different media i.e., ½ R2A broth, ½ TSB, ½NB in sea water and Marine broth in distilled were used for culturing. Bacterial cultures were grown for 72 h and after every 24 h optical density (OD) was checked and antifungal activity was assessed against *P. capsici*, *Py. ultimum* using the disc diffusion method. The effect of temperature was optimized within the range of temperatures (20–40°C) in ½ R2A broth. For pH optimization, different ranges of pH values (5–12) were used for the growth and antifungal compound production in ½ R2A broth.

LC-MS Analysis of Bacterial Culture

In order to assess the actual presence of antimicrobial products in bacterial cultures that had been previously screened and shown to be biologically active we performed LC-MS analyses of the bacterial supernatants. A Bacterial culture (5 ml) was placed on −80°C for 5 min, and then transfer to 37°C water bath for 5 min and this procedure was repeated 5 times. It was centrifuged at 15,000 g for 10 min and transfer 3 ml supernatant fluid was transferred to a tube. To it was added 12 ml of acetonitrile and vortexed for 30 s. It was centrifuged again at 15,000 g for 10 min and 300 μl supernatant was taken for LC-MS metabolomic analysis. The injection volume was 3 μl and samples were analyzed on an Agilent 6540B TOF/Q-TOF Mass Spectrometer coupled with an Agilent 1290 UPLC and Dual AJS ESI ion source. An ACQUITY UPLC HSS T3 (100 × 2.1mm, 1.8 um) column and pre-column (Phenomenex Security Guard™) was used to separate the sample. The column temperature was set to 45°C and flow rate was 0.5 ml/min. The acquisition range was from 50 m/z to 1500 m/z and the scan rate was 1.00 spec/sec. MS conditions were set as follow: capillary voltage 3500 V, nebulizer pressure 35 psi, drying gas 10 L/min, gas temperature 325°C, vaporizer 200 V, voltage charge 1000 V; negative-ion mode capillary voltage 3500 V, corona negative 15.0 V, fragmentor 175 V, skimmer1 65.0 V, octopole RF Peak 750 V; positive ion mode capillary voltage 3500 V, corona positive 4.0 V, fragmentor 175 V, skimmer1 65.0 V, and octopole RF Peak 750 V. Raw data was imported to the Agilent MassHunter Qualitative Analysis B.06.00 software. Metabolites were identified by using National Institute of Standards and Technology database (NIST) database. Further bioactive metabolites from other secondary metabolites in culture extract were identified using different public databases such as PubChem (http://pubchem.ncbi.nlm.nih.gov/), ChemSpider (http://www.chemspider.com/), SciFinder (http://www.cas.org/products/scifinder), and ChEMBL (https://www.ebi.ac.uk/chembl/).

Nucleotide Sequence Numbers

Nucleotide sequences of the antagonistic bacteria isolated from the halophyte have been deposited in the GenBank database under accession numbers KY436424–KY436445.

RESULTS

Isolation of Rhizospheric and Endophytic Bacteria

In this study, rhizospheric and endophytic bacteria were isolated from the halophyte, *S. imbricata* growing in a selected site on the Saudi Arabian peninsula. A total of 168 rhizospheric and endophytic bacteria were isolated from soil, roots, and leaves samples. Various methods were used to acquire these bacteria including the use of four different culturing media. It turns out that both high and low nutrient medium content favored the growth of different groups of bacteria. It has been noted that the ½ R2A in SW is a favorable medium for the isolation of bacteria from the marine environment. Thus, all isolated bacteria culture on different media were sub-cultured on ½ R2A in SW. In total 168 bacteria were isolated from different parts of halophyte and soil as well (**Table 1**). From soil, 85 rhizobacteria were isolated by culturing on different media listed above for isolation. Roots samples used for isolation of endophytic bacteria yielded 48 endophytic bacteria. While from leaves 35 bacteria were recovered. Details of bacteria number their antagonistic activity and dominance of different bacterial phyla is mentioned in **Table 1**.

Screening of Bacteria against Pathogenic Fungi

All isolated bacteria were tested for their antagonism against plant pathogenic oomycetes. Of the 168 bacteria isolated, 22 (13%) displayed inhibition to both oomycete plant pathogens that was the established bioassay test. The percentage of antagonistic bacteria varied in different parts of halophyte, being highest in roots ($n = 9$; 5.3%), followed by soil ($n = 8$; 4.7%), and leaves ($n = 5$; 2.9%). These antagonistic bacterial isolates were tested further against three other fungal pathogens. Different groups of antagonistic bacteria were identified from *S. imbricata*, and *Actinobacteria* was the dominant phylum (**Table 1**. Both rhizospheric and endophytic antagonistic *Actinobacteria* i.e., *Nocardiopsis dassonvillei* (EA52), *Pseudonocardia* sp. (EA58), *Arthrobacter* sp. (EA59), and *Streptomyces* sp. (EA64) showed strong inhibition against the oomycetes (**Table 2**). Some of the antagonistic isolates (EA56, EA62, EA68-EA70) showed inhibition against oomycetes but did not show inhibition against other pathogenic fungi used (**Table 2**).

Identification and Phylogenetic Analysis of Rhizopheric and Endophytic Antagonistic Bacteria

In total, 22 antagonistic bacteria were identified by using 16S rRNA gene sequence analysis. Result of sequencing showed that eight different genera of bacteria were identified belonging to four major classes: *Actinobacteria* ($n = 15$; 68%), *Firmicutes* ($n = 5$; 23%), *γ-Proteobacteria* ($n = 1$; 4.5%), and *β-Proteobacteria* ($n = 1$; 4.5%). Sequence identities of antagonistic bacteria were recorded from 95.7−100% (**Table 1**). Sequencing results indicated *Actinobacteria* as the dominant phylum and 26% of

TABLE 1 | Distribution of rhizopheric and endophytic antagonistic bacteria isolated from halophyte.

Isolation source[a]	Number of isolates[b]	Number of antagonist[c]	Antagonists (%)[d]	Dominant phylum[e]
Rhizopheric (Soil)	85	8	4.7	*Actinobacteria*
Endophytes (Roots)	48	9	5.4	*Actinobacteria*
Endophytes (Leaves)	35	5	2.9	*Firmicutes*
	168	22	13%	*Actinobacteria*

[a] *Isolation source of rhizopheric and endophytic bacteria.*
[b] *Total number of rhizopheric and endophytic bacteria isolated from soil, roots and leaves of halophyte.*
[c] *Total number of antagonistic rhizopheric and endophytic bacteria.*
[d] *Percentage of antagonistic rhizopheric and endophytic bacteria.*
[e] *Dominant phylum in all rhizopheric and endophytic antagonistic bacteria.*

TABLE 2 | Taxonomic identification, antifungal acitivity, and enzymes production of rhizo and endophytic bacteria from halophyte.

				Antifungal activity against[a]					Enzymatic activities[b]				Detection of		
Lab no	Closely related type strain	Accession number	% identity	*P. ultimum*	*P. capsici*	*M. grisea*	*A. mali*	*F. moniliforme*	Protease	Amylase	Lipase	Cellulase	NRPS	PKS-I	PKS-II
Salsola imbricate															
RHIZOPHERIC (SOIL)															
EA51	*Nocardioides luteus* KCTC 9575^T	KY436424	99.8	W	W	−	−	−	++++	−	++++	−	+	−	−
EA52	*Nocardiopsis dassonvillei subsp. dassonvillei* DSM 43111^T	KY436425	99.7	++++	+++	+	++	−	++++	−	−	+++	+	−	+
EA53	*Bordetella avium* ATCC 35086 ^{T}	KY436426	98.9	++++	++++	+++	−	−	−	−	−	−	−	−	−
EA54	*Pseudomonas boreopolis* ATCC 33662^T	KY436427	99.8	+	+	+	−	−	−	−	−	−	−	+	−
EA55	*Nocardioides luteus* KCTC 9575^T	KY436428	100	+	W	−	+	−	−	−	++++	−	−	+	−
EA56	*Nocardioides deserti* SC8A-24 ^{T}	KY436429	98.9	+	+	−	−	−	−	−	−	−	−	−	−
EA57	*Arthrobacter subterraneus* CH7 ^{T}	KY436430	99.3	+	W	−	+	−	++++	++++	−	−	+	−	−
EA58	*Pseudonocardia carboxydivorans* Y8^T	KY436431	100	++++	++++	+++	++++	+	−	−	−	−	−	+	−
ENDOPHYTES (ROOTS)															
EA59	*Arthrobacter subterraneus* CH7 ^{T}	KY436432	99.9	++++	+++	+	−	−	++++	+++++	++++	−	−	−	−
EA60	*Bacillus licheniformis* ATCC 14580^T	KY436433	99.4	+++	++	+	+++	++	++++	+++++	−	−	−	−	−
EA61	*Streptomyces enissocaesilis* NBRC 100763^T	KY436434	99.4	+++	+	+	+++	−	+++	+++++	−	−	+	−	+
EA62	*Streptomyces phaeopurpureus* NRRL B-2260^T	KY436435	98.8	+	W	−	−	−	−	−	−	−	−	−	−
EA63	*Streptomyces geysiriensis* NBRC 15413^T	KY436436	100	+	++	−	−	−	+++	W	−	−	+	−	+
EA64	*Streptomyces violaceochromogenes* NBRC 13100 ^{T}	KY436437	99.3	+++	+++	+++	−	−	−	−	++++	−	+	−	+
EA65	*Streptomyces diastatochromogenes* ATCC 12309^T	KY436438	97.3	++	++	+	−	+	−	−	−	−	−	−	+
EA66	*Bacillus sonorensis* NBRC 101234^T	KY436439	95.7	++	+	+	+++	+++	−	−	++++	−	+	−	−
EA67	*Streptomyces europaeiscabiei* KACC 20186^T	KY436440	98.2	++	+	++	+++	+	+++	−	−	−	−	−	+
ENDOPHYTES (LEAVES)															
EA68	*Bacillus tequilensis* KCTC 13622^T	KY436441	100	+	W	−	−	−	−	−	−	−	−	−	−
EA69	*Nocardioides luteus* KCTC 9575^T	KY436442	99.1	+	+	−	−	−	−	+++++	++++	−	−	−	−
EA70	*Agromyces indicus* NIO-1018 ^{T}	KY436443	100	++	+	−	−	−	−	++	−	−	−	−	−
EA71	*Bacillus halosaccharovorans* E33^T	KY436444	98.9	+++	+	+	−	+++	−	−	−	−	−	−	−
EA72	*Bacillus subtilis subsp. inaquosorum* KCTC 13429^T	KY436445	99.3	+	++	++	+	−	+++	−	+++	−	+	+	+

[a]*Antagonistic activity of all bacteria isolated in this study. The activity was measured after 3–5 days incubation at 28°C by measuring the clear zone of mycelial growth inhibition: W, weak; –, Negative; +, 3 mm; ++, between 4 and 6 mm; +++, between 7 and 9 mm; ++++, between 10 and 12 mm.*

[b]*Production of protease, amylase, lipase, and cellulase was determined by plate assay. Enzymatic activity was estimated as zone of halo formed around bacterial colonies: W, weak; –, Negative; +, 3 mm; ++, between 4 and 6 mm; +++, between 7 and 9 mm; ++++, between 10 and 12.*

the antagonistic isolates belonged to the genus *Streptomyces* (**Table 1**). Using 16S rRNA gene data, a neighbor Joining (NJ) phylogenetic tree was constructed (**Figure 1**). High bootstrap values were recovered in phylogenetic tree. A separate cluster has been generated for isolates of class *Actinobacteria*. Antagonistic strains in this cluster were identified with high bootstraps values (51–100%). Representatives of this class belong to four different genera i.e., *Nocardioides, Nocardiopsis, Arthrobacter,* and *Streptomyces.* One strain of *β-Proteobacteria* was identified with separate cluster also showing high bootstrap value (100%). One strain of *γ-Proteobacteria* made a distinct cluster with closely related type strain *Pseudomonas* sp. (EA54) with high a bootstrap value of 100 %. Antagonistic bacterial strains of class *Firmicutes* were placed in separate cluster belonging to the genus *Bacillus*. In this study, two antagonistic endophytic bacterial strains were also identified showing low 16S rRNA gene sequence similarity (<98%). One strain of *Actinobacteria* i.e., *Streptomyces* sp. (EA64) and *one* strain belonging to *Firmicutes* i.e., *Bacillus* sp. (EA68) were identified with similarities of <98% to the closest type strains (**Table 3**). Four antagonistic *Actinobacteria* were selected for secondary metabolites identification depending on their antagonistic activity against oomycetes.

Enzymatic Activities of Antagonistic Bacteria

Antagonistic bacteria were evaluated for their ability to produce fungal cell wall lytic enzymes. Protease, amylase, lipase, and cellulase activities of these bacteria were examined (**Table 2**). The number of bacteria exhibiting protease activity ($n = 9$; 41%) was high as compared to other enzymatic activities. Most of protease producing bacteria were endophytes ($n = 6$) and recovered from leaves and roots of halophyte. Amylase activity was observed for 7 (32%) antagonistic bacteria. Mostly strains of *Actinobacteria* showed amylase activity. Endophytic strains of *Actinobacteria* exhibited high production of amylase. Lipase activity was observed in 7 (32%) antagonistic bacteria. Strains of *Actinobacteria* and *Firmicutes* showed high production of protease. Cellulase activity was observed in only 1 (4.5%) bacterial strain. A strain of *N. dassonvillei* (EA52) exhibited cellulase activity belonged to *Actinobacteria*.

Detection of PKS and NRPS Genes

Bacteria possess NRPS/PKS gene clusters especially common in the phyla *Proteobacteria, Actinobacteria* and *Firmicutes*. Antagonistic bacterial isolates from this study showed that of

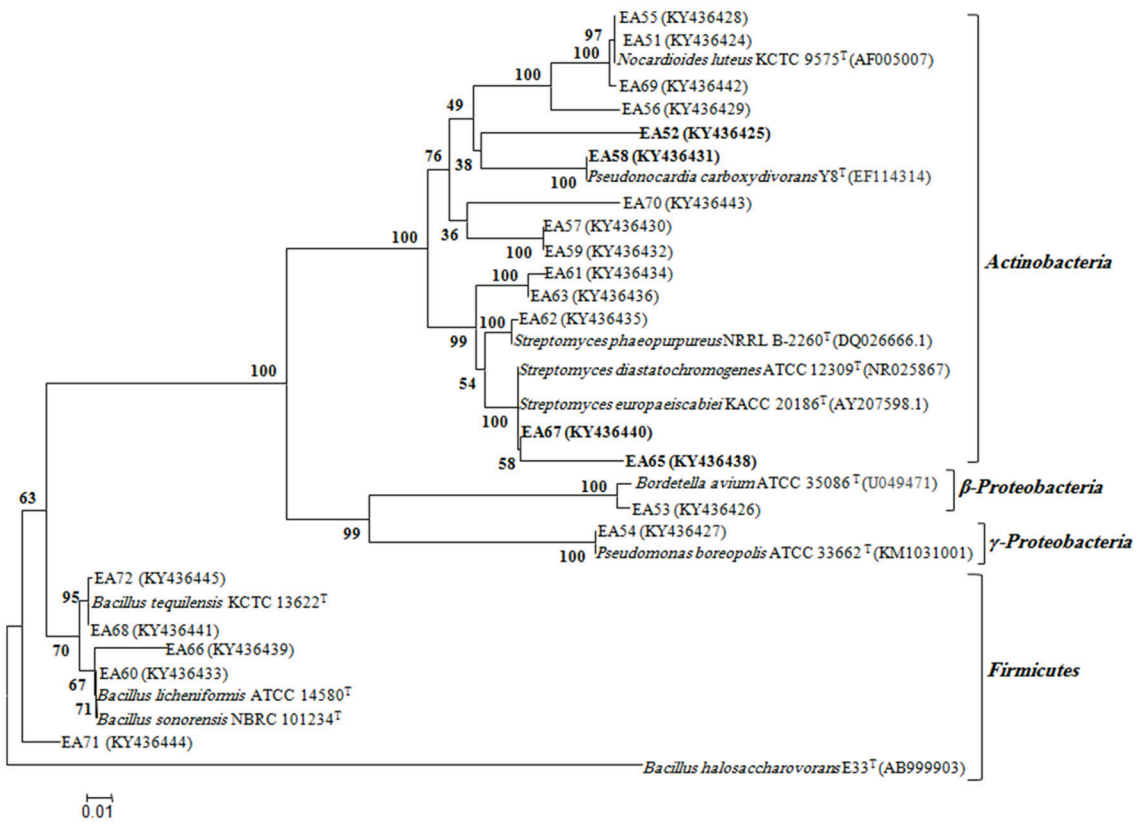

FIGURE 1 | Phylogenetic distribution of antagonistic bacteria isolated from *S.imbricata* on the basis of 16S rRNA gene sequences obtained from bacteria and closely related sequences of the type strains of other species. The phylogenetic relationships were inferred from the 16S rRNA gene sequences (1 kb) by using the neighbor-joining method from distances computed with the Jukes-Cantor algorithm. Bootstrap values (1,000 replicates) are shown next to the branches. GenBank accession numbers for each sequence are shown in parentheses. Bar, 0.01 accumulated changes per nucleotide. Isolates selected for bioactive metabolites identification are highlighted.

TABLE 3 | Tentative novel taxa based on partial 16S rRNA gene sequence analyses from this study.

Source	Similarity	Identity%	Family	Gram stain
Rhizosphere (Soil)	*Bordetella avium* ATCC 35086 ^{T}	98.9	*Alcaligenaceae*	Gram-negative
Rhizosphere (Soil)	*Nocardioides deserti* SC8A-24 ^{T}	98.9	*Nocardioidaceae*	Gram-positive
Endophytic (Root)	*Streptomyces phaeopurpureus* NRRL B-2260 ^{T}	98.8	*Streptomycetaceae*	Gram-positive
Endophytic (Root)	*Streptomyces diastatochromogenes* ATCC 12309^{T}	97.3	*Streptomycetaceae*	Gram-positive
Endophytic (Root)	*Bacillus sonorensis* NBRC 101234 ^{T}	95.7	*Bacillaceae*	Gram-positive
Endophytic (Leaf)	*Streptomyces europaeiscabiei* KACC 20186^{T}	98.2	*Streptomycetaceae*	Gram-positive
Endophytic (Leaf)	*Bacillus halosaccharovorans* E33^{T}	98.9	*Bacillaceae*	Gram-positive

22 isolates, PCR amplification revealed that 13 strains (59%) were positive for at least one of the biosynthetic genes checked (**Table 2**). For the NRPS gene, 8 (36%) strains were positive. NRPS genes were detected in both classes of *Actinobacteria* and *Firmicutes*. The PKS-I gene was detected in only 4 (18%) antagonistic bacterial strains. While PKS-II gene was detected in 7 (32%) strains. All three genes PKS-I and PKS-II and NRPS were detected in strain *Bacillus* sp. (EA72). Three isolates belong to *Actinobacteria, Streptomyces enissocaesilis* sp. (EA52), *Streptomyces* sp. (EA63), and *Streptomyces* sp. (EA64) were positive for the presence of both NRPS and PKS-II.

Identification of Strain EA52, EA58 (Rhizobacteria), and EA65, EA67 (Endobacteria) Metabolites by LC-MS

On the basis of high inhibition activity against fungal pathogens we have selected four antagonistic bacterial strains from our study to identify secondary metabolites. These four antagonistic isolates belong to class *Actinobacteria* i.e., *Nocardiopsis* sp. (EA52) and *Pseudonocardia* sp. (EA58) were rhizobacteria while *Streptomyces* sp. (EA65) and *Streptomyces* sp. (EA67) are endophytes. Production of antifungal compounds was influenced by culturing in R2A as culture medium using optimum culture conditions. The maximum antifungal activity was observed after 48 h of growth at 28°C with pH 7.5. Metabolites in cultures extract of selected bacteria were analyzed using the LC-MS. LC-MS technique confirms presence of various bioactive compounds although not novel but already known for their bioactivity. Identification of metabolites was determined by LC-MS analysis and comparing results from NIST database. LC-MS analysis of culture extract of these selected strains identified different bioactive metabolites (**Table 4**). Strain of *Nocardiopsis* sp. (EA52) produced 7 identified peaks of different bioactive metabolites in both the positive- and negative-ion mode (**Figures 2A,B**). These compounds include Thiabendazole, Sulfamethoxypyridazine, N-Nitrosodiethylamine, Ibuprofen, Laberalol, Isoxsuprine, and Oxibendazole. For *Pseudonocardia carboxydivorans* (EA58), 2 bioactive compounds have been detected among hundreds of peaks known for other different metabolites. These three compounds include Sulfamethoxypyridazine and Dimetridazole (**Figures 2C,D**). Strain EA65 genetically closely associated as a *Streptomyces diastatochromogenes* showed presence of nine different bioactive compounds in the culture extract i.e., Sulfamethoxypyridazine, Dimetridazole, Salbutero, Moxidectin, Atenolol, Timolol, Benzydamine, Acebutolol, and Bambuterol (**Figures 2E,F**). These bioactive secondary metabolites are already known for their biological activities. LC-MS analysis of strain EA67, *Streptomyces europaeiscabiei* showed the presence of six bioactive compounds among several compounds detected. These bioactive compounds include, Sulfamethoxypyridazine, Benzamide, Sulfamerazine, Dimetridazole, Metronidazole-oh, and Carazolol (**Figures 2G,H**). These compounds are already known for antimicrobial, antiphytopathogenic and biocontrol properties.

DISCUSSION

There is a need for the discovery of new drugs due to the emergence of resistant bacteria to different antibiotics and cancer cells to anticancer drugs. Currently, marine habitats have been identified as a source of discovering many important novel therapeutic agents (Malve, 2016). *S.imbricata*, is a halophyte containing various chemical compounds of medicinal use and it is widely spread in the sea shore areas in Saudi Arabia. Generally halophytes with traditional health benefits in folk medicine are a source of attraction for modern comprehensive microbial and chemical investigations. In our previous study (Bibi et al., 2017) we have isolated two rhizobacteria from *Salsola* exhibiting cellulase and amylase activity on culturing media. One strain EA151 with cellulase activity belonged to *Firmicutes* while other strain EA152 exhibited amylase activity closely related to type strain of *Actinobacteria.*

In this study, rhizopheric and endophytic bacterial populations with potent antagonistic activity against phytopathogens were studied. One hundred and sixty eight rhizopheric and endophytic bacteria were isolated and only 22 were found to be potent antagonists of oomycetes. Different cultivation media were used to culture bacteria to isolate novel isolates with unique bioactivities. A study has reported that high nutrient medium favors growth of diverse groups of bacteria, while low nutrient media increase number of bacteria (Vishnivetskaya et al., 2000). To our knowledge, this work is the first study on the isolation, screening, characterization and metabolites identification of both rhizospheric and endophytic bacteria from *S. imbricata*. In this study, culture-dependent approach was used to study the diversity of antagonistic bacteria

TABLE 4 | Secondary metabolites found in crude extract of selected four *Actinobacteria* strains.

No	Name	Formula	RT	m/z	MASS	Score	Diff(DB, ppm)	Mode	Height	Area
STRAIN EA52										
1	Thiabendazole	C10 H7 N3 S	0.962	201.037	201.0366	44.89	−2.4	Negative	36878	184023
2	Sulfamethoxypyridazine	C11 H12 N4 O3 S	1.125	279.0582	280.0655	75.22	−9.01	Negative	44633	255086
3	N-Nitrosodiethylamine	C4 H10 N2 O	1.579	215.0641	102.0784	46.98	8.48	Negative	23538	232204
4	Ibuprofen	C13 H18 O2	2.042	189.127	206.1303	92.03	2.07	Positive	62696	509785
5	Laberalol	C19 H24 N2 O3	2.8	329.1839	328.1766	81.9	6.46	Positive	54189	463656
6	Isoxsuprine	C18 H23 N O3	2.892	302.1733	301.1658	82.96	6.73	Positive	139154	2034111
7	Oxibendazole	C12 H15 N3 O3	3.533	250.1187	249.1114	97.67	−0.32	Positive	126155	961667
STRAIN EA58										
8	Sulfamethoxypyridazine	C11 H12 N4 O3 S	1.117	279.0575	280.0647	67.12	−6.21	Negative	15321	77965
9	Sulfadiazin	C10 H10 N4 O2 S	2.683	249.0475	250.055	72.64	−10.1	Negative	29954	210781
10	Dimetridazole	C5 H7 N3 O2	4.045	159.0876	141.0527	70.14	8.14	Positive	111793	948315
STRAIN EA65										
11	Sulfamethoxypyridazine	C11 H12 N4 O3 S	1.126	279.0574	280.0649	80.47	−6.78	Negative	47164	293920
12	Sulfadiazin	C10 H10 N4 O2 S	2.686	249.0474	250.0548	74.78	−9.52	Negative	41010	344049
13	Sulfacetamide	C8 H10 N2 O3 S	1.793	215.0474	214.0401	72.66	5.06	Positive	68031	563882
14	Dimetridazole	C5 H7 N3 O2	4.039	159.0881	141.0535	82.65	2.25	Positive	211556	1889105
15	Salbuterol	C13 H21 N O3	15.786	239.1764	239.1532	47.28	−4.28	Positive	1181230	8578877
16	Moxidectin	C37 H53 N O8	16.671	662.3704	639.3739	49.37	4.97	Positive	95729	1314871
17	Atenolol	C14 H22 N2 O3	17.318	267.1729	266.1657	77.62	−10.11	Positive	907768	7927165
18	Timolol	C13 H24 N4 O3 S	21.415	317.1641	316.1571	73.26	−0.54	Positive	67063	538273
19	Benzydamine	C19 H23 N3 O	21.419	310.1884	309.1797	21.59	14.35	Positive	89361	929352
20	Acebutolol	C18 H28 N2 O4	21.711	336.2052	336.2045	59.81	1.09	Positive	88941	887397
21	Bambuterol	C18 H29 N3 O5	21.714	367.2376	367.2098	51.49	2.53	Positive	58314	570222
STRAIN EA67										
22	Sulfamethoxypyridazine	C11 H12 N4 O3 S	1.122	279.0576	280.0651	80.42	−7.33	Negative	30594	156782
23	Sulfadiazin	C10 H10 N4 O2 S	2.682	249.0471	250.0545	78.19	−8.26	Negative	44098	367641
24	Sulfanilamide	C6 H8 N2 O2 S	0.748	173.0376	172.0307	91.12	−0.47	Positive	95732	1075748
25	Sulfadiazin	C10 H10 N4 O2 S	1.223	251.058	250.0508	65.17	6.67	Positive	99002	476334
26	Sulfacetamide	C8 H10 N2 O3 S	1.8	215.0475	214.0404	90.98	3.98	Positive	142535	1446212
27	Benzamide	C7 H7 N O	2.72	103.0426	121.0525	37.43	1.82	Positive	641812	5370210
28	Sulfamerazine	C11 H12 N4 O2 S	2.991	265.0734	264.0662	65	7.1	Positive	59659	389438
29	Dimetridazole	C5 H7 N3 O2	4.059	159.0879	141.0531	75.55	5.01	Positive	149866	1286924
30	Metronidazole–oh	C6 H9 N3 O4	4.06	188.0669	187.0597	95.68	−1.89	Positive	1776794	17824964
31	Carazolol	C18 H22 N2 O2	20.924	303.1474	298.1688	42.24	−2.22	Positive	81193	668836

from halophyte against oomycetes plant pathogens. Screening of 168 rhizospheric and endophytic bacteria and their 16S rRNA gene sequencing resulted in 22 different bacteria belong to four major classes. Bioactive compounds production by rhizo and endophytic bacteria is one of the phenomenon used by bacteria to defend the host against different pathogens.

Antagonistic bacteria belong to four major classes, *Firmicutes*, *γ-Proteobacteria,* and *β-Proteobacteria*. Our results show that *Actinobacteria* is recognized as a dominant phylum among these antagonitistic bacteria isolated from halophyte (**Table 1**). Actinobacteria are distributed in various ecological habitats especially in terrestrial and aquatic ecosystems where they play a potential role in decomposition of plants and animals remains. They are biotechnologically important due to their ability to produce secondary metabolites (Berdy, 2005). Therefore, we have selected four different strains of *Actinobacteria* for further study of bioactive metabolites produced by these antagonistic rhizopheric and endophytic bacteria. Results of 16S rRNA sequence analyses showed a diversity of different genera of *Actinobacteria* and the genus *Streptomyces* remain which are dominant in our study. Results of this study are also compatible with previous studies where many novel *Actinobacteria* have been recovered from different unexplored marine sources (Fiedler et al., 2005; Bull and Stach, 2007). Halophytes growing near the sea shore in tropical and subtropical areas have become valuable sources for the isolation of microbial flora especially in the group *Actinobacteria* because of the chemodiversity of environmental factors prevailing there. The presence of major numbers of *Streptomyces* strains amongst antagonists from the halophyte in this study are in accordance with the results reported

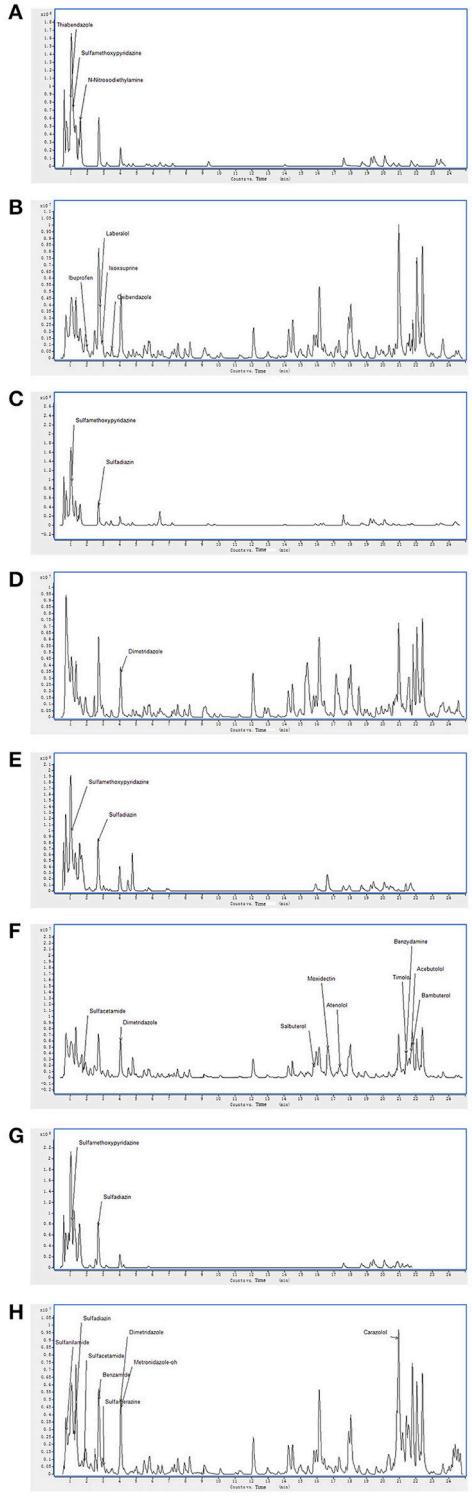

FIGURE 2 | Spectra of LC/MS analysis showing detection of various bioactive metabolites. *Nocardiopsis* sp. (EA52) **(A)** negative mode LC/MS analysis **(B)** positive mode LC/MS analysis **(C)** negative mode LC/MS analysis of *Pseudonocardia* sp. (EA58) **(D)** positive mode LC/MS analysis **(E)** negative mode LC/MS analysis of *Streptomyces* sp. (EA65) **(F)** positive mode LC/MS analysis **(G)** negative mode LC/MS analysis of *Streptomyces* sp. (EA67) **(H)** positive mode LC/MS analysis.

previously (Eccleston et al., 2008; Ravikumar et al., 2012). In *Actinibacteria*, the most active bacterial strains in addition to *Streptomyces* are, *Nocardiopsis*, *Pseudonocardia,* and *Arthrobacter* which have been reported for their inhibitory activities and production of antimicrobial metabolites (Anandan et al., 2016).

The metabolic diversity of *Streptomycetes* is remarkable and novel strains with new bioactive compounds including antibiotics have been isolated from extreme habitats (Fiedler et al., 2005). Until now, several studies have reported diverse groups of antimicrobial compounds by bacteria isolated from halophytic endophytes, such as steroids, peptides, alkaloids, terpenoids, quinines, flavonoids, and phenols (Newman and Cragg, 2007). Our results showed that most of the endophytes in this study belonging to *Streptomyces* and were recovered from root samples. This finding is supported by previous results where maximum number of endophytes were recovered from roots specimens (Taechowisan et al., 2003; Passari et al., 2015) This may be due to the reason that the rhizosphere has a high concentration of nutrients and various bacteria enter different root tissues of the host plant through the rhizosphere and become endophytic in their mode of life (Rosenblueth and Martinez-Romero, 2006).

The second dominant class of bacteria in this study was *Firmicutes*. The group of bacteria belonging to the class *Firmicutes* are commonly found as they are easily culturable. Also, from marine sources, species of *Bacillus* are dominant for the production of antibacterial, antifungal antibiotics, surfactants, and lytic enzymes (Mondol et al., 2013). Endophytic bacterial strains of *Bacillus* from halophytes are already reported to show inhibition against bacterial and fungal plant pathogens (Menpara and Chanda, 2013). In this study, only two antagonistic rhizopheric and endophytic bacteria were related to the class *Proteobacteria* belonging to two different classes *γ-Proteobacteria* and *β-Proteobacteria.* Members of class *γ-Protobacteria* from marine source produce the highest number of secondary metabolites (Long and Azam, 2001).

Hydrolytic enzymes from marine bacteria are produced and used by them in catalyzing different biochemical processes in the marine environment (Thatoi et al., 2013). In addition to production of useful secondary metabolites and antibiotics Actinobacteria from marine sources are also known for the production of lytic enzymes of industrial use (Anandan et al., 2016). In this study, mostly strains of *Actinobacteria* and *Firmicutes* were active candidates for production of different enzymes. Protease, lipase and amylase activity was mostly observed (**Table 2**). Only one strain EA59 exhibited protease, amylase and lipase activity. This strain is endophytic and belongs to *Actinobacteria*. It seems as if the productions of these hydrolytic enzymes is necessary for antagonism as well as for intracellular colonization of bacteria in the host plant. Only four strain of rhizobacteria belong to *Actinobacteria* were able to produce at least one type of enzyme tested in this study. While most of the endophytic bacteria were able to show enzymatic activities belong to *Actinobacteria*. Marine *Actinobacteria* have the potential to produce extracellular enzymes of industrials use. These extracellular enzymes are usually used by endophytic bacteria for their antagonism against different pathogens invading host plants (Zhao et al., 2016).

Polyketide synthetase (PKS) and nonribosomal polyketide synthetase (NRPS) genes are considered as hallmarks for the production of secondary metabolites. These biosynthetic genes of bacterial secondary metabolites are involved in the biosynthesis of many bioactive products. The presence of the biosynthetic genes PKS and NRPS in rhizospheric and endophytic bacteria associated with this halophyte suggests that these beneficial bacteria are dominant producers of bioactive natural products. Amplification of these three biosynthetic gene clusters domains is an indirect approach to test the knowledge that bioactive products are being produced by the organism. Of 22 isolates screened for detection of PKS-I, PKS-II, and NRPS gene, only 13 strains were positive for at least one of the biosynthetic genes tested. Several species of *Streptomyces* possess two types of PKSs. One is responsible for antibiotic synthesis, while the other is involved in spore pigments production in various *Streptomyces* species (Bergh and Uhlén, 1992). In this study, PKS-II and NRPS were detected in most of the *Streptomyces*. Among them, strains EA52, EA61, EA63, and EA64 were positive for the presence of NRPS and PKS-II, and also exhibited antifungal activity against tested pathogens (**Table 2**). While strains EA53, EA56, EA59, EA60, EA62, EA68-EA71 were negative for the presence of PKS-I, PKS-II, and NRPS genes and positive for antifungal activity. Therefore, there is no correlation between presence of PKS and NRPS gene and antimicrobial properties (Qin et al., 2009). It is evident that NRPS PKS-I and PKS-II gene screening using PCR in different bacterial strain help to find out potential strain of bacteria producing important secondary metabolites.

Strains of *Streptomycetes* are active producers of antimicrobial compounds and more than 70% of antibiotics in the world are produced by them. Endophytic *Streptomycetes* occupy a biologically important niche in tissues of the host plant, taking nutrition from host and in turn providing protection to the plant. These endophytes produce important metabolites that are not toxic to their host therefore, can be considered as important bioactive metabolites in drug discovery (Castillo et al., 2007). Selection of *Actinobacteria* for identification of active secondary metabolites resulted in a number of known synthetic bioactive compounds not of microbial origin (**Table 4**). Based upon antifungal activity and low 16S rRNA gene similarity four out of twenty-two rhizopheric and endophytic strains were ultimately selected for LC-MS analyses. Production of antifungal compounds was influenced by culturing the organism in R2A as a culture medium using optimum culture conditions. The maximum antifungal activity was observed after 48 h of growth with pH 7.5 at 28°C. Metabolites in cultures extracts of selected bacteria were analyzed using LC-MS. Two rhizopheric strains namely EA52 closely related to *Nocardiopsis* sp. and EA58 showed similarity with *Pseudonocardia* sp. in producing diverse metabolites including Thiabendazole, Sulfamethoxypyridazine, N-Nitrosodiethylamine, Ibuprofen, Laberalol, Isoxsuprine, Oxibendazole, and Dimetridazole. Strain EA52 showed close similarity to *Nocardiopsis sp*. This marine non-Streptomycete genus of *Actinobacteria*, contained a variety of bioactive compounds and showed a wide range of activities including cytotoxicity, antibacterial, antifungal, and anti-angiogenesis. *Nocardiopsis sp*. contained α-pyrone (nocapyrone S1) and (4-aminophenyl) acetic acid, N-(2- hydroxyphenyl)-acetamide, cyclo-(L-Pro-L-Val), cyclo-(L-Pro-L-Leu), and cyclo-(L-Pro-L-Ile) when evaluated for secondary metabolite production (Zou et al., 2017) but different secondary metabolites have been detected in closely related strain EA58 in the present study (**Figures 2A,B**). Antimicrobial activity of strains of *Pseudonocardia* is already evident from previous studies but no such antifungal activity and metabolites detected in our study were produced from the closely related strain EA58 (**Figures 2C,D**; Tanvir et al., 2016).

Two endophytic *Streptomycetes* EA65 and EA67 were analyzed by LC-MS and they exhibited spectra for 15 bioactive compounds including antibacterial, antifungal, antiprotozoal and anthelmintic compounds. Strain EA65 showed close similarity at the 97% level to a rhizobacterium *S. diastatochromogenes,* a promising source of antifungal metabolites and novel natural antibiotics and used as biocontrol agents (Shentu et al., 2016). *S. diastatochromogenes* reportedly produces polyketomycin, momofulvenoneA, azdimycin, toyocamycin, concanamycin, and oligomycins. Strain EA65 exhibited the presence of bioactive compounds not reported in *S. diastatochromogenes* including Sulfamethoxypyridazine, Dimetridazole, Salbutero, Moxidectin, Atenolol, Timolol, Benzydamine, Acebutolol, and Bambuterol (**Figures 2E,F**). Another endophytic strain EA67 related to *S. europaeiscabiei* showed the presence of bioactive compounds such as Sulfamethoxypyridazine, Benzamide, Sulfamerazine, Dimetridazole, Metronidazole-oh, and Carazolol (**Figures 2G,H**). These compounds are already known for antimicrobial, antiphytopathogenic and biocontrol properties. Sulfamethoxypyridazine, Nitroimidazole including Dimetridazole and Metronidazole-oh and Moxidectin are major antimicrobial compounds detected in all four strains in this study. Sulfamethoxypyridazine was detected in all four strains in our study. These antibacterial sulfonamides are synthetic antimicrobial agents used as antibiotics in different inflammatory diseases (Vicente and Pérez-Trallero, 2010). This is first study showing the isolation and screening of antagonistic bacteria from *S. imbricata* and the identification of active metabolites from crude extracts. Compounds produced by these strains although not new, have been not reported in literature from other organisms and most of them are synthetic so this is the first reported of these *Actinobacteria* as a natural source for production of these antimicrobial compounds.

In conclusions, present study of antagonistic bacteria from *S. imbricata* exhibited wide range antifungal and enzymatic activities indicating their industrial, biotechnological and agricultural potential. Further analyses of bioactive metabolites from selected *Actinobacteria* exhibited production of known antibiotics and bioactive compounds of synthetic nature not reported from bacteria before. These results suggest that halophytes are potential source of bioactive metabolites via their associated bacterial microflora. This study points to an enormous potential of discovering novel bioactive compounds using the methodology described. Currently we are working on some

selected novel strains for deep bioactive analysis and genome sequencing that will be presented in our future work.

AUTHOR CONTRIBUTIONS

FB and GS: designed the study and write manuscript; MN and EA: identified the plant samples and help in collection of sample and experimental work; MY and AK collect and analyzed the data. All authors read and approved the final manuscript.

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Isolation, Diversity, and Antimicrobial and Immunomodulatory Activities of Endophytic Actinobacteria From Tea Cultivars Zijuan and Yunkang-10 (*Camellia sinensis* var. *assamica*)

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Endophytic actinobacteria exist widely in plant tissues and are considered as a potential bioresource library of natural products. Tea plants play important roles in human health and in the lifestyles of Asians, especially the Chinese. However, little is known about the endophytic actinobacteria of tea plants. In this study, 16 actinobacteria of 7 different genera and 28 actinobacteria of 8 genera were isolated and analyzed by 16S rRNA gene sequencing from tea cultivars of Zijuan and Yunkang-10 (*Camellia sinensis* var. *assamica*), respectively. The diversity of actinobacteria species from Zijuan were higher in July than December (6 vs. 3 genera), but the diversity of species from Yunkang-10 were higher in December than July (7 vs. 3 genera). No actinobacteria isolates were obtained from any tea cultivar in September. Ten isolates from Yunkang-10 exhibited antimicrobial activity against at least one human pathogenic microorganism (*Staphylococcus epidermidis*, *Shigella flexneri*, and *Escherichia coli*), but none of the isolates from Zijuan exhibited antimicrobial activities. Fourteen strains were further exammined the genes of polyketide synthetase (*PKS*)-*I* and *PKS-II* and non-ribosomal peptide synthetase (*NRPS*). *Brevibacterium* sp. YXT131 from Yunkang-10 showed strong inhibitory activity against *S. epidermidis*, *Sh. flexneri*, and *E. coli*, and *PKS-I* and *PKS-II* and *NRPS* genes were obtained from the strain. In *in vitro* assays, extracts from 14 actinobacteria that were tested for antibiotic biosynthetic genes showed no inhibition of concanavalin A (ConA)-induced murine splenocyte proliferation. In *in vivo* assays, the crude extract of YXT131 modulated the immune response by decreasing the proinflammatory cytokines interleukin (IL)-12/IL-23 p40 and tumor necrosis factor (TNF)-α in the serum of mice. These results confirm that endophytic actinobacteria from tea plants might be an undeveloped bioresource library for active compounds.

Keywords: *Camellia sinensis*, endophytic actinobacteria, diversity, antimicrobial activity, immunomodulatory activities

INTRODUCTION

Actinobacteria are aerobic, gram-positive bacteria and are well-known producers of a vast array of secondary metabolites, including antibiotics, immunosuppressive agents, antitumor agents, and enzymes, many of which are of great importance to the pharmaceutical and agricultural industries (Saini et al., 2015; Landwehr et al., 2016; Salcedo et al., 2016; Yang et al., 2016). Endophytes are microorganisms that ubiquitously colonize the internal tissues of plants without causing any negative effects, and some endophytes are able to control plant pathogens and promote the growth of plants (Santoyo et al., 2016; Kandel et al., 2017). Although numerous species of actinobacteria occur in the soil, other microbial habitats, such as leaf litter and plants, are potential sources of actinobacteria for the isolation of biologically active compounds (Sardi et al., 1992; Takahashi and Omura, 2003; Gos et al., 2017). In recent years, endophytic actinobacteria have been isolated from many crop plants (such as wheat, rice, and potatoes) (Coombs and Franco, 2003; Sessitsch et al., 2004; Tian et al., 2007) and medicinal plants (Qin et al., 2009; Gos et al., 2017). Endophytic actinobacteria are a potential source for the production of secondary metabolites that are used in the direct antagonism of pests and diseases (Cao et al., 2005) as well as various natural products with antimicrobial, antitumor, and anti-infection activities (Qin et al., 2011; Gos et al., 2017). Endophytic actinobacteria can also confer salt tolerance to host plants and promote host-plant growth (Qin et al., 2017).

As a popular non-alcoholic beverage, tea and tea drinks play important roles in human health and lifestyle, such as by reducing cardiovascular mortality and treating digestive disorders (Yang C.S. et al., 2009; Persson et al., 2010; Begas et al., 2017). As the same plant species, the tea cultivars of Zijuan and Yunkang-10 belong to the taxonomic species of *Camellia sinensis* var. *assamica*, which originated from the Yunnan province of China (Wang et al., 2017; Zhu et al., 2017). The two closely related cultivars of Zijuan and Yunkang-10 are the major raw materials for Pu'er tea (a kind of dark tea) produced in Yunnan. Catechins are the major secondary metabolic products in tea leaves (especially in green tea), and polyphenols are known to confer health benefits (Khan and Mukhtar, 2007). Anthocyanin is another functional flavonoid with health benefits and is widely distributed in higher plants (e.g., vegetables, flowers, fruits, cereals, and tea plants) (Joshi et al., 2015; Fabroni et al., 2016; Chen et al., 2017). The total anthocyanin content in Zijuan has been reported to be almost three times that found in other Chinese tea cultivars (including Yunkang-10), and thus Zijuan is considered to be an anthocyanin-rich cultivar (Yang X.R. et al., 2009). However, only a few studies have observed on tea plant endophytes, and those studies have mainly been performed on endophytic fungi and for plant disease protection (Rabha et al., 2014). For example, the endophytic fungus *Colletotrichum gloeosporioides* from healthy tea plant leaves shows strong inhibitory activity against tea pathogens of *Pestalotiopsis theae* and *Colletotrichum camelliae*; the inhibitory factors may be the highly efficient fungal chitinase and protease (Rabha et al., 2014). Compared to endophytic fungi, endophytic actinobacteria have received almost no attention in tea plants, and the interactions between endophytic actinobacteria and tea plants have not been investigated. Based on recent research progress in other plants, the study of endophytic actinobacteria culture and bioactivity is of great significance for developing bioresource libraries. In this study, the endophytic actinobacteria from tea plants of Zijuan and Yunkang-10 were isolated at different seasons, and the bioactivities of antimicrobial and immunomodulatory were screened and further evaluated for the isolates.

MATERIALS AND METHODS

Tea Sample Collection

Three-year-old tea plants of Zijuan and Yunkang-10 (*Camellia sinensis* var. *assamica*) were obtained from Dechang tea plantation (Shucheng China, 31°11' N, 116°47' E). The leaf samples of Zijuan and Yunkang-10 were collected on July 5, September 10, and December 5 of 2015. At the each time of sampling, 15 branches with the same growth and no pest damage were selected for each tea cultivar (considered as 1 sample). The healthy branches were placed in fully soaked floral foam and transported to the laboratory within 2 h for actinobacteria isolation; the isolation procedures were performed within 96 h.

Endophytic Actinobacteria Isolation

The leaf samples were pretreated following the method described by Qin et al. (2009) with minor modifications. The samples were air-dried for 48 h at room temperature and then washed with ultrasonic cleaning (160 W, 15 min). After drying, the samples were sterilized in the following order: a 6-min wash in 5% NaOCl, followed by a 10-min wash in 2.5% $Na_2S_2O_3$, a 5-min wash in 70% ethanol, a 5-min wash in sterile water, and a final rinse in 10% $NaHCO_3$ for 10 min. The sterilized tissues were imprinted on nutrient agar (NA, Difco) and tryptic soy agar (TSA, Difco), and incubated at 28°C for 2 weeks to ensure the sterilization effectiveness. After surface sterilization and thorough drying under aseptic conditions, the samples were cut up in a sterile mortar and ground to a homogenate, followed by dilution to 10^{-1} to 10^{-3} with sterile water. Aliquots of 200 μL of the dilutions were spread-plated onto a series of isolation media as indicated in **Table 1** and incubated at 28°C for 2–3 weeks for actinobacteria cultivation. The pH of the selected media was adjusted to 7.2. Each isolation medium was amended with nalidixic acid (50 mg/L) and nystatin (100 mg/L) to prevent the growth of gram-negative bacteria and fungi. As colonies appeared on the plates, candidate colonies were observed and selected carefully according to phenotypic characteristics.

DNA Extraction, 16S rRNA Gene Sequencing, and Phylogenetic Analysis

The obtained isolates were subjected to 16S rRNA gene sequence analysis for genus and species identification. The genomic DNA was extracted using the method of Li et al. (2007). The 16S rRNA gene of each isolate was amplified using primer pairs 27F and 1492R (**Table 2**), and polymerase chain reaction (PCR) amplification was carried out as described by Li et al. (2007).

TABLE 1 | Culture medium composition for endophytic actinobacteria isolation.

Medium	Composition (per 1000 mL)	Reference
GAUZE's medium	5 g K_2HPO_4, 20 g soluble starch, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 1 g KNO_3, 0.5 g NaCl, 18 g agar	Ma et al., 2017
TWYE	0.5 g K_2HPO_4, 0.25 g yeast extract, 18 g agar	Crawford et al., 1993
YECD	2 g K_2HPO_4, 0.3 g yeast extract, 0.3 g glucose, 18 g agar	Coombs and Franco, 2003
Humic acid-vitamin agar (HV)	0.02 g $CaCO_3$, 0.5 g Na_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 1 g Humic acid, 1.7 g KCl, 0.5 mg VB_6, 0.5 mg p-aminobenzoic acid, 0.5 mg riboflavin, 0.5 mg thiamine, 0.5 mg inositol, 0.5 mg pantothenic acid, 0.5 mg nicotinic acid, 0.25 mg biotin, 18 g agar	Hayakawa and Nonomura, 1989
Glucose-Asparagine modified media (GA)	1 g K_2HPO_4, 1 g asparagine, 0.01 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 10 g glucose, 0.01 g $MnCl_2 \cdot 4H_2O$, 18 g agar	Shirling and Gottlieb, 1966

TABLE 2 | Polymerase chain reaction (PCR) primers used in this study.

Primer name	Sequence (5′—3′)	Target gene	Length (bp)	Reference
27F	5′-AGAGTTTGATCCTGGCTCAG-3′	*16S rRNA*	1400–1500	Li et al., 2007
1492R	5′-ACGGTTACCTTGTTACGACTT-3′			
K1F	5′-TSAAGTCSAACATCGGBCA-3′	*PKS-I*	1200–1400	Ayuso-Sacido and Genilloud, 2005
M6R	5′-CGCAGGTTSCSGTACCAGTA-3′			
KS_α	5′-TSGCSTGCTTGGAYGCSATC-3′	*PKS-II*	600	Metsa-Ketela et al., 1999
KS_β	5′-TGGAANCCGCCGAABCCTCT-3′			
A3F	5′-GCSTACSYSATSTACACSTCSGG-3′	*NRPS*	700–800	Ayuso-Sacido and Genilloud, 2005
A7R	5′-SASGTCVCCSGTSCGGTAS-3′			

The reagents for PCR reaction were purchased from TaKaRa (Dalian, China). The PCR -products were separated by agarose gel electrophoresis, purified using QIA quick gel extraction kits (Qiagen, Hilden, Germany), then ligated into a pMD-19T vector (TaKaRa). Positive clones were screened further, and insert DNA sequencing was performed by Invitrogen (Shanghai, China) on an Applied Biosystems PRISM 3730 DNA sequencer.

The 16S rRNA gene analysis was performed by BLAST searches in the National Center for Biotechnology Information database[1] and EzBioCloud[2]. Multiple sequence alignment of selected 16S rDNA sequence was carried out using CLUSTAL_X (version 2.0) (Thompson et al., 1997), and a phylogenetic tree was constructed using MEGA v6.0 (Tamura et al., 2013). Distances (distance options according to the Kimura two-parameter model) (Kimura, 1980) and clustering were based on the neighbor-joining (Saitou and Nei, 1987) method. Bootstrap analysis based on 1000 resamplings was used to evaluate the topology of the neighbor-joining tree (Felsenstein, 1985). The 16S rRNA gene sequences of the 44 isolates have been deposited in GenBank under the accession numbers (MH298662–MH298705).

Detection of *PKS-I*, *PKS-II*, and *NRPS* Genes

Three sets of degenerate primers for amplification of the genes encoding polyketide synthases I and II (*PKS-I* and *PKS-II*) and non-ribosomal peptide synthetase (*NRPS*) were selected (**Table 2**), and amplification was carried out as recommended by Metsa-Ketela et al. (1999) and Ayuso-Sacido and Genilloud (2005). The reaction mixture contained 2.5 U of *Taq* DNA polymerase, 1 mM $MgCl_2$, 0.4 mM deoxynucleoside triphosphates, 2 μM each primer, and 5% dimethyl sulfoxide in a 50-μL reaction volume. A reaction mixture with no actinobacterial DNA template was used as a negative control. Thermocycling conditions consisted of 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 KS_α–KS_β) for 1 min, and 72°C for 2 min; and a final extension at 72°C for 5 min.

Active Compound Extraction and Bioactivity Evaluation

The endophytic isolates were cultured in GAUZE's liquid medium at 28°C and 180 r/min (Rehacek, 1959). After 7–12 days of cultivation, the 100 mL culture broth was collected by centrifugation at 12,000 × *g* for 10 min and extracted by 100 mL ethyl acetate for three times. The organic phase was evaporated under reduced pressure to yield a dry extract. The dry extract was resuspended by 5 mL sterile water and used for antimicrobial screening. The antimicrobial susceptibility was examined by placing antimicrobial testing disks (7 mm diameter) containing 25 μL test extract suspension onto LB plates (Mearns-Spragg et al., 1998). The tested plates were incubated at 37°C, and the diameters of the inhibition zones were measured after 24 h. A 25-μL volume of sterile water was used as a negative control. The pathogenic bacteria *Staphylococcus epidermidis*, *Shigella flexneri*, *Escherichia coli*, and *Bacillus cereus* were used as the indicator microorganisms for antimicrobial determination. The pathogenic microorganisms were obtained from the Institute of Quality and Standard for Agro-products, Zhejiang Academy of Agricultural Sciences.

[1] https://blast.ncbi.nlm.nih.gov/Blast.cgi

[2] https://www.ezbiocloud.net/

Animal Experiments and Physiological Tests

Female BALB/c mice at 8–9 weeks of age were purchased from the Zhejiang Laboratory Animal Center (Hangzhou, China). The mice were maintained in pathogen-free conditions with standard laboratory chow and water *ad libitum*. Animal experiments were approved and performed in accordance with the guidelines of the Animal Care Committee of Zhejiang province, China. Dried extracts from the test endophytic actinobacteria were resuspended by saline with concentration of 2 mg/mL and injected i.p. 100 μL per mouse once daily for 2 weeks. The same volume (100 μL per mouse) of saline was given as a vehicle control. Three independent experiments were repeated, and in each experiment five mice were used. Clinical signs of poisoning were assessed and the weights of mice were recorded daily. Blood samples (∼500 μL per mouse) were taken from the retro-orbital venous plexus at the end of the experiment and incubated at 4°C for 30 min. Serum was collected by centrifugation at 4500 × *g* for 10 min and stored at −20°C until analysis. Cytokines [interleukin-2 (IL-2), interleukin-6 (IL-6), the shared p40 subunit of IL-12 and IL-23 (IL-12/IL-23 p40), and tumor necrosis factor-alpha (TNF-α)] in the serum were determined with sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Dakewe Biotech, Shenzhen, China).

Splenocyte Proliferation

Freshly isolated splenocytes were obtained from BALB/c mice and incubated in 200 μL RPMI 1640 with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 μg/mL of concanavalin A (ConA) in a humidified, 37°C, 5% CO_2-containing incubator for 48 h in the presence or absence of extracts. Cyclosporin A (CsA) (500 ng/mL) was used as a positive control. Three independent experiments were repeated, and in each experiment five wells of splenocytes were used. RPMI 1640 medium was purchased from Gibco, Thermo Fisher Scientific (Waltham, MA, United States). ConA and CsA were purchased from Sigma (St. Louis, MO, United States). The cell number was determined by a Millipore Guava easyCyte 8HT Flow Cytometer (Millipore, Billerica, MA, United States).

Statistical Analysis

Data are presented as means ± standard deviation. Statistical analyzes were performed using Student's *t* test. $P < 0.05$ was considered statistically significant.

Ethics Statement

This study was carried out in accordance with the guidelines of the Animal Care Committee of Zhejiang province, China (Government Decree No. 263). The protocol was approved by the Committee on the Ethics of Animal Experiments of Zhejiang Academy of Agricultural Science.

RESULTS

Evaluation of Surface Sterilization

Surface sterilization is critical for the study of plant endophytic actinobacteria. In this study, the surface-sterilized leaves were examined by NA and TSA, and no microbial colony was observed after 2 weeks of incubation at 28°C. This indicated that the surface-sterilization protocol modified from Qin et al. (2009) was effective in removing phyllospheric microorganisms of tea plants.

Selective Isolation of Culturable Endophytic Actinobacteria From Zijuan and Yunkang-10

To obtain as many endophytic actinobacteria as possible, five selective isolation media were used simultaneously in this study (**Table 1**). Endophytic actinobacteria were isolated on all of five media. In total, 44 actinobacterial strains (28 from Yunkang-10 and 16 from Zijuan) were isolated from 3 samples of Zijuan and 3 samples of Yunkang-10 (**Table 3**).

Diversity of Endophytic Actinobacteria Analyzed by 16S rRNA Gene Sequencing

The endophytic actinobacteria obtained from Zijuan were distributed among 7 genera [i.e., *Brachybacterium* sp. (3 isolates), *Brevibacterium* sp. (1 isolate), *Kocuria* sp. (4 isolates), *Leucobacter* sp. (1 isolate), *Micrococcus* sp. (4 isolates), *Microbacterium* sp. (1 isolate), and *Streptomyces* sp. (2 isolates)] within the class actinobacteria (**Figure 1**). Among them, *Brachybacterium* sp. and *Kocuria* sp. were two mutual groups both isolated in July and December, while the others were only isolated in July or December, and no endophytic actinobacteria were obtained in September. The diversity of endophytic actinobacteria from the Zijuan cultivar was in the order of July (6 genera) > December (3 genera) > September (0 genera). The 28 endophytic actinobacteria isolated from Yunkan-10 were distributed among 8 genera [i.e., *Brevibacterium* sp. (10 isolates), *Micrococcus* sp. (3 isolates), *Mycobacterium* sp. (3 isolates), *Pseudarthrobacter* sp. (1 isolate), *Brachybacterium* sp. (1 isolate), *Kocuria* sp. (4 isolates), *Microbacterium* sp. (5 isolates), and *Saccharomonospora* sp. (1 isolate)] within the class actinobacteria (**Figure 1**). Isolates of *Brevibacterium* sp. and *Micrococcus* sp. were obtained from both July and December specimens, and isolates of *Mycobacterium* sp. were found only in July. The isolates from other genera were only obtained in December, while no endophytic actinobacteria were obtained in September. The diversity of endophytic actinobacteria from Yunkang-10 was in the order of December (7 genera) > July (3 genera) > September (0 genera). In comparing the two cultivars, isolates of *Leucobacter* sp. and *Streptomyces* sp. were endemic actinobacterial groups to the Zijuan cultivar, while *Mycobacterium* sp., *Pseudarthrobacter* sp., and *Saccharomonospora* sp. were endemic to Yunkang-10.

Endophyte Antimicrobial Activity and Sequencing of *PKS* and *NRPS* Genes

In antimicrobial screening test, several culture media indicated in **Table 1** were preliminary evaluated, and GAUZE's medium

TABLE 3 | The isolated endophytic actinobacteria of Zijuan and Yunkang-10.

Tea cultivars	July		September		December	
	Species	**Number**	**Species**	**Number**	**Species**	**Number**
Zijuan	*Brachybacterium* sp.	1			*Brachybacterium* sp.	2
	Brevibacterium sp.	1			*Kocuria* sp.	3
	Kocuria sp.	1			*Microbacterium* sp.	1
	Leucobacter sp.	1				
	Micrococcus sp.	4				
	Streptomyces sp.	2				
	Total number	10	Total number	0	Total number	6
Yunkang-10	*Brevibacterium* sp.	4			*Pseudarthrobacter* sp.	1
	Micrococcus sp.	1			*Brachybacterium* sp.	1
	Mycobacterium sp.	3			*Brevibacterium* sp.	6
					Kocuria sp.	4
					Microbacterium sp.	5
					Micrococcus sp.	2
					Saccharomonospora sp.	1
	Total number	8	Total number	0	Total number	20

was the best one for the majority of actinobacteria isolates from Zijuan and Yunkang-10. The extracts from GAUZE's medium showed no obvious antimicrobial difference from other media, but all the tested strains could be cultured by the GAUZE's medium. The 44 isolates were screened for antimicrobial activities against the pathogenic bacteria *S. epidermidis*, *Sh. flexneri*, *E. coli*, and *B. cereus*. Ten of the 28 isolates (35.7%) from Yunkang-10 exhibited activity against at least one of the tested pathogenic microorganisms. Surprising, none of the 16 isolates from Zijuan showed obvious antimicrobial activity.

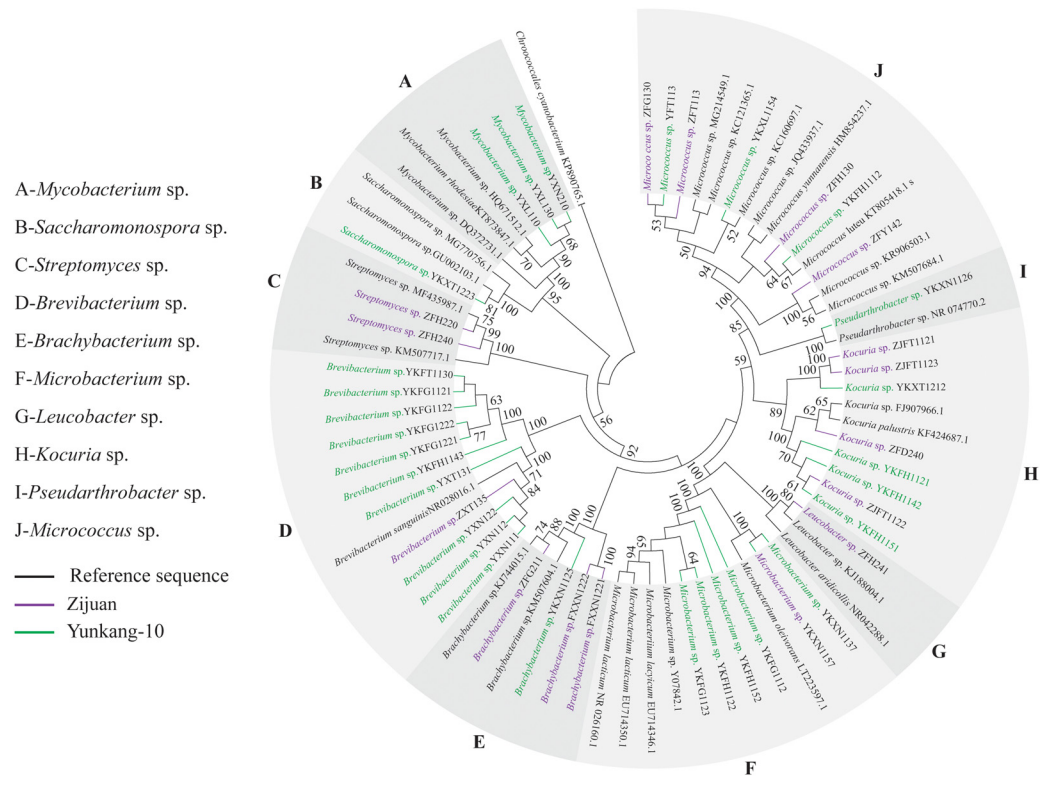

FIGURE 1 | Phylogenetic analysis of endophytic actinobacteria in different seasons from Zijuan and Yunkang-10. The phylogenetic tree was constructed by using 73 16S rRNA gene sequences.

TABLE 4 | Endophytic actinobacteria isolated from Zijuan and Yunkang-10, and similarity values for 16S rRNA gene sequences.

Isolate no.	Sequence length	Closest cultivated species	Similarity (%)	Query coverage (%)
YFT113	1345	*Micrococcus yunnanensis* (FJ214355)	99.33	93.0
YXN120	1334	*Brevibacterium bullata* (D12785)	99.77	96.5
YXN111	1441	*Brevibacterium celere* (AY228463)	99.44	97.8
YXN112	1441	*Brevibacterium celere* (AY228463)	99.44	97.9
YXT131	1395	*Brevibacterium celere* (AY228463)	99.78	96.1
YKFG1221	1531	*Brevibacterium casei* (X76564)	99.72	100
YKFG1121	1489	*Brevibacterium casei* (X76564)	99.24	100
YKFG1122	1489	*Brevibacterium casei* (X76564)	99.31	100
YKFT1130	1492	*Brevibacterium casei* (X76564)	99.72	100
YKFH1122	1487	*Microbacterium lacticum* (X77441)	99.73	100
YKFG1112	1487	*Microbacterium lacticum* (X77441)	99.59	100
ZFY142	1428	*Micrococcus endophyticus* (EU005372)	97.88	99.0
ZFG130	1366	*Micrococcus yunnanensis* (FJ214355)	99.19	94.5
ZJFT1121	1459	*Kocuria marina* (AY211385)	99.86	100

A total of 14 isolates selected (10 antimicrobial positive strains and four negative strains) were selected for the determination of antibiotic biosynthetic gene sequences of *PKS-I*, *PKS-II*, and *NRPS* by PCR amplification using specific primer sets K1F–M6R, KS_{α}–KS_{β}, and A3F–A7R, respectively (**Table 4**). As shown by primary screening, the inhibitory effect on *Sh. flexneri* was the most frequent detected antimicrobial activity in this study. Eight isolates were active against *S. epidermidis*, and 7 isolates were found to inhibit two or more pathogenic microorganisms. However, none of the isolates of this study exhibited activity

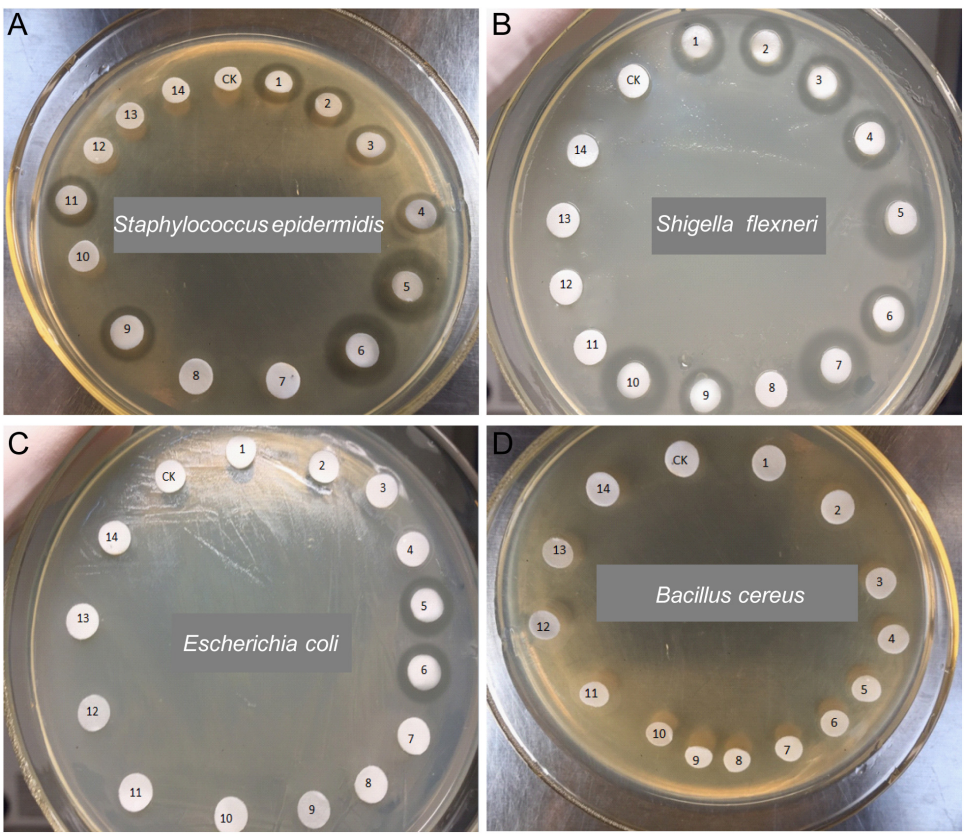

FIGURE 2 | Antimicrobial activities of endophytic actinobacteria against pathogenic bacteria. *Staphylococcus epidermidis* **(A)**, *Shigella flexneri* **(B)**, *Escherichia coli* **(C)**, and *Bacillus cereus* **(D)** were the tested strains. CK-Vehicle, 1-YFT113, 2-YXN120, 3-YXN111, 4-YXN112, 5-YXT131, 6-YKFG1221, 7-YKFG1121, 8-YKFG1122, 9-YKFT1130, 10-YKFH1122, 11-YKFG1112, 12-ZFY142, 13-ZFG130, 14-ZJFT1121.

against *B. cereus*. Two isolates, YXT131 and YKFG1221, which belong to the genus *Brevibacterium*, appeared to have a broad spectrum of antimicrobial activity (three pathogenic microorganisms). *Brevibacterium* sp. YXT131 exhibited high inhibitory effects against *S. epidermidis*, *Sh. flexneri*, and *E. coli* (**Figure 2** and **Table 5**).

The *PKS-I* sequence was detected in 9 isolates (64.3%), while the *PKS-II* and *NRPS* sequences were detected in 6 and 4 of the 14 strains (**Table 5** and **Supplementary Figure S1**), respectively. The isolates YXT131 and YKFG1221, which have broad spectrum antimicrobial activity, gave positive amplification products with *PKS-I*, *PKS-II*, and *NRPS* primers. The isolates from Yunkang-10 that exhibited antimicrobial activity against pathogenic microorganisms also gave positive amplification products for at least one of the *PKS-I*, *PKS-II*, and *NRPS* genes. The isolate YKFG1122 neither exhibited antimicrobial activity to the four tested pathogenic microorganisms nor provided any positive amplification products for the three biosynthetic genes. The three antimicrobial negative isolates from Zijuan still provided positive amplification products *PKS-I* or *PKS-II*.

Immunomodulatory Activity in Selected Actinobacteria

Chattopadhyay et al. (2012) showed that black tea has potential anti-inflammatory and immunomodulatory effects in animal models and in human peripheral mononuclear cells, and actinobacteria can produce secondary metabolites with immunosuppressive activity by suppressing cytokine expression and T cell proliferation (Yang et al., 2016). In *in vitro* splenocyte proliferation assays, extracts from 14 actinobacteria that were tested for antibiotic biosynthetic genes showed no inhibition of ConA-induced murine splenocyte proliferation (**Figure 3**), indicating that extracts from actinobacteria might not directly affect splenocyte proliferation. In the animal model, six isolates, YFT113, YXN111, YXN112, YXT131, YKFG1221, and YKFT1130, were examined for their potential immunomodulatory activity. Compared with the control group, BALB/c mice treated by fermentation extracts of the six isolates showed no obvious clinical signs of poisoning or other atypical signs throughout the trial. No significant differences in body weight were observed between control and fermentation extract-treated mice during the 2 weeks of testing (data not shown). $CD4^+$ T cells are the key components of the adaptive immune system, and naïve $CD4^+$ T cells can differentiate into effector T helper cell subsets (e.g., Th1, Th2, or Th17) by the coordinated functioning of distinct cytokines, including IL-6, IL-12, IL-23, and IL-2 (Murphy and Stockinger, 2010). TNF-α is a multifunctional cytokine that coordinates tissue homeostasis by regulating cytokine production, cell survival, and cell death (Annibaldi and Meier, 2018). In this study, no significant differences in IL-2 and IL-6 concentrations in serum were observed between control and fermentation extract-treated mice (**Figures 4A,B**). IL-12 and IL-23 are heterodimeric cytokines that share a common p40 subunit. To our surprise, the serum levels of IL-12/IL-23 p40 and TNF-α in *Brevibacterium* sp. YXT131 fermentation extract-treated mice were significantly lower than in the control group, but other fermentation extract-treated groups showed no significant differences (**Figures 4C,D**). These results indicated that the isolate YXT131 appeared to have immunosuppressive activity.

DISCUSSION

As described by previous studies, the natural characteristics and planting environment of Zijuan and Yunkang-10 are similar, and the two cultivars both originate from the Yunnan province of China (Wang et al., 2017; Zhu et al., 2017).

TABLE 5 | Antimicrobial activities and *PKS/NRPS* genes of culturable actinobacteria from Zijuan and Yunkang-10.

Isolate no.	Activity[a] against				Presence[b] of gene		
	S. epidermidis	*Sh. flexneri*	*E. coli*	*B. cereus*	*PKS-I*	*PKS-II*	*NRPS*
YFT113	++	++	−	−	+	−	−
YXN120	++	++	−	−	+	−	−
YXN111	++	++	−	−	+	−	−
YXN112	+	++	−	−	+	−	−
YXT131	+++	+++	++	−	+	+	+
YKFG1221	+++	+	++	−	+	+	+
YKFG1121	−	+++	−	−	+	+	−
YKFG1122	−	−	−	−	−	−	−
YKFT1130	+	+	−	−	+	+	−
YKFH1122	−	++	−	−	−	−	+
YKFG1112	+	−	−	−	−	−	+
ZFY142	−	−	−	−	−	+	−
ZFG130	−	−	−	−	+	−	−
ZJFT1121	−	−	−	−	−	+	−

[a]*Estimated by measuring the diameter of the clear zone of growth inhibition. Symbols: −, no activity; +, ++, and +++, weak activity, moderate activity, and strong activity, respectively.* [b]*+, present; −, absent.*

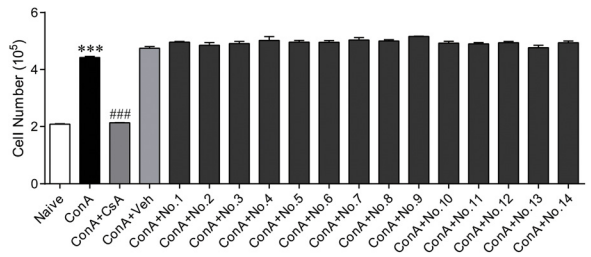

FIGURE 3 | Splenocyte proliferation in ConA-induced splenocytes treated with extracts of endophytic actinobacteria from Zijuan and Yunkang-10. Naive mouse splenocytes were stimulated with 5 μg/mL of ConA in the presence of different extracts, and 500 ng/mL CsA was set as a positive control. No.1-YFT113, No.2-YXN120, No.3-YXN111, No.4-YXN112, No.5-YXT131, No.6-YKFG1221, No.7-YKFG1121, No.8-YKFG1122, No.9-YKFT1130, No.10-YKFH1122, No.11-YKFG1112, No.12-ZFY142, No.13-ZFG130, 14-ZJFT1121. Data are means ± SD ($n = 5$), $^{***}p < 0.001$ vs. naive, $^{###}p < 0.001$ vs. ConA.

The high anthocyanin content of Zijuan is one of the most important differences between the two cultivars (Yang X.R. et al., 2009). Previous studies have indicated that plants secondary metabolites such as alkaloids, phenolics, and terpenoids can interfere with cancer cells, bacteria, and fungi (Wink et al., 2012), and that anthocyanins act as antimicrobial agents of natural plant origin (Cisowska et al., 2011). The different anthocyanin content of Zijuan and Yunkang-10 may influence the microbial community, diversity and bioactivity of endophytic actinobacteria. In this study, the endophytic actinobacteria from Zijuan and Yunkang-10 were isolated and compared for their diversity and antimicrobial and immunomodulatory activities.

In our study, 44 isolates were isolated in July and December, but none of the endophytic actinobacteria was obtained in September. This result might be an incidental, alternatively, endophytic actinobacteria community in Zijuan and Yunkang-10 changed in September and is unculturable on these media. Many studies indicated that the endophytes of plant tissues [such as maple tree sap (Filteau et al., 2010), the buds of Scots pine trees (Pirttilä et al., 2005) and the grape endosphere (Baldan et al., 2014; Bulgari et al., 2014)] were shown to be sensitive to seasonal changes. Other than culture-dependent method, a variety culture independent methods including T-RFLP, PCR fingerprinting and 16S rRNA specific probes were used to investigate the seasonal community changes, and these results were consistent to the present culture-dependent investigation from tea plant (Shen and Fulthorpe, 2015).

In recent years, the antibiotics abuse has been resulted serious bacterial resistance, and become a heavy threat to public health. For instance, methicillin-resistant staphylococcal infections are an important cause of catheter-associated disease, and 75–90% among hospital isolates are *S. epidermidis* (Otto, 2009). Antibiotic resistance by *Shigella* species is also a global issue now (Lampl, 2015). *E. coli* and *B. cereus* are abundant in nature, and many factors make them a potential threat for the food industry (Gundogan and Avci, 2014). Endophytic actinobacteria are well-known producers of a vast array of secondary metabolites, including antibiotics. In our study, all of 44 isolates were screened for antimicrobial activities against *S. epidermidis*, *Sh. flexneri*, *E. coli*, and *B. cereus*. Ten isolates from Yunkang-10 exhibited antimicrobial activity against at least one of the tested pathogenic microorganisms, but none of the isolates from Zijuan showed obvious activity. The high inhibitory activity and broad antimicrobial spectrum of these tested strains suggested

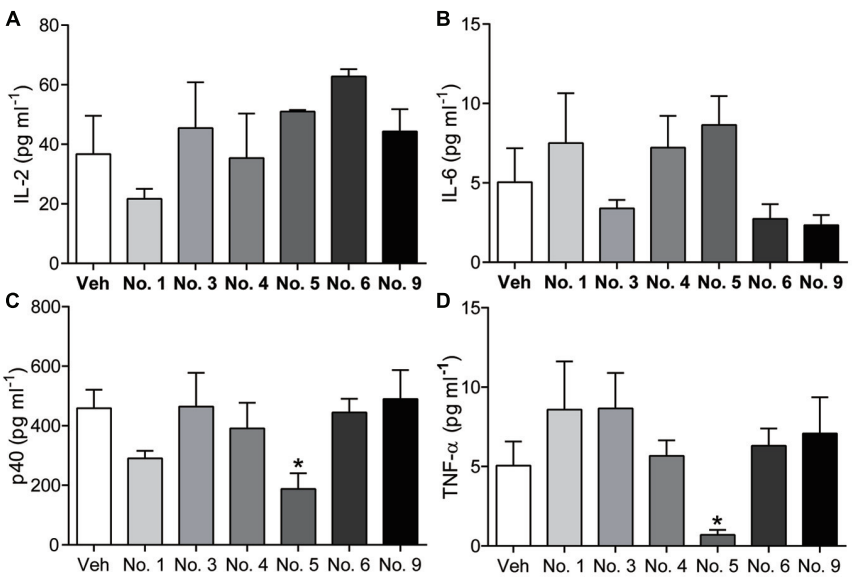

FIGURE 4 | Cytokine levels in sera of mice treated by extracts of endophytic actinobacteria. IL-2 **(A)**, IL-6 **(B)**, IL-12/IL-23 p40 **(C)**, and TNF-α **(D)** levels in serum were measured by ELISA. No.1-YFT113, No.3-YXN111, No.4-YXN112, No.5-YXT131, No.6-YKFG1221, No.9-YKFT1130. Data are means ± SD ($n = 5$), $^{*}p < 0.05$ vs. vehicle.

that the endophytic actinobacteria from tea cultivar Yunkang-10 are potential candidates for novel antimicrobial agents. For *PKS-I*, *PKS-II*, and *NRPS* screening, the antimicrobial activity results and the biosynthetic genes seemed to be positively correlated in isolates from Yunkang-10, but 3 isolates from Zijuan that showed no antimicrobial activity still provided positive amplification products for *PKS-I* or *PKS-II*. The 3 isolates from Zijuan that showed negative antimicrobial results in this study might produce the antimicrobial agents to other pathogenic microorganisms. The 6 isolates with a broad spectrum of antimicrobial activity and high inhibitory effects were then selected for potential immunomodulatory activities in *in vivo* test. The isolate *Brevibacterium* sp. YXT131, which have broad spectrum antimicrobial activity, gave positive amplification products with *PKS-I*, *PKS-II*, and *NRPS* primers, also exhibited high inhibitory effects of the serum levels of IL-12/IL-23 p40 and TNF-α. These results indicated that endophytic actinobacteria from Yunkang-10 might be an undeveloped bioresource library for active compounds.

CONCLUSION

In this study, we found that the endophytic actinobacterial communities in the tea cultivars Zijuan and Yunkang-10 were quite different. The isolates of *Leucobacter* sp. and *Streptomyces* sp. were endemic actinobacterial groups for the Zijuan cultivar, while *Mycobacterium* sp., *Pseudarthrobacter* sp., and *Saccharomonospora* sp. were endemic actinobacterial groups for Yunkang-10. Ten of the 28 isolates (35.7%) from Yunkang-10 exhibited activity against at least one of the tested pathogenic microorganisms, but none of the 16 isolates from Zijuan showed obvious antimicrobial activity. *Brevibacterium* sp. YXT131 and *Brevibacterium* sp. YKFG1221 from Yunkang-10 appeared to have broad-spectrum antimicrobial activity (against *S. epidermidis*, *Sh. flexneri*, and *E. coli*) and gave positive amplification products for the *PKS-I*, *PKS-II*, and *NRPS* genes. The crude extract from *Brevibacterium* sp. YXT131 showed no inhibition of ConA-induced splenocyte proliferation but decreased IL-12/IL-23 p40 and TNF-α levels in the serum of a mouse model, indicating that *Brevibacterium* sp. YXT131 had immunosuppressive activity. Endophytic actinobacteria from Yunkang-10 might be an undeveloped bioresource library for active compounds.

AUTHOR CONTRIBUTIONS

JX, XW, and YZ conceived of and designed the experiments. WW, XW, FC, XY, and YL performed the experiments. WW drafted the manuscript. WW, YZ, JX, CW, and XC analyzed the data. JX and XW revised 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: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01304/full#supplementary-material

FIGURE S1 | DNA gel electrophoresis of *PKS-I*, *PKS-II*, and *NRPS*. The PCR amplification products were resolved using electrophoresis in 1.5% agarose gels. M-Marker, 1-YFT113, 2-YXN120, 3-YXN111, 4-YXN112, 5-YXT131, 6-YKFG1221, 7-YKFG1121, 8-YKFG1122, 9-YKFT1130, 10-YKFH1122, 11-YKFG1112, 12-ZFY142, 13-ZFG130, 14-ZJFT1121.

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Actinobacteria Associated With Arbuscular Mycorrhizal *Funneliformis mosseae* Spores, Taxonomic Characterization and Their Beneficial Traits to Plants: Evidence Obtained From Mung Bean (*Vigna radiata*) and Thai Jasmine Rice (*Oryza sativa*)

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In this study, we report on the isolation of actinobacteria obtained from spores of *Funneliformis mosseae* and provide evidence for their potential in agricultural uses as plant growth promoters *in vitro* and *in vivo*. Actinobacteria were isolated from spores of *F. mosseae* using the dilution plate technique and media designed for the selective isolation of members of specific actinobacterial taxa. Six strains namely 48, S1, S3, S4, S4-1 and SP, were isolated and identified based on16S rRNA gene sequences. Phylogenetic analysis showed that isolate SP belonged to the genus *Pseudonocardia* with *P. nantongensis* KLBMP 1282^T as its closest neighbor. The remaining isolates belonged to the genus *Streptomyces*. Two isolates, 48 and S3 were most closely related to *S. thermocarboxydus* DSM 44293^T. Isolates S4 and S4-1 shared the highest 16S RNA gene similarity with *S. pilosus* NBRC 127772^T. Isolate S1 showed its closest relationship with the type strain of *S. spinoverrucosus* NBRC14228^T. The ability of these isolates to produce indole-3-acetic acid (IAA), siderophores and the ability to solubilize phosphate *in vitro* were examined. All isolates produced siderophores, four isolates produced IAA and two isolates solubilized inorganic phosphate at varying levels. *S. thermocarboxydus* isolate S3 showed the highest IAA production with high activities of phosphate solubilization and siderophore production. The inoculation of mung beans (*Vigna radiata*) with this strain resulted in a significant increase in fresh weight, root length and total length as an effect of IAA production. In an experiment with rice (*Oryza sativa*), *S. thermocarboxydus* isolate S3 promoted the growth of rice plants grown in low nutritional soil under induced drought stress. This report supports the view that the inoculation of rice with plant growth promoting actinobacteria mitigates some adverse effects of low nutrient and drought stress on rice.

Keywords: actinobacteria, arbuscular mycorrhizal spore, *Funneliformis mosseae*, plant growth-promoting activity, indole-3-acetic acid (IAA), rice (*Oryza sativa*), drought stress, low nutritional soil

INTRODUCTION

Actinobacteria are Gram-positive mostly filamentous bacteria with high %G+C content in their genomes. They are prolific producers of useful bioactive metabolites with a wide spectrum of uses in particular antibiotics (Bérdy, 2005; Kurtboke, 2012; Tiwari and Gupta, 2012). It is also well accepted that some actinobacteria are considered potential plant growth promoting bacteria (Doumbou et al., 2001; Hameda and Mohammadipanah, 2015; Qin et al., 2017). Their ability to produce phytohormones (IAA), siderophores and to solubilize inorganic phosphates has been well-documented (Manulis et al., 1994; Rungin et al., 2012; Sadeghi et al., 2012; Lin and Xu, 2013; Anwar et al., 2016; Passari et al., 2016). The growth promotion of several plants by actinobacteria was reported in peas (Tokala et al., 2002), beans (Nassar et al., 2003), alfalfa (Le et al., 2016), wheat (Sadeghi et al., 2012; Anwar et al., 2016), and rice (Rungin et al., 2012; Gopalakrishnan et al., 2014).

Arbuscular mycorrhizal fungi (AMF) are well-known plant growth promoters and have been used in the agricultural industry worldwide (Smith and Smith, 2011). AMF can only be grown with plants as obligate symbionts (Owen et al., 2015). This symbiosis improves the plant's nutrient uptake and provides AM fungi with carbon sources. *Funneliformis* is a member of AMF in the family *Glomeraceae*, which form symbiotic relationships with plant roots. It was formerly known as *Glomus mosseae* until the genus *Funneliformis* was established in 2010 with *F. mosseae* as a type species (Schubler and Walker, 2010). *F. mosseae* is widely used in horticulture as a bioinoculant (Kruger et al., 2012). This symbiosis improves the plant's nutrient uptake and provides AM fungi with carbon sources. Recently, microorganisms associated with AM fungal spores have been reported, including several bacterial species that are both Gram-negative (Xavier and Germida, 2003; Bharadwaj et al., 2008a,b; Battini et al., 2016; Lasudee et al., 2017) and Gram-positive bacteria including actinobacteria of the genus *Streptomyces* (Schrey et al., 2012; Mohandas et al., 2013; Poovarasan et al., 2013; Battini et al., 2016). These mycorrhizal associated bacteria have shown interesting properties such as those associated with biocontrol and plant growth promoting activities (Mohandas et al., 2013; Poovarasan et al., 2013; Battini et al., 2016). The beneficial properties of these bacteria are considered attractive for sustainable agricultural practices. However, few research studies have been published on actinobacteria that are associated with mycorrhizal spores. This has led to our interest in mycorrhizal-associated actinobacteria, which may be a good source of novel taxa for the purposes of agricultural bioprospecting. Hence, it is the aim of this study to isolate actinobacteria from *F. mosseae* spores and to screen for their plant growth promoting properties both *in vitro* and *in planta* using mung beans (*Vigna radiata*) and Thai jasmine rice (*Oryza sativa*). We have given special attention to testing the ability of these actinobacteria to promote rice growth under drought conditions in soil possessing low nutritional content. Their taxonomic positions were also identified.

MATERIALS AND METHODS

Selective Isolation of Actinobacteria From *Funneliformis mosseae* Spores

Funneliformis mosseae CMU-RYA08 was used for the isolation of actinobacteria in this study. It was originally isolated from the soil of a *Aquilaria crassna* plantation in Rayong Province, Thailand (Chaiyasen et al., 2014) and was kindly supplied to us by Dr. Amornrat Chaiyasen, Department of Biology, Faculty of Science, Chiang Mai University. Briefly, spores of *F. mosseae* were surface sterilized by vortex mixing them sequentially in 500 μl of 2% (v/v) sodium hypochlorite solution for 1 min, followed by 500 μl of 70% (v/v) ethanol for 1 min. After that, spores were washed with 500 μl of distilled water for 1 min (three times) and used for isolation of actinobacteria. The efficiency ofsurface sterilization method was determined by spreading the final washing water onto the nutrient agar as well as the International *Streptomyces* Project (ISP) medium 2 agar (Shirling and Gottlieb, 1966), and the plates were incubated at 30°C for 48 h. Twenty surface sterilized spores of *F. mosseae* were used for each isolation. Aseptically, 20 surface sterilized spores were added to microcentrifuge tubes containing 200 μl of soil extract broth and were ground by sterile micropestle. Soil extract was prepared according to the method of Taylor and Lochhead (1938). One kilogram of garden soil was sterilized in 1 l of tap water for 20 min at 121°C. The suspension was allowed to settle and the supernatant was filtered through Whatman's No. 41 filter paper. One gram of glucose was added to the filtrate and the pH value was adjusted to 6.8–7.0 prior to being autoclaved. The resultant solution was used as a soil extract broth for enrichment. The resulting spore suspension was enriched by shaking at 120 rpm for 1 h at room temperature. Two hundred microliters of the sample were spread on starch casein agar (Küster and Williams, 1964) and humic acid-vitamin agar (Hayakawa and Nonomura, 1987) supplemented with 25 μl/ml nalidixic acid and 100 μg/ml ketoconazole. Plates were then incubated at 30°C for up to 4 weeks. All isolates were routinely cultivated on International *Streptomyces* Project (ISP) medium 2 agar (Shirling and Gottlieb, 1966) at 30°C and spore suspension was maintained in 20% (w/v) glycerol at −20°C for long term preservation.

Molecular Identification by 16S rRNA Gene Sequencing

Biomass for molecular study was prepared by growing the isolates on ISP medium 2 agar for 7 days. Genomic DNA was extracted using FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favogen) according to the manufacturer's protocol. All isolates were subjected to amplification of 16S rRNA gene using universal primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARCC-3′) (Lane, 1991). PCR was carried out in a GeneAmp PCR System 9700 with the following reactions. The initial denaturation step was done at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s and final extensions at 72°C for 7 min. PCR products of expected size were confirmed by 1% (w/v) agarose gel electrophoresis. The purified PCR products were sequenced

commercially at 1st BASE DNA Sequencing Division, Malaysia. The obtained sequences were compared with related sequences in the EzBioCloud database[1] using BLAST program. Phylogenetic analysis based on a neighbor-joining method (Saitou and Nei, 1987) was carried out using MEGA version 5.2 program (Tamura et al., 2011). The resultant tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbor-joining data based on 1000 re-sampled datasets

In Vitro Plant Growth Promoting Potential

Indole-3-Acetic Acid (IAA) Production

The production of IAA of all isolates was determined by the colorimetric method as has been described previously (Khamna et al., 2010). Agar plug (5 mm diameter) of actinobacteria grown on ISP medium 2 agar was inoculated into 5 ml of ISP medium 2 broth containing 2 mg ml^{-1} of L-tryptophan (Gordon and Weber, 1951). Tubes were incubated at 28 ± 0.2°C with shaking at 110 rpm for 7 days. The supernatant was collected by centrifugation at 11,000 rpm for 5 min. IAA production was detected by mixing one ml of the supernatant with 2 ml of Salkowski's reagent (Glickmann and Dessaux, 1994). The appearance of a pink color after incubation at room temperature for 30 min in the dark was an indicator of IAA production. The optical density was measured at 530 nm using a spectrophotometer (GENESYS TM 20 Visible Spectrophometer, Thermo Fishcer Scientific). The IAA concentration in the culture broth was estimated based on a calibration curve of pure IAA standard. The confirmation of IAA production was also confirmed by TLC analysis with 10 mg/ml IAA standard (Mohite, 2013). In addition, the *iaaM*, the key IAA biosynthetic gene of the highest IAA producing isolate, was examined by PCR amplification (Lin and Xu, 2013). The amplified gene (1,698 bp) was cloned and sequenced at 1st BASE DNA Sequencing Division, Malaysia. The obtained sequences were compared with related sequences in the GenBank database using BLAST program.

Phosphate Solubilization

Phosphate solubilizing activity of all isolates was determined on Pikovskaya (PVK) agar (Pikovskaya, 1948) containing 0.5% (w/v) tricalcium phosphate. The agar plug (5 mm diameter) of actinobacterial growth on ISP medium 2 agar was transferred to PVK agar plates and incubated at 28 ± 0.2°C for 7 days. The occurrence of a clear zone around the agar plug was considered a positive indication of phosphate solubilization (Nautiyal, 1999). Quantitative analysis of tricalcium phosphate solubilization in liquid medium was also carried out using the Fiske and Subbarow (1925). Briefly, 5 agar plugs (5 mm diameter) of fully grown actinobacteria were inoculated into 50 ml of Pikovskaya's broth and were incubated with shaking at 120 rpm for 7 days at 28 ± 0.2°C. The supernantant was collected by centrifugation at 12,000 rpm for 15 min. The resultant supernatant (500 µl) was mixed with 500 µl of 10% (w/v) trichloroacetic acid in a test tube. Four milliliters of the color reagent (1:1:1:2 ratio of 3M H_2SO_4, 2.5% ammonium molybdate (w/v), 10% ascorbic acid (w/v) and sterile distilled water) was added and the mixture was incubated at room temperature for 15 min. The pH value of the culture broth was also measured on day 0 and day 7. Sterile PVK medium served as a control. The optical density was read at 820 nm using a spectrophotometer (GENESYS TM 20 Visible Spectrophometer, Thermo Fishcer Scientific). Soluble phosphorus was estimated using a standard curve.

Siderophore Production

Siderophore production in all isolates was determined by chrome azurol S (CAS) assay (Schwyn and Neilands, 1987). Actinobacteria were grown on ISP medium 2 agar for 5 days at 28 ± 0.2°C. Agar plugs (5 mm diameter) of actinobacterial growth that were grown on ISP medium 2 agar were transferred to CAS agar and incubated for 7 days at 28 ± 0.2°C in a dark room. A yellow to orange zone around the actinobacterial disks indicated siderophore production. Siderophore quantity and types were determined and quantified by ferric perchlorate assay (Atkin et al., 1970) for hydroxamate type and Arnow assay (Arnow, 1937) for catecholate type.

In Vitro Assay for Drought Tolerance in Actinobacteria

All actinobacteria were grown on ISP medium 22 agar for 7 days. Agar plugs (5 mm diameter) of fully grown actinobacteria were transferred to 10% Tryptic soy agar (TSA) that was supplemented with 0 gL^{-1}; 85 gL^{-1}; 285 gL^{-1}; 405 gL^{-1}; 520 gL^{-1}, and 660 gL^{-1} of sorbitol to adjust the a_w value of the media in order to simulate water stress at 40°C for 7 days. The final a_w value of the media were 0.998, 0.986, 0.957, 0.919, 0.897, and 0.844, respectively (Hallsworth et al., 1998). Growth recorded at a_w of 0.919 was considered a drought-tolerant property.

Growth Promotion of *Vigna radiata* by Selected Actinobacteria

Actinobacteria isolate S3 was selected based on its *in vitro* plant growth promoting properties. Mung beans (*Vigna radiata*) were used as a representative of dicotyledon plants. Mung bean seeds (Raithip Brand, Thai Cereal World Company, Thailand) were surface sterilized by being immersed sequentially in 2% (v/v) sodium hypochlorite for 1 min, 95% (v/v) ethanol for 1 min, 70% (v/v) ethanol for 1 min and then washed with sterile distilled water for 1 min (five times). Five decontaminated seeds were randomly selected to check for surface sterility on nutrient agar. Cell suspension was prepared from 7-day-old culture of isolate S3 grown in ISP medium 2 broth. Surface sterile seeds were mixed with 10^8 CFU ml^{-1} cell suspension in 25 ml sterile water on a shaker at 120 rpm for 3 h before being sown in a sand pot. The following treatments, each with three hundred replicates of mung bean seeds, were conducted: (1) control (without bacterial inoculation), (2) cell suspension of isolate S3 (10^8 CFU ml^{-1}), and (3) standard IAA solution adjusting the concentration to 11.12 µg ml^{-1}. The experiment was carried out in 2.5 cm × 2.5 cm pots and kept in a greenhouse at 30°C for 7 days. Pots were arranged in a completely randomized

[1]https://www.ezbiocloud.net/taxonomy

arrangement. After 7 days, the following growth parameters were recorded: fresh weight (g), root length (cm), seed germination (%) and total length (cm). Seed germination was calculated as a percentage of the number of germinated seeds from a total of 100 sown seeds per pot.

Growth Promotion of Rice Under Drought Conditions in Low Nutritional Soil by Selected Actinobacteria

In this experiment, Thai jasmine rice (*Oryza sativa*) KDML105 was used as a representative monocotyledon plant. The soil used in the experiment was of a sandy loam type and had poor nutritional value. The soil was bought from Lan Sai Company, Chiang Mai, Thailand. Soil properties were determined by Central Laboratory, Faculty of Agriculture, Chiang Mai University. The soil was sterilized by being autoclaved twice for 20 min over two consecutive days. The sterility of the soil was determined by spreading the autoclaved soil suspension on ISP medium 2 and nutrient agar.

Rice seeds were kindly supplied by Chiang Mai Rice Seed Center, Rice Seed Division, Rice Department, Ministry of Agriculture and Cooperatives, Thailand. Seeds were surface sterilized by being immersed sequentially in 2% (v/v) sodium hypochlorite for 1 min, 95% (v/v) ethanol for 1 min and 70% (v/v) ethanol for 1 min, and then being washed with sterile distilled water for 1 min (three times). Five decontaminated seeds were randomly selected to check for surface sterility on ISP medium 2 and nutrient agar. Surface sterile seeds were mixed with 10^8 CFU ml^{-1} of isolate S3 and incubated on a rotary shaker at 120 rpm at 30°C for 16–18 h before being sown. Seedlings were prepared by sowing 100 surface sterile seeds in a tray (25 cm × 35 cm × 9 cm) containing 1 kg of sterile soil. Seedlings were watered once a day with sterile distilled water for 7 days at room temperature. Seed germination was calculated as a percentage of the number of germinated seeds from a total of 100 sown seeds per tray. Seven-day-old seedlings having two leaves were transferred to a bigger pot for further growth promoting experimentation.

The experiment was carried out in pots (20 cm diameter × 15 cm height) containing 3 kg of sterile soil and kept in a greenhouse at ambient light and temperature for 45 days with five replicates per treatment and five seedlings per pot (n = 25). Pots were arranged in a completely randomized arrangement in a greenhouse. Tap water was supplied once a day to full container capacity and kept at 1 cm above soil level. Fertilizer was not added in order to maintain the low nutritional value of the soil. The following treatments were investigated: (1) control (without bacterial inoculation) and (2) drought-induced treatment [seeds mixed with cell suspension of isolate S3 (10^8 CFU ml^{-1})]. In addition, a well-watered treatment [seeds mixed with cell suspension of isolate S3 (10^8 CFU ml^{-1})] with its control (without bacterial inoculation) was also carried out for comparison purposes. Drought stress was induced by completely withholding water starting on day 36 for 10 days. At the end of the experiment (day 46 after being sown), the following growth parameters were recorded: fresh weight (g), dry weight (g), root length (cm), total length (cm), height (cm), stem diameter (mm), number of leaves, relative water content (RWC), chlorophyll content (mg g^{-1}) and proline content (μmol g^{-1}). Proline content was determined by rapid colorimetric method (Bates et al., 1973). Chlorophyll content was determined spectrophotometrically based on the standard method of Arnon (1949). RWC of rice leaves (n = 5) was calculated from the formula RWC (%) = [(FM-DM)/(TM-DM)] × 100 according to Oukarroum et al. (2007).

Colonization of rice root by isolate S3 was confirmed by dilution spread-plate and microscope examination. Briefly, the root was ground in TE buffer and shaken on a rotary shaker for 3 h at 180 rpm. The root suspension (1 ml) was serially diluted and spread on ISP medium 2 agar that was supplemented with 25 μl/ml nalidixic acid and 100 μg/ml ketoconazole. The suspension was then incubated at 30°C for 7 days. 16S rRNA gene sequencing was also performed on the obtained isolate.

Isolate S3 was also screened for 1-aminocyclopropane-1-carboxylate (ACC) deaminase production as described by Palaniyandi et al. (2014). Inoculation was done on DF minimal salts agar (Dworkin and Foster, 1958) with no nitrogen source and DF minimal salts agar that had been supplemented with either 5 mmol l^{-1} of ammonium sulfate or ACC. Plates were then incubated at 30°C for 10 days. The level of growth of the isolates on three different media was compared. Strains that were able to utilize ACC as a nitrogen source (ACC deaminase positive) exhibited a level of growth equal to the growth on $(NH_4)_2SO_4$ containing medium, whereas strains that could not utilize ACC showed no growth, which was compatible to that of the medium without a nitrogen source.

Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequences of all actinobacterial isolates were deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession numbers LC062606-LC062608, LC065388-LC065389 and LC207997.

Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple range tests (TMRT). The detection for all treatments in this study was calculated using SPSS (version 16.0) at p = 0.05.

RESULTS

Selective Isolation of Actinobacteria From *Funneliformis mosseae* Spores

In this section, we tried to isolate cultivable actinobacteria that are associated with the spores of arbuscular mycorrhiza, *Funneliformis mosseae*. The first attempt at isolation without

employing an enrichment step in the soil extract broth resulted in no actinobacterial growth on both selective media. Six presumptive actinobacteria were successfully isolated from the spores of *F. mosseae* using an enrichment step. Isolates 48, S1 and S3 were recovered from starch casein agar, whereas the remaining isolates were grown on humic acid vitamin agar (**Table 1**). The total actinobacterial count was 5×10^2 CFU/20 spores on the starch casein agar. No microorganisms were grown on the nutrient agar and the ISP medium 2 agar obtained from the final washing water.

Identification of Actinobacteria Based on 16S rRNA Gene Analysis

The 16S rRNA genes obtained from all isolates were amplified with primers 27F and 1525R and sequenced. The resultant sequences ranged from 1200 to 1422 bp were identified using the BLAST program in the EzBioCloud database. Their closest phylogenetic neighbors are shown in **Table 1**. Five isolates were identified as members of the genus *Streptomyces*. Isolates 48 (99.86% similarity) and S3 (99.93% similarity) were closely related to *S. thermocarboxydus* DSM 44293^T, whereas isolates S4 and S4-1 shared 99.75% and 99.79% similarity with *S. pilosus* NBRC 127772^T. Isolate S1 was closely related to *S. spinoverrucosus* NBRC14228^T (99.28% similarity). The remaining isolate, isolate SP was identified as *Pseudonocardia* and closely related to *P. nantongensis* KLBMP 1282^T (98.56% sequence similarity). Phylogenetic analysis based on an almost complete sequence of the *Streptomyces* isolates is showed in **Figure 1A**. Isolates 48 and S3 were in the *S. thermocarboxydus* clade and supported by 88% bootstrap value, whereas isolates S4 and S4-1 fell into the *S. pilosus* clade with 68% bootstrap support. Isolate S1 was clustered with *S. spinoverrucosus*. The *Pseudonocardia* isolate SP shared a clade with *P. nantongensis* KLBMP 1282^T, a relationship supported by a 100% bootstrap value (**Figure 1B**). The sequences from all isolates were deposited in the DDBJ database under the accession numbers shown in **Table 1**.

In Vitro Plant Growth Promoting Potential

IAA Production

All 5 isolates of *Streptomyces* (48, S1, S3, S4, S4-1) were positive for IAA production in a range of 0.74 – 11.12 μg ml^{-1} (**Table 2**). Only the *Pseudonocardia* isolate SP showed a negative result. Isolate S3 produced the highest IAA value of 11.12 μg ml^{-1}. This isolate also produced 1.12 μg ml^{-1} without L-tryptophan supplement. In addition, during periods of reduced water activity, isolate S3 still produced IAA at up to a_w = 0.919 (data not shown). TLC analysis of the IAA sample showed a pink color spot at similar Rf when compared with the chemical IAA standard (Supplementary Figure S1). PCR amplification of the IAA gene in isolate S3, the highest IAA producer, showed a product of approximately 1600 bp and was similar to that of the positive control, *S. coelicolor* A3(2). Sequence analysis of this PCR fragment using the BLAST program (blastx) revealed a similarity to the amino oxidase gene in *Streptomyces coelicolor* A3(2) (NP_625735).

Phosphate Solubilization

Half of the isolates (isolates 48, S1, S3) were found to produce clear zones around their colonies on Pikovskaya's agar (**Table 2**). The largest clear zone diameter of 7.00 ± 0.05 mm was observed (Supplementary Figure S2a). Quantitative estimation of phosphate solubilization in the culture broth was in a range of 215.6 – 224.27 mg L^{-1}, with the highest value obtained from isolate S3 (224.27 ± 0.06 mg L^{-1}). A decline in the pH value of the culture filtrate was observed in all these positive isolates. The lowest pH value was recorded at pH 4.46 ± 0.25 for isolate S3.

TABLE 1 | Identification of actinobacteria associated with *F. mosseae*'s spores based on 16S rRNA gene sequence analysis.

Isolates no.	Isolation media	Length (bp)	Accession number	Similarity (%)	Closest match
48	SC	1412	LC062606	99.86	*Streptomyces thermocarboxydus* DSM 44293^T
S1	SC	1392	LC207997	99.28	*Streptomyces spinoverrucosus* NBRC 14228^T
S3	SC	1410	LC062607	99.93	*Streptomyces thermocarboxydus* DSM 44293^T
S4	HV	1200	LC062608	99.75	*Streptomyces pilosus* NBRC 127772^T
S4-1	HV	1422	LC065389	99.79	*Streptomyces pilosus* NBRC 127772^T
SP	HV	1421	LC065388	98.56	*Pseudonocardia nantongensis* KLBMP 1282^T

SC, starch casein agar; HV, humic acid-vitamin agar.

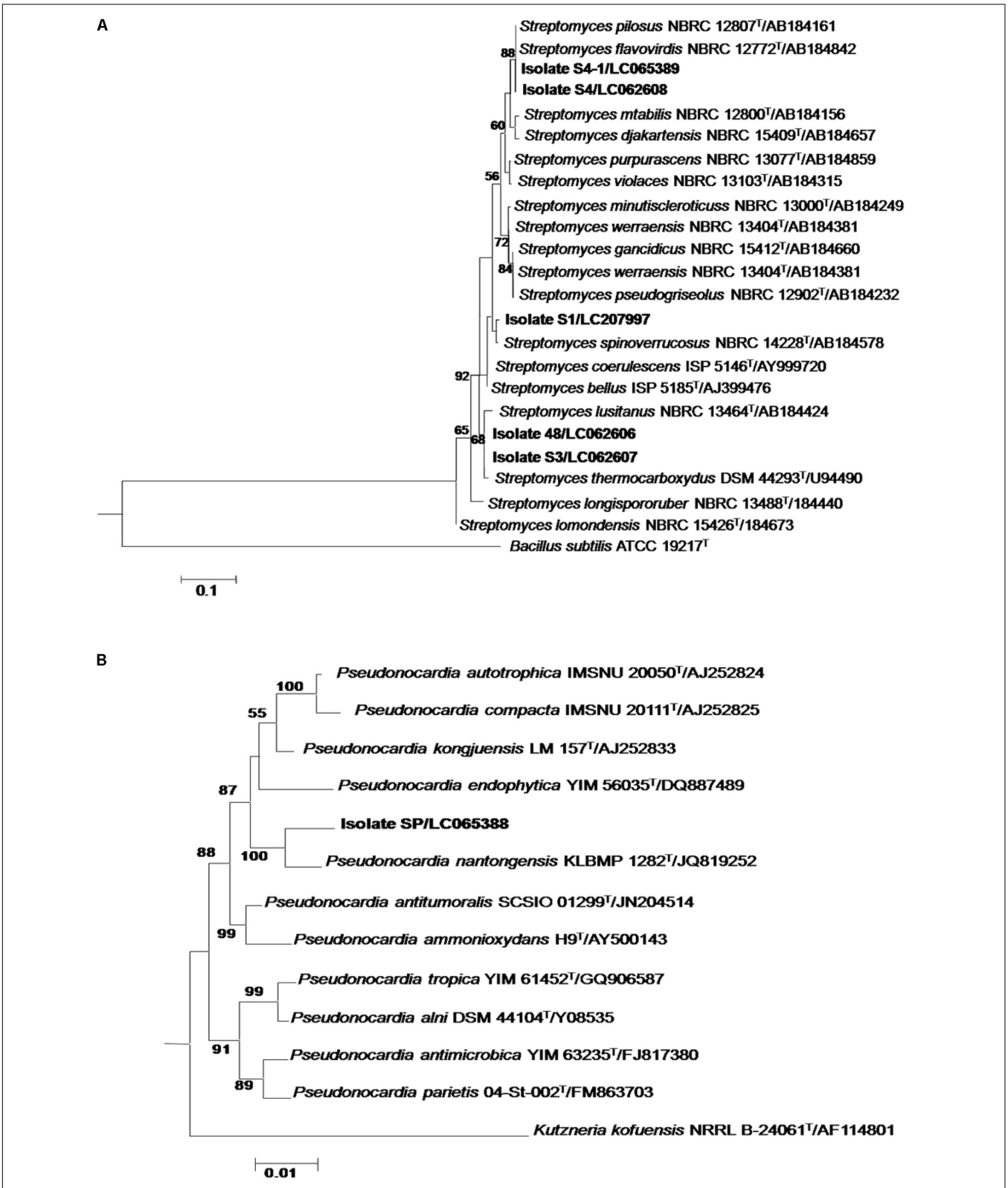

FIGURE 1 | Neighbor-joining phylogenetic tree based on an almost complete 16S rRNA gene sequence of *Streptomyces* **(A)** and *Pseudonocardia* **(B)** isolates representative of their related taxa. Bootstrap values are based on 1,000 re-samplings. Only values of >50% are shown at the nodes.

Siderophore Production

All isolates could produce siderophore on CAS agar as an orange halo zone was observed around the agar plugs (**Table 3**). Isolate 48 produced the largest zone of siderophore production (17.83 ± 0.58 mm) followed by isolate S3 (15.83 ± 0.58 mm) (Supplementary Figure S2b). All isolates could produce both catecholate and hydroxamate type siderophores at various levels. Isolate S3 produced the highest catecholate type siderophore (39.17 ± 0.002 μmol L^{-1}), whereas isolate S1 produced the highest hydroxamate type siderophore (97.50 ± 3.63 μmol L^{-1}) (**Table 2**).

In Vitro Assay for Drought Tolerance in Actinobacteria

Two *Streptomyces* isolates, S1 and S3, showed a high tolerance to drought *in vitro*, as they were able to grow in medium with reduced water availability (a_w = 0.919). The remaining isolates could grow up to a_w of 0.957 (**Table 3**). Though both isolates could grow under water stressed conditions, isolate S3 was selected as a candidate for rice growth promotion under drought conditions due to its better performance with regard to *in vitro* plant growth promoting properties.

Growth Promotion of *Vigna radiata* by Selected Actinobacteria

The effects of *S. thermocarboxydus* isolate S3 inoculation on mung bean seeds were observed. The percentage of seed germination in all treatments involving isolate S3 was in a range of 95–98%, which was statistically higher than the control. The highest value of enhancement of all growth parameters was obtained from Treatments 2 and 3 (**Figure 2A**). Treatments with isolate S3 showed higher fresh weight, root length and total length when compared to the control. The increases in fresh weight, root length and total length were not significantly different in Treatments 2 and 3. In addition, though root intensity was not directly observed, it is evident from **Figure 2B** that Treatments 2 and 3 displayed more intense roots than the control.

Growth Promotion of Rice Under Drought Conditions in Soil With Low Nutritional Value by Selected Actinobacteria

The soil used in this study was of poor quality as can be seen in **Table 4**. The organic matter content was only 0.75%, which determined it to be a low nutritional type of soil for rice cultivation as recommended by the Rice Department, Ministry of Agriculture and Cooperatives, Thailand. In addition, the nitrogen, phosphate and potassium levels were also low and this was especially true for nitrogen. The effects of *S. thermocarboxydus* isolate S3 inoculation on Thai jasmine rice KDML105 is presented in **Figures 3–5**. Seed germination was recorded between 98 and 99% for all treatments, which was higher than the control (96%). In the well-watered treatment, the positive effects of *S. thermocarboxydus* isolate S3 was seen in the rice as indicated by the following observed growth parameters, namely fresh weight, dry weight, root length and total length, which were all significantly higher than in the control (**Figure 3A**). However, the chlorophyll and proline contents were similar between the two treatments (**Figure 3B**). The RWC value of the rice leaves obtained from the treatment using isolate S3 was 96.1% compared to 81.0% in the control.

For the drought-induced treatment, a similar level of growth enhancement was observed in all tested parameters. The root, stem and total length measurements of the rice in *S. thermocarboxydus* isolate S3 treatment were higher than in the control (**Figure 4A**). The proline and chlorophyll contents, as well as the fresh and dry weight, were also higher than in the control (**Figure 4B**). At the end of the experiment, all rice plants in the control treatment withered and died, whereas in the treatment with *S. thermocarboxydus* isolate S3, some plants were still green (**Figures 5A,B**). The RWC value of the rice leaves in the inoculated rice with isolate S3 was 48.3% as compared to 43.3% in the control under drought stress conditions. *S. thermocarboxydus* isolate S3 was re-isolated from the rice root after harvesting. A count of 2×10^3 cfu/g fresh weight was obtained. The re-isolated strain showed 100% 16S rRNA gene sequence, which was identical to that of the *S. thermocarboxydus* isolate S3. In addition, filamentous cells of *S. thermocarboxydus* isolate S3 were observed under a light microscope as is shown in the Supplementary Figure S3. ACC deaminase activity was detected in isolate S3 as it could grow on DF salt minimal agar that was supplemented with 5 mmol l^{-1} of ACC as the nitrogen source.

DISCUSSION

Isolation and Identification of Actinobacteria From Spores of *Funneliformis mosseae*

Cultivable actinobacteria associated with arbuscular mycorrhizal spores were reported to be an interesting source of both biocontrol and plant growth-promoting activities (Schrey et al., 2012; Mohandas et al., 2013; Poovarasan et al., 2013; Battini et al., 2016). These findings encouraged us to explore actinobacteria that was obtained from the spores of *Funneliformis mosseae*. In the present study, we successfully isolated 6 actinobacteria from the spores of *Funneliformis mosseae*. The predominant isolates were members of the genus *Streptomyces*, a finding consistent with previous reports (Schrey et al., 2012; Mohandas et al., 2013; Poovarasan et al., 2013; Battini et al., 2016). The fact that we could isolate actinobacteria from surface sterilized spores suggested that they should be obtained from inside the spores. No bacterial growth was observed on nutrient agar and ISP2 agar obtained from the final wash water. These findings suggested that these actinobacteria are likely to be endophyte.

The interaction between actinobacteria and their mycorrhizal symbiosis have not been well-documented. Most research studies were conducted on Gram-negative bacteria such as *Pseudomonas*. However, several researchers have reported on the enhanced spore germination or mycorrhiza formation of various AM fungi in the presence of actinobacteria (Frey-Klett et al., 2007).

TABLE 2 | Indole-3-acetic acid (IAA) production, phosphate solubilization and siderophore production of actinobacteria associated with *F. mosseae*'s spores.

Isolate	IAA production ($\mu g\ ml^{-1}$)	Phosphate solubilization			Siderophore production		
		Clear zone on PVK agar (mm)	P released in PVK broth ($mg\ L^{-1}$)	pH	Color zone on CAS agar (mm)	Hydroxamate-Type ($\mu mol\ L^{-1}$)	Catecholate-Type ($\mu mol\ L^{-1}$)
48	4.44 ± 0.04	5.17 ± 0.29	217.56 ± 0.56	5.09 ± 0.09	17.83 ± 0.58	7.37 ± 0.009	17.50 ± 0.008
S1	9.08 ± 1.79	5.60 ± 0.76	215.60 ± 0.65	5.53 ± 0.72	13.00 ± 0.00	97.5 ± 3.63	7.54 ± 3.50
S3	11.12 ± 0.02	7.00 ± 0.50	224.27 ± 0.76	4.46 ± 0.25	15.83 ± 0.76	5.09 ± 0.003	39.17 ± 0.002
S4	0.74 ± 0.00	0	0	0	13.50 ± 1.50	1.40 ± 0.001	30.87 ± 0.005
S4-1	6.04 ± 0.05	0	0	0	14.00 ± 0.50	1.05 ± 0.000	17.50 ± 0.001
SP	0	0	0	0	13.83 ± 0.29	12.45 ± 0.003	2.12 ± 0.006

PVK, Pikovskaya's agar; CAS, Chrome Azurol Sulphonate agar. Data represent mean values of three replicates ± SD. Different letters indicate the existence of significant differences according to Tukey test (P = 0.05).

TABLE 3 | Growth of actinobacteria associated with *F. mosseae*'s spores under reduced water activity.

Sorbitol (g/L)	Water activity (a_w)	Isolates					
		48	S1	S3	S4	S4-1	SP
0	0.998	+	+	+	+	+	+
85	0.986	+	+	+	+	+	+
285	0.957	+	+	+	+	+	+
405	0.919	−	+	+	−	−	−
520	0.897	−	−	−	−	−	−
660	0.844	−	−	−	−	−	−

+, growth; −, no growth.

Tylka et al. (1991) found that volatiles obtained from S. *orientalis* stimulated the germination of both *Gigaspora margarita* and *G. mosseae* spores on water agar. Similarly, the spore germination of *G. margarita* was found to be stimulated by volatiles of soil-isolated actinobacteria, especially those with straight spore chains and 2-methylisoborneol (MIB) producers (Carpenter-Boggs et al., 1995). The same authors speculated that the stimulation of AM spore germination enhanced the probability of the formation of mycorrhizal associated with nearby plants. Inoculation of *S. coelicolor* 2389 with *G. intraradices* increased the intensity of mycorrhizal root colonization and arbuscular formation in sorghum (Abdel-Fattah and Mohamedin, 2000). *Streptomyces* was also found to enhance mycorrhiza formation in the ectomycorrhizal fungus of *Amanita muscaria* as a result of the promotion of fungal growth (Schrey et al., 2005). In addition, mycorrhiza-associated bacteria together with AM fungi contributed to protection against root pathogens (Frey-Klett et al., 2007). For example, mycorrhizal associated streptomycetes obtained from Norway spruce trees produced secondary metabolites displaying antifungal activity against the growth of several plant pathogenic fungi including *Fusarium oxysporum* (Schrey et al., 2012). *Streptomyces* obtained from *G. mossaea* spores also showed strong antifungal activity against *F. oxysporum* and *Alternaria solani* (Mohandas et al., 2013). Considering the plant growth promoting ability of the isolated actinobacteria, it is possible that these actinobacteria are "mycorrhiza helper bacteria" that can contribute to nutrient mobilization from the soil and impact root structure through the production of growth promoters, as has been suggested by Frey-Klett et al. (2007). However, the possibility of the isolated actinobacteria being involved in the process of promoting mycorrhization could not be ruled out and requires further study.

It is interesting to note that a member of the genus *Pseudonocardia* was isolated from mycorrhiza spores for the first time in this study. It was previously only detected in the spores of *Gigaspora margarita* and *Gigaspora rosea* by DGGE (Long et al., 2008). Currently, DGGE analysis of *F. mosseae* spores has revealed the presence of actinobacteria in the genera *Amycolatopsis*, *Arthrobacter,* and *Propionibacterium* (Agnolucci et al., 2015). *Glomus mosseae* spores were reported to harbor cultivable actinobacteria of the genus *Arthrobacter, Curtobacterium, Liefsonia, Microbacterium, Micrococcus,* and *Streptomyces* (Bharadwaj et al., 2008b; Mohandas et al., 2013; Poovarasan et al., 2013; Selvakumar et al., 2016).

It is also worth mentioning that we could not isolate any actinobacteria without an enrichment step in the soil extract solution. Enrichment is a common practice for effectively increasing the population of target microorganisms (Kamagata, 2015). Soil extract was successfully used to isolate novel actinomycetes from the soil (Hamaki et al., 2005). Conventional cultures and isolation media that are generally nutritionally rich usually result in a failure to cultivate most bacteria in nature. Our

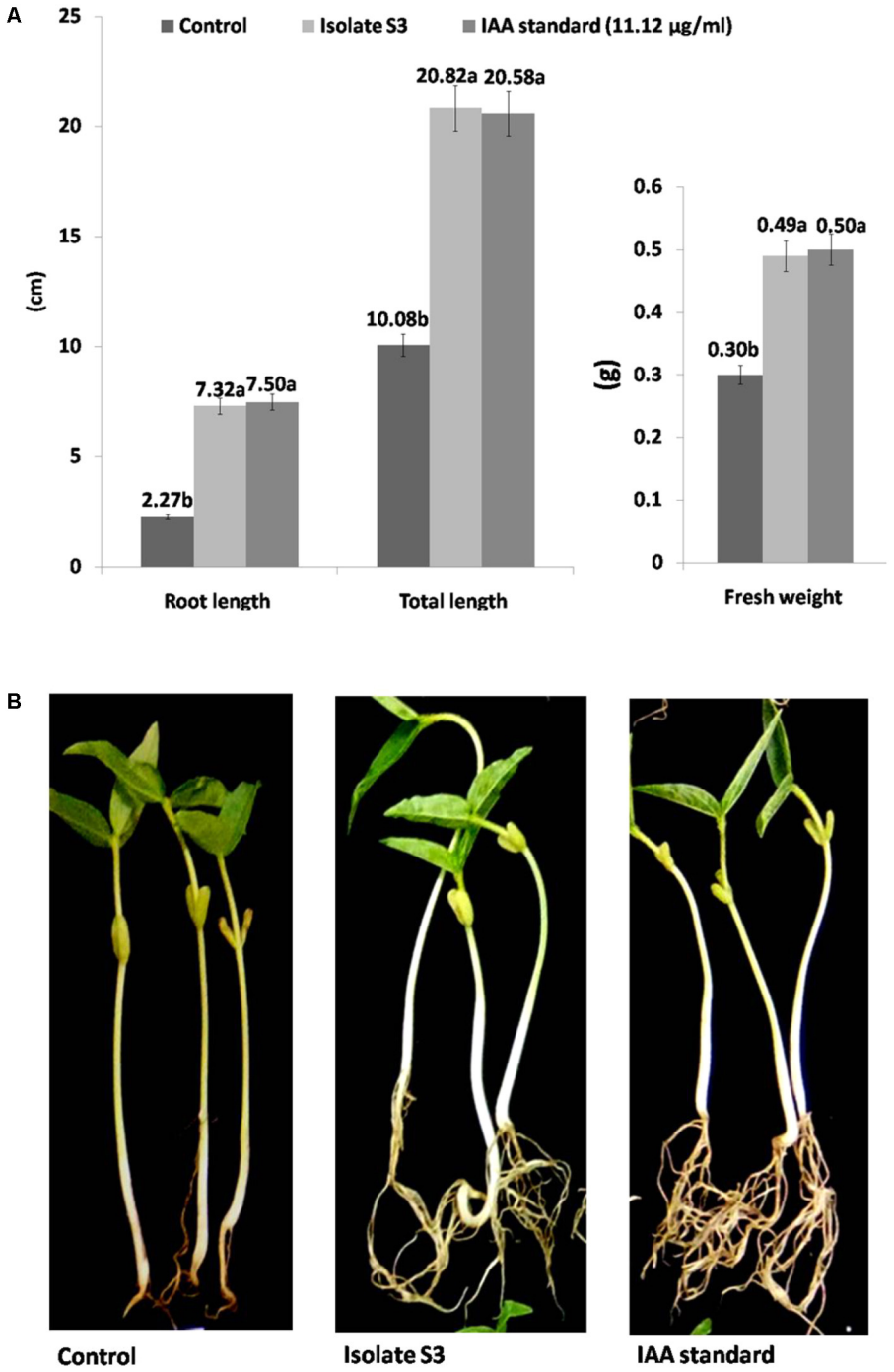

FIGURE 2 | Growth promotion of mung beans (*Vigna radiata*) by *S. thermocarboxydus* isolate S3 **(A)**, root intensity **(B)**.

results suggest that the addition of the soil extract could support the growth of actinobacteria, which represents a small proportion of the bacteria that is associated with mycorrhizal spores and can increase the numbers to a detectable level on the isolation plate.

It is evident from the 16S rRNA gene sequences and the phylogenetic analysis that 5 isolates were members of the genus *Streptomyces*. These isolates were closely related to *S. thermocarboxydus*, *S. pilosus,* and *S. spinoverrucosus* (**Table 1** and **Figure 1A**). Based on this data, we identified isolates 48 and S3 as *S. thermocarboxydus*, isolates S4 and S4-1 as *S. pilosus* and isolate S1 as *S. spinoverrucosus*. These three *Streptomyces* species have never been isolated from mycorrhizal spores. Isolate SP was identified as *Pseudonocardia* and is closely related to *P. nantongensis* KLBMP 1282^{T} (**Table 1**). It shared a well support

TABLE 4 | Characteristics of soil used in plant growth promotion experiment.

No	Properties	Amount
1	Electrical Conduction (μS/cm)	56.80
2	Exchangeable Potassium (mg/kg)	79.83
3	Organic Matter (%)	0.75
4	Total Nitrogen (%)	0.04
5	Available Phosphate (mg/kg)	10.20
6	Exchangeable Magnesium (mg/kg)	98.12
7	Available Iron (mg/kg)	35.04
8	Extractable Sulfur (mg/kg)	23.93

branch with *P. nantongensis* KLBMP 1282^T (**Figure 1B**). To the best of our knowledge, this is the first *Pseudonocardia* strain isolated from mycorrhizal spores. This strain shared only 98.56% similarity with *P. nantongensis* KLBMP 1282^T, a value below the proposed 98.7% (Stackebrandt and Ebers, 2006) or 98.65% (Kim et al., 2014) cut-off values for the delineation of novel species. It is likely that isolate SP will represent a novel species within the genus *Pseudonocardia* though a further characterization using the polyphasic approach is needed. However, the detailed formal description of this strain as a novel species will be the subject of another publication.

In Vitro Plant Growth Promoting Activities

Indole-3-acetic acid (IAA) is a phytohormone of the auxin type, which helps promote plant growth and development especially in the root system. The production of IAA is common in actinobacteria. Several *Streptomyces* species are known to produce IAA including *S. coelicolor*, *S. griseus,* or *S. scabies* (Manulis et al., 1994). A high proportion (70%) of IAA producing actinobacteria that are associated with the mycorrhizal spores of *R. intraradices* was also reported (Battini et al., 2016). In the present study, all *Streptomyces* isolates were able to produce IAA in the presence of L-tryptophan (0.74 – 11.12 μg ml^{-1}, **Table 2**). *Streptomyces* and *Leifsonia poae* isolated from *G. mosseae* spores were reported to produce IAA at similar levels to our isolates (5.0–10.1 μg ml^{-1}) (Mohandas et al., 2013). A higher level of IAA production of 43.8 μg ml^{-1} compared to *S. thermocarboxydus* isolate S3 (11.12 μg ml^{-1}) was recorded in *S. thermocarboxydus* DBT219 using endophytic actinobacteria associated with tomatos (*Solanum lycopersicum*) (Passari et al., 2016). However, those authors did not indicate the amount of supplemented L-tryptophan in the experiment. *Streptomyces* isolates obtained from Thai medicinal plants produced IAA in a range of 11-144 μg ml^{-1}, while *S. viridis* CMUH-009 could produce IAA at a level as high as 300 μg ml^{-1} under optimized conditions (Khamna et al., 2010). The expected PCR product of approximately 1,600 bp from *S. thermocarboxydus* isolate S3, as well as *S. coelicolor* A3(2), indicated the presence of the IAA encoding gene. However, BLAST identification of this PCR product as an amino oxidase gene in isolate S3 suggested that this *Streptomyces* may use the tryptamine (TAM) pathway in IAA synthesis instead of the indole-3-acetamide (IAM) pathway, as was the case with several *Streptomyces* species including *S. coelicolor* A3(2) and *S. scabies* (Manulis et al., 1994).

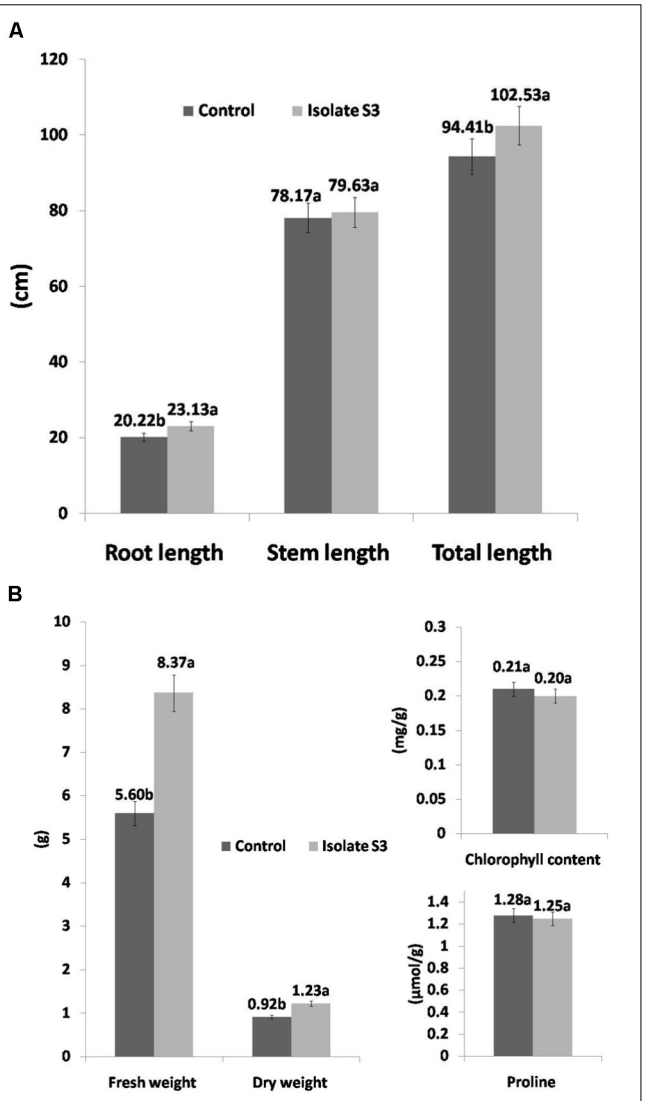

FIGURE 3 | Growth promotion of Thai jasmine rice (*O. sativa*) KDML105 by *S. thermocarboxydus* isolate S3 under well-watered conditions. **(A)** Root, stem and total length. **(B)** Fresh weight, dry weight, chlorophyll and proline contents.

Several actinobacteria associated with mycorrhizal spores were reported to have phosphate solubilization properties. Three of our *Streptomyces* isolates, namely isolates 48, S3 and S1, produced a clear zone of tricalcium phosphate solubilization on Pikovskaya's agar. *Streptomyces* and *Leifsonia* isolated from *G. mosseae* spores were also found to produce clear zones of tricalcium phosphate solubilizing activity (Mohandas et al., 2013). Similarly, *Arthrobacter ilicis* obtained from the spores of *G. intraradices* could solubilize phosphate on Pikovskaya's

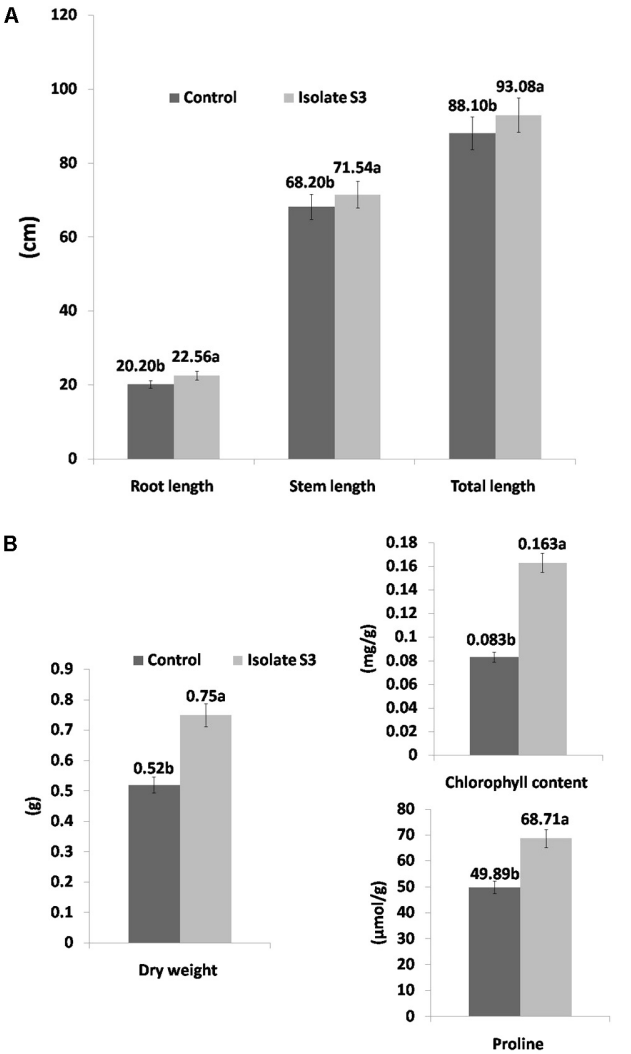

FIGURE 4 | Growth promotion of Thai jasmine rice (*O. sativa*) KDML105 by *S. thermocarboxydus* isolate S3 under drought conditions. **(A)** Root, stem, and total length. **(B)** Dry weight, chlorophyll and proline contents.

agar (Bharadwaj et al., 2008a). However, the quantitative determination of phosphate solubilization in the culture broth was not reported in these previous research studies. High phosphate solubilization in the range of 215.6 – 224.27 mg L^{-1} as was recorded in this study was higher than that of the *Streptomyces* isolates obtained from the sediment of Chorao Island, India (89.3 – 112.1 µg mL^{-1}) (Dastager and Damare, 2013). Nevertheless, a higher degree of phosphate solubilizing activity of 72.13 mg/100 ml was reported in *Streptomyces* obtained from the wheat rhizosphere soil in Pakistan (Anwar et al., 2016). The ability to solubilize phosphate in actinobacteria obviously varies from strain to strain and species to species, as exemplified by our results and the previous research reports.

The acidification of the culture filtrate was observed in all isolates that could release soluble phosphate in the culture broth. This observation suggested that the solubilization process was involved with the production of organic acids. Microbial solubilization of mineral phosphates is proposed to either be due to the production of organic acids (Surapat et al., 2013) or the production of chelating substances such as siderophores (Hamdali et al., 2008). A preliminary investigation on the quality and quantity of organic acids in the culture broth by HPLC analysis confirmed the presence of several organic acids including gluconic acid, malonic acid, oxalic acid and propionic acid (data not shown). Lactic and 2-ketogluconic acids were the predominant acids found in phosphate-solubilizing *Streptomyces* (Banik and Dey, 1982). Additionally, phosphate-solubilizing actinobacteria, *Micromonospora endolithica*, was reported to be capable of producing organic acids namely citric, oxalic, gluconic, malic, succinic, acetic, and lactic acids (El-Tarabily et al., 2008).

All actinobacteria that were isolated from the spores of *F. mosseae* were able to produce siderophores in both hydroxamate and catecholate types at varying concentrations. Siderophores are small molecules of iron chelating compounds produced by several microorganisms including actinobacteria under iron starvation (Wang et al., 2014). Siderophores are known to promote plant growth and inhibit plant pathogens (Verma et al., 2011; Sadeghi et al., 2012). Several *Streptomyces* species have been found to be able to produce desferrioxamine (hydroxamte type siderophores) including *S. pilosus* (Wang et al., 2014). Catecholate types are not common among siderophore-producing actinobacteria. However, *Streptomyces* were found to be able to secrete enterobactin, a catecholate type siderophore of *Enterobacteriaceae* (Lee et al., 2012). All actinobacteria obtained from *G. mosseae* spores including *Streptomyces* were also able to produce siderophores on CAS agar (Mohandas et al., 2013). Similarly, 79% of the cultivable actinobacteria obtained from *R. intraradices* spores could produce siderophores (Battini et al., 2016). The siderophore-producing endophytic *Streptomyces* was clearly shown to be able to promote the growth of Thai jasmine rice by significantly increasing root and shoot biomass, as well as root length, in comparison with the siderophore-deficient mutant (Rungin et al., 2012).

It is worth mentioning that three of our *Streptomyces* isolates (48, S1, S3) displayed all of the plant growth-promoting activities we investigated. The observation that the actinobacteria associated with the spores of *F. mosseae* are equipped with plant growth promoting properties *in vitro* suggests that these actinobacteria are potential candidates for *in planta* testing. This strategy has been used successfully to identify potential actinobacteria that can promote the growth of several plant species, e.g., guava (Mohandas et al., 2013), rice (Rungin et al., 2012; Gopalakrishnan et al., 2014) tomato (Passari et al., 2016) and wheat (Anwar et al., 2016).

In Vitro Assay for Drought Tolerance in Actinobacteria

Bacteria in general prefer a high a_w value for growth. A minimum a_w for bacterial growth was defined at 0.900a_w for a majority of bacteria (Grant, 2004). In the present study, *Streptomyces* isolates S1 and S3 grew up to 0.919 a_w which could indicate xerotolerance. Drought is a water deficit condition that does

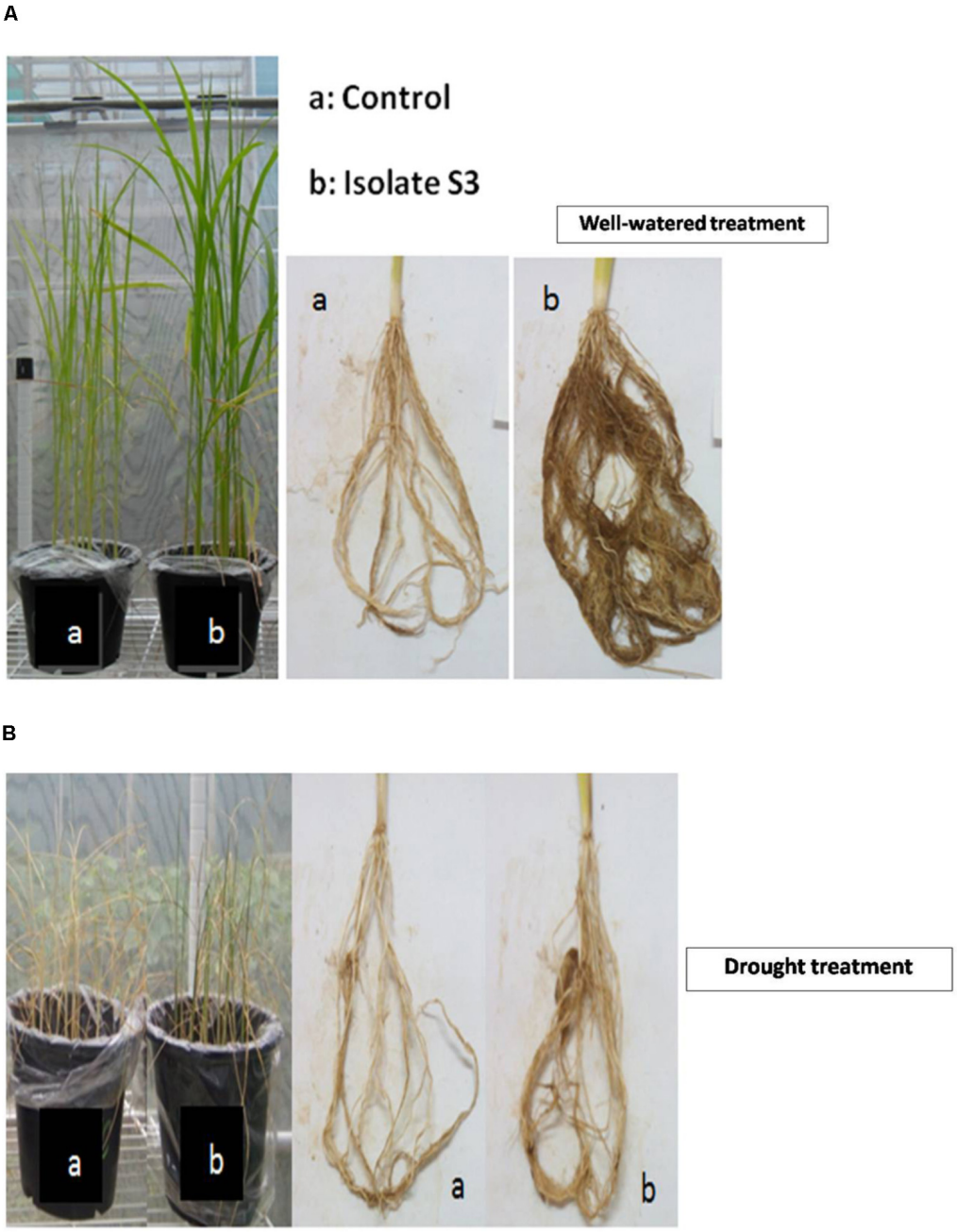

FIGURE 5 | Growth of Thai jasmine rice (*O. sativa*) KDML105 inoculated with *S. thermocarboxydus* isolate S3 under well-watered conditions **(A)** and drought **(B)**. a: control; b: isolate S3.

not allow for the growth of most plants. To use plant growth promoting bacteria to alleviate drought stress for plants, one basic requirement is the growth of plant growth-promoting bacteria under such condition. The ability to grow during periods of low water availability is an important criterion for selecting potential actinobacterial isolates when testing the growth promotion of the plant under the conditions of drought. This screening approach was successfully used to obtain rhizosphere bacteria from cacti for the growth promotion of *Zea mays* L. under drought conditions (Kavamura et al., 2013). The ability of actinobacteria to grow at low a_w is not surprising as several species of actinobacteria, including several *Streptomyces* species, have been isolated from even the driest places on earth like the Atacama desert (Okoro et al., 2009). This report and our results strongly support the conclusion that these xerotolerant actinobacteria could survive under extreme water deficit environments and we recommended them as promising materials for plant growth-promoting bacteria under conditions of drought stress.

Plant Growth Promotion

Growth Promotion of *Vigna radiata* by Selected Actinobacteria

This experiment was designed to test the ability of the selected *S. thermocarboxydus* isolate S3 to promote plant growth *in vivo*. Mung beans (*V. radiata*) have been widely cultivated throughout Southeast Asia. This plant species has been used as a test plant due to its short life cycle. The ability of isolate S3 to promote the growth of mung beans was evident (**Figure 2**). Similar results from treatment 2 (cell suspension and supernatant) and treatment 3 (addition of 11.12 $\mu g\ ml^{-1}$IAA) indicated that the observed level of growth enhancement in the root and total length might be a result of the contribution of IAA that was produced by *S. thermocarboxydus* isolate S3 as this isolate was found to produce 11.12 $\mu g\ ml^{-1}$ IAA *in vitro* (**Table 2**). More developed root systems were also observed in the treatment with isolate S3. This observation suggested the effect of IAA production by isolate S3. IAA is known for its effect on the production of lateral roots and the promotion of root-length (Etesami et al., 2015). Similar incidences of the promotion of plant growth that occurred due to the production of plant phytohormones by *G. mosseae* spores associated with actinobacteria in pomegranate seedlings have been reported (Poovarasan et al., 2013). *S. canus* which produced 10.10 $\mu g\ ml^{-1}$ of IAA *in vitro* was found to increase shoot and root growth and plant dry matter in 6-month-old pomegranate seedlings. The production of bacterial phytohormones such as IAA is considered to be a major mechanism for drought endurance and resilience in microbial strains (Kaushal and Wani, 2016; Forni et al., 2017). This is the reason that *S. thermocarboxydus* isolate S3 was selected for further experimentation on rice growth promotion under induced drought conditions in the greenhouse.

Growth Promotion of Rice Under Drought Conditions in Soil With Low Nutritional Content by Selected Actinobacteria

It is evident that in soil having low nutritional content and under non-stress conditions, inoculation with isolate S3 improved the growth of rice seedlings as was observed in terms of root length, stem length, and fresh and dry weight (**Figure 3**). Our results provide evidence that the most prominent beneficial effects of inoculation with a potential PGPR is to be expected in poor soil when the development of the indigenous microbial community is inhibited (Ramos Solana et al., 2006). Similar results have been reported under other unfavorable conditions such as in rice (Yuwono et al., 2005) or wheat (Yandigeri et al., 2012) under conditions of drought and in tomatos (Palaniyandi et al., 2014) under conditions of salinity.

Rice is more susceptible to drought stress than most crop plants. According to the International Rice Research Institute (IRRI), when rice plants experience drought, they have a reduced ability to extract nutrients from the soil (Lafitte and Bell, 2017). Thai jasmine rice KDML105 is a dominant rice variety that has been widely cultivated in Thailand and is sensitive to drought (Cha-um et al., 2010). Therefore, it has been selected as a test plant in the investigation of the ability of plant growth promoting *S. thermocarboxydus* isolate S3 to protect KDML105 under conditions of induced drought stress in soil of low nutritional value. In this study, we induced conditions of drought stress in rice during the early vegetative stage, which requires large amounts of water for the development of a complete panicle formation[2]. This drought stress induces a reduction in rice growth and development as was observed in many growth parameters in this study. With the use of soil having low nutritional content in this study, the adverse effects on rice growth was expected to be more severe as was seen in reductions of fresh and dry weights (**Figures 3B**, **4B**).

The growth and development of rice was observed to decline due to the fact that drought stress induces damage to biochemical and physiological mechanisms (Pandey and Shukla, 2015). This stress could be alleviated through the application of a microbial inoculant that induces biochemical and physiological changes in plants to sustain their growth (Yang et al., 2009; Yandigeri et al., 2012; Vurukonda et al., 2016; Rubin et al., 2017). This study provides evidence that induced drought stress conditions could be relieved by inoculation with plant growth promoting *S. thermocarboxydus* isolate S3.

Fresh weight is another common morphological growth parameter that is severely affected by drought (Jaleel et al., 2009). Reduced biomass was reported in rice under conditions of water stress (Manickavelu et al., 2006; Farooq et al., 2009, 2010). Many studies have reported a significant decrease in the fresh and dry weights of the shoots (Centritto et al., 2009; Mostajeran and Rahimi-Eichi, 2009) and roots (Ji et al., 2012) under conditions of drought. Our results revealed a reduction in both the fresh (3.18 g) and dry (0.52 g) weights of drought-induced rice compared to well-watered rice in treatments without *S. thermocarboxydus* isolate S3 (5.6 g, 0.92 g). However, the fresh and dry weight measurements in drought-induced rice that has been inoculated with *S. thermocarboxydus* isolate S3 were higher than in the control. This observation implies the ability of plant growth promoting *S. thermocarboxydus* isolate S3 to reduce the damaging effects caused by drought stress.

Drought stress also affects various physiological processes in plants. One common adverse effect is on photosynthetic pigments. Chlorophyll is the most important pigment for photosynthesis in plants and thus affects their growth. Since severe drought stress may cause termination in photosynthesis. A reduction in chlorophyll content has been reported in KDML105 rice under conditions of drought stress (Cha-um et al., 2010) and in other rice varieties (Maisura et al., 2014; Yang et al., 2014). In the present study, the chlorophyll content in the well-watered treatment was similar between treatment with *S. thermocarboxydus* isolate S3 (1.25 mg/g) and the control (1.28 mg/g). However, a remarkable difference was observed in the drought-induced treatments. In the treatment with *S. thermocarboxydus* S3, the chlorophyll content (0.163 mg/g) revealed a 96.4% increase when compared to the control treatment (0.083 mg/g) without bacterial inoculation. This observation strongly suggests that

[2]http://ricepedia.org/rice-as-a-plant/growth-phases

S. thermocarboxydus isolate S3 could prevent the deleterious effects of drought on the chlorophyll content in Thai jasmine rice KDML105.

Proline accumulation in crop species has been established as a good indicator of water stress. Changes in proline concentrations in rice have been observed. Proline content in rice KDML105 increased under conditions of drought stress (Cha-um et al., 2010). An increase in proline levels is regarded as one mechanism of drought tolerance in certain plants including rice (Pandey and Shukla, 2015). Proline can act as both an osmo-protectant agent and a hydroxyl radical scavenger to protect plants from certain kinds of stress (Kaushal and Wani, 2016; Forni et al., 2017). It is evident from our results that *S. thermocarboxydus* isolate S3 could induce the accumulation of proline in Thai jasmine rice KDML105 that has been subjected to conditions of drought stress (**Figure 4B**).

Relative water content in plant leaves is considered one of the best criterion for measuring plant water status as drought is known to decrease the RWC in the leaves of plants under conditions of stress (Ngumbi and Kloepper, 2016). In this study, the leaves of rice seedlings inoculated with *S. thermocarboxydus* isolate S3 showed an 11% higher RWC than the un-inoculated leaves under drought conditions. Our results are in line with the previous findings by Grover et al. (2014) who observed that sorghum plants that were inoculated with PGPR *Bacillus* spp. strain KB129 showed a 24% increase in RWC over the control plants under drought stress conditions. Similar results were reported in maize seedlings that had been inoculated with several plant growth-promoting rhizobacteria (Sandhya et al., 2010; Vardharajula et al., 2011; Garcia et al., 2017). The data from those findings and this study suggest that plant growth promoting rhizobacteria could increase RWC and improve the survival rate of plants under conditions of drought stress. An increase in RWC should be considered an important drought tolerance strategy in plants.

Bacteria producing phytohormones such as auxins were reported to be able to improve the drought resistance of host plants (Yang et al., 2009; Vurukonda et al., 2016). Root growth enhancement by IAA promotes water and nutrient uptake, hence increase the tolerance of rice to conditions of drought (Dimpka et al., 2009). Root proliferation improvement in rice plants under conditions of drought stress has been shown to be a result of IAA production by inoculated rhizobacteria (Yuwono et al., 2005). *S. thermocarboxydus* isolate S3 was able to produce IAA at lower water availability ($0.919a_w$; data not shown) which could promote the growth of rice seedlings under drought conditions. These results suggest that the level of drought tolerance observed in this study may be partly mediated by IAA.

Ethylene is a gaseous plant hormone, which is also known as a plant stress hormone. Under conditions of drought stress, plants produce ethylene to regulate homeostasis, which also causes reductions in root and shoot growth (Yang et al., 2009). 1-aminocyclopropane-1-carboxylate (ACC) is an immediate precursor of ethylene in higher plants. Bacteria containing enzyme ACC deaminase, which are able to degrade ACC to ammonia and α-ketobutyrate, could mitigate this deleterious effect of ethylene. ACC deaminase-producing bacteria confer tolerance against water deficiency in rice (Bal et al., 2013) and tomato plants (Palaniyandi et al., 2014).

The importance of IAA production and ACC deaminase activity in promoting plant-growth under conditions of drought has been recognized (Saleem et al., 2007; Dimpka et al., 2009; Kaushal and Wani, 2016; Ngumbi and Kloepper, 2016; Vurukonda et al., 2016). IAA is involved in ethylene biosynthesis through stimulation of enzyme ACC synthetase activity to convert *S*-adenosyl-methionine (SAM) to ACC (Saleem et al., 2007). Microbial uptake and hydrolysis of ACC by ACC deaminase-producing bacteria provide a level of equilibrium between the internal and external ACC levels. This lower ACC level reduced ethylene biosynthesis within the plant. In a relationship between IAA and the ethylene precursor, ACC supports the positive effects of IAA on root growth through a reduction of ethylene levels (Lugtenberg and Kamilova, 2009). El-Tarabily (2008) demonstrated that *Streptomyces filipinensis* no. 15, ACC-deaminase and an IAA producer, could reduce the endogenous levels of ACC, an ethylene precursor, in both the roots and shoots and subsequently enhanced tomato growth under greenhouse conditions. Similarly, ACC deaminase and IAA producing bacteria from the rhizosphere were shown to promote the growth of rice seedlings, especially with regard to root and shoot growth (Bal et al., 2013). Palaniyandi et al. (2014) reported on the plant growth-promoting and stress-alleviating activities from a halo-tolerant and ACC deaminase-producing *Streptomyces* sp. strain PGPA39 that was applied to tomato (*Solanum lycopersicum*) plants under conditions of salinity stress. Our results imply that *S. thermocarboxydus* isolate S3 uses its ability for IAA and ACC deaminase production as a mechanism to promote the growth of KDML105 rice under induced drought conditions.

The ability of *S. thermocarboxydus* isolate S3 to produce siderophores and solubilize phosphate is also beneficial for rice growth as phosphorus and iron are essential for plant growth. Enhanced plant nutritional uptake is an example of a direct mechanism that could stimulate plant growth (Bharadwaj et al., 2008a). *Streptomyces* from *G. mosseae* with phosphate solubilizing and siderophore-producing abilities were able to promote the growth of guava (Mohandas et al., 2013). Similarly, phosphate solubilizing rhizosphere *M. endolithica* was also able to promote the growth of *Phaseolus vulgaris* beans (El-Tarabily et al., 2008).

The successful re-isolation of *S. thermocarboxydus* isolate S3 from the roots along with the observation of the typical filamentous cells of *Streptomyces* on the root surface has suggested that this isolate has the ability to colonize the roots of rice. In addition, the substantial number of isolate S3 that was found in the roots after being harvested was suggestive of the survival of this strain within the root tissues. Similar results of bacterial colonization have been reported in previous studies (Etesami et al., 2014; Qin et al., 2017). This root colonization is an important factor in both plant-growth promoting activities and the survival of bacteria under

conditions of abiotic stress such as drought (Gontia-Mishra et al., 2016).

CONCLUSION

Our results provide evidence that actinobacteria were associated with arbuscular mycorrhizal spores of *F. mosseae*. One new genus of actinobacteria, *Pseudonocardia*, was added to the list of cultivable actinobacteria associated with arbuscular mycorrhizal spores as well as the species of *S. pilosus*, *S. spinoverrucosus,* and *S. thermocarboxydus*. The inoculation of *S. thermocarboxydus* isolate S3 could promote the growth of Thai jasmine rice in soil of low nutritional content and under induced conditions of drought stress. The positive effects of *S. thermocarboxydus* isolate S3 inoculation may be due to its PGP properties, in particular IAA production and ACC deaminase activity. The ability of this organism to produce plant growth promoting agents supports the possibility of using this actinobacteria for agricultural purposes especially in areas affected by water deficit stress or in arid and semi-arid habitats.

AUTHOR CONTRIBUTIONS

WP-A, KL, and SL designed the research and project outline. KL performed all the experiments. KL, ST, SL, and WP-A drafted 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: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01247/full#supplementary-material

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A Phylogenomic and Molecular Markers Based Analysis of the Class *Acidimicrobiia*

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Recent metagenomic surveys of microbial community suggested that species associated with the class *Acidimicrobiia* are abundant in diverse aquatic environments such as acidic mine water, waste water sludge, freshwater, or marine habitats, but very few species have been cultivated and characterized. The current taxonomic framework of *Acidimicrobiia* is solely based on 16S rRNA sequence analysis of few cultivable representatives, and no molecular, biochemical, or physiological characteristics are known that can distinguish species of this class from the other bacteria. This study reports the phylogenomic analysis for 20 sequenced members of this class and reveals another three major lineages in addition to the two recognized families. Comparative analysis of the sequenced *Acidimicrobiia* species identified 15 conserved signature indels (CSIs) in widely distributed proteins and 26 conserved signature proteins (CSPs) that are either specific to this class as a whole or to its major lineages. This study represents the most comprehensive phylogenetic analysis of the class *Acidimicrobiia* and the identified CSIs and CSPs provide useful molecular markers for the identification and delineation of species belonging to this class or its subgroups.

Keywords: *Acidimicrobiia*, marine *Acidimicrobiia*, phylogenomics, molecular signatures, conserved signature indels, conserved signature proteins

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INTRODUCTION

The class *Acidimicrobiia* is a deep-rooting lineage within the phylum *Actinobacteria*. This class is comprised of few cultivable representatives that were mostly isolated from extremely acidic environments (Zhi et al., 2009; Gao and Gupta, 2012b; Ludwig et al., 2012). Four type species of this class namely, *Acidimicrobium ferrooxidans*, *Acidithrix ferrooxidans*, *Ferrimicrobium acidiphilum,* and *Ferrithrix thermotolerans* are extremely acidophilic, with optimal growth pH at around 2.0, and are able to oxidize ferrous iron at relatively fast rates (Cleaver et al., 2007; Ludwig et al., 2012; Norris, 2012). These species were mainly isolated from acidic mine waters or geothermal sites, and were responsible for the regeneration of ferric iron within the acidic ecosystem (Clark and Norris, 1996; Johnson et al., 2009; Jones and Johnson, 2015). In contrast, other members of this class were not acidophiles and inhabited more diverse aquatic environments. For example, neutrophilic *Iamia majanohamensis* was isolated from the abdominal epidermis of a sea cucumber, filamentous "*Candidatus* Microthrix parvicella" (henceforth called M. parvicella) from wastewater sludge, while members of the genus *Ilumatobacter* from estuary sediment or seashore sand (Kurahashi et al., 2009; Matsumoto et al., 2009; Mcllroy et al., 2013).

In spite of few cultivable *Acidimicrobiia* species, metagenomic analyses have revealed that there were many uncultured actinobacterial species belonging to the class *Acidimicrobiia* in freshwater and marine samples (Rheims et al., 1996; Jensen and Lauro, 2008; Ghai et al., 2014). Warnecke et al. (2004) analyzed actinobacterial 16S rRNA genes from freshwater habitats and suggested four most prominent lineages, one of which "acIV lineage" is associated with the order *Acidimicrobiales*. An extensive microbial community composition survey of northwestern Sargasso Sea identified that a marine clade closely related to M. parvicella were abundant in the deep chlorophyll maximum (DCM), with occasional blooms during summer stratification period (Treusch et al., 2009). More recently, Chen et al. (2016) investigated the actinobacterial diversity in the deep sea along the Southwest Indian Ridge and discovered that *Acidimicrobiales* is one of the two most widely distributed and abundant actinobacterial orders in all nine samples from deep sea environments. In addition to the species diversity analyses based on 16S rRNA sequences, further genomic data mining of uncultured *Acidimicrobiia* species suggested that the ecological and metabolic diversity of this class is far underestimated by the culture-dependent species characterization. A metagenomic analysis of Mediterranean DCM assembled four nearly complete genomes for marine *Acidimicrobiales*, and pathway analysis indicated that these species have the capability to assimilate C2 compounds and also derive energy from dimethylsulfoniopropionate, sulfonate, and carbon monoxide (Mizuno et al., 2015). In addition, one of the genomes encodes acidirhodopsin, a novel rhodopsin clade related to freshwater actinorhodopsins (Mizuno et al., 2015).

Although the metagenomic data greatly expanded our knowledge of the species diversity of *Acidimicrobiia*, the current taxonomic frame of this class contains only one order *Acidimicrobiales*, and two families *Acidimicrobiaceae* and *Iamiaceae* with few genera (Ludwig et al., 2012). The taxonomic ranks were determined solely based on 16S rRNA gene sequence analyses and taxon-specific 16S rRNA signature nucleotides using limited representative isolates. To date, despite the availability of seven complete genomes from cultivated *Acidimicrobiia* species and many incomplete genomes from metagenomic data, there is

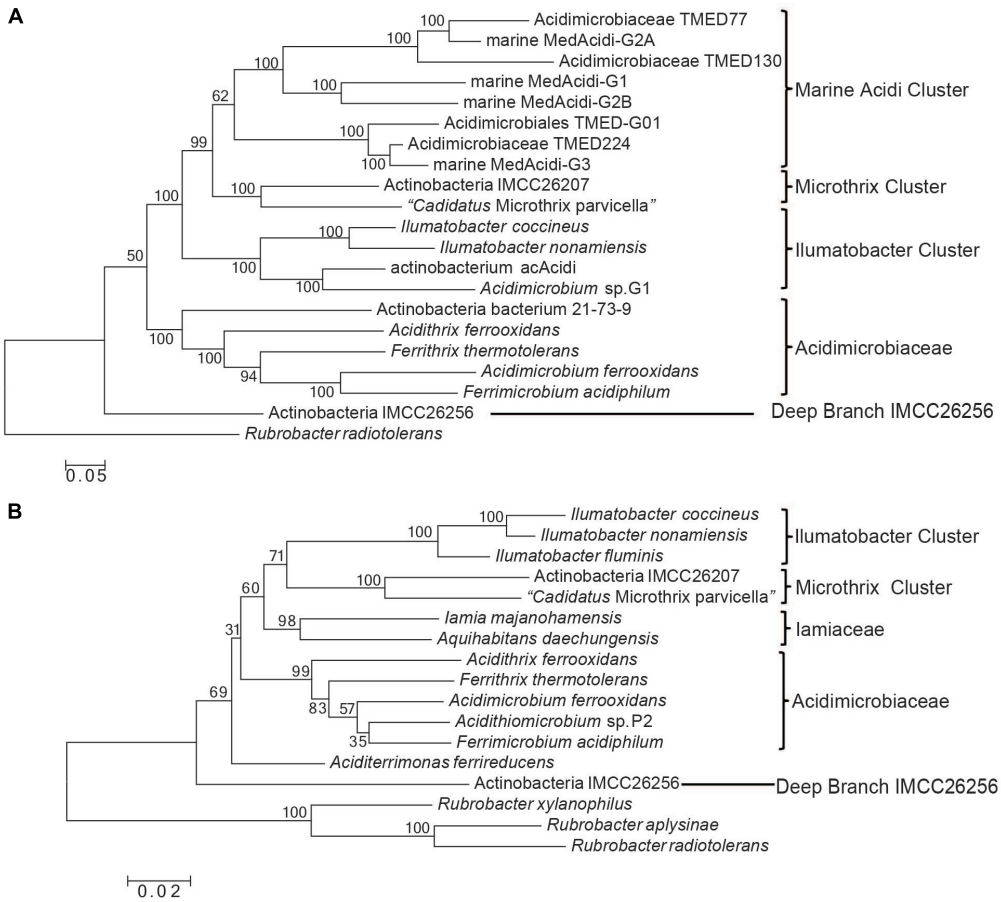

FIGURE 1 | Phylogenetic tree analysis of class *Acidimicrobiia*. **(A)** ML tree for 20 *Acidimicrobiia* species based upon concatenated sequences of 30 conserved proteins. **(B)** Neighbor-joining tree based on full length 16S rRNA gene sequences of all type species within the class *Acidimicrobiia.* Bootstrap values (%) are shown at each node and different clusters that are consistently observed in both phylogenetic trees are marked.

no comprehensive phylogenetic analysis performed to examine the evolutionary relationship within this class. As such, no detailed evolutionary relationship of the uncultured species can be assigned with relation to the known type species of the class *Acidimicrobiia*. (Warnecke et al., 2004; Ghai et al., 2014; Mizuno et al., 2015). In addition, except the branching pattern of these species in phylogenetic trees, no molecular, biochemical or physiological characteristics are known that can clearly distinguish *Acidimicrobiia* species from other *Actinobacteria* (Norris, 2012).

Comparative genomic analyses can lead to discovery of molecular markers that are specific to different higher taxon (e.g., genus level and above), which cannot be easily derived from culture-dependent phenotypic characterization (Gupta and Gao, 2010; Gao and Gupta, 2012a). One important category of these molecular markers is conserved signature indels (CSIs) that are uniquely found in the genes/proteins homologs from a specific group of organisms. Another type of molecular markers are conserved signature proteins (CSPs) that are uniquely shared by a monophyletic group of prokaryotes. The two molecular marker types represent highly reliable characteristics of specific groups of organisms, and they provide novel methods for the identification or delineation of prokaryotic taxonomic units in clear molecular terms (Gao and Gupta, 2012b; Ho et al., 2016; Zhang et al., 2016; Alnajar and Gupta, 2017).

In the present work, a robust phylogenetic tree was constructed for 20 sequenced members of class *Acidimicrobiia* based on 30 universal conserved proteins. The tree clearly showed another three major clusters in addition to the two recognized families within this class, and these clusters may comprise separate families. Besides, comparative analysis of the sequenced *Acidimicrobiia* species identified 15 CSIs in universal proteins and 26 CSPs, which are either specific for this class as a whole or to its major lineages. This study represents the most comprehensive phylogenetic analysis of the class *Acidimicrobiia* and the identified CSIs and CSPs provide useful molecular markers for the identification and demarcation of the members belonging to this class or its subgroups.

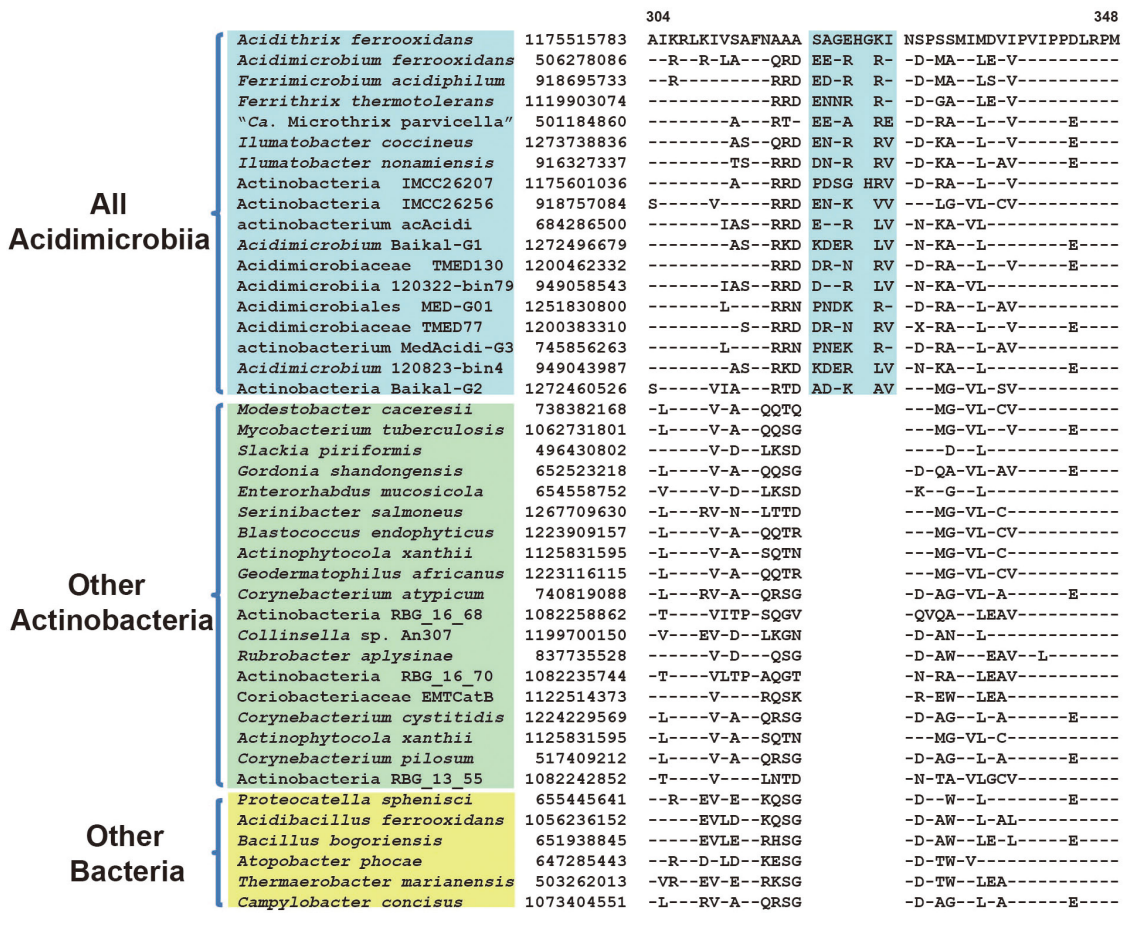

FIGURE 2 | Conserved signature indel (CSI) specific to all *Acidimicrobiia* species. Partial sequence alignment of the protein DNA-directed RNA polymerase subunit beta' showing a 6 ~ 8aa insertion in a conserved region that is specific for members of the class *Acidimicrobiia*. The dashes in this alignment as well as all other alignments indicate identity with the amino acid on the top line. The GenBank identification numbers of the protein sequences are shown, and the topmost numbers indicate the position of this sequence in the species shown on the top line. Information for other CSIs that are also specific to the class *Acidimicrobiia* are presented in **Table 1** and Supplementary Figures S1,S2.

MATERIALS AND METHODS

Phylogenetic Analysis

A phylogenetic tree for 20 genome-sequenced members of class *Acidimicrobiia* (Supplementary Table S1) was constructed based on the concatenation of 30 protein sequences, selected from a set of 92 single copy orthologous proteins (Na et al., 2018) and can be retrieved for the most assembled genomes of this class (Supplementary Table S2). Sequences from *Rubrobacter radiotolerans* was used as outgroup to root the tree. Multiple sequence alignments for each protein were performed using the Clustal X 2.1 program (Larkin et al., 2007) and concatenated to produce a single alignment file. The poorly aligned regions of the sequence alignment were removed by the Gblocks 0.91b program (Talavera and Castresana, 2007). The resulting alignment containing 7600 aligned amino acids was used for phylogenetic analysis. A maximum-likelihood (ML) tree was constructed by MEGA 6.0 (Tamura et al., 2013) with the Whelan and Goldman substitution model based on 1000 bootstrap replicates. Another ML tree including more assembled genomes from freshwater *Acidimicrobiia* was constructed based on concatenation of 10 ribosomal protein sequences (Supplementary Table S3). The method applied here was the same as done earlier and the final combined protein alignment used for phylogenetic analysis include 1814 amino acids.

A neighbor-joining (NJ) tree based on sequence alignment of 16S rRNA gene sequences was constructed for the representative strains of cultured *Acidimicrobiia* and some assembled genomes. Full length 16S rRNA sequences were retrieved from Ribosomal Database Project (Cole et al., 2014) or NCBI GenBank, and accession number of each 16S rRNA sequences were summarized in Supplementary Table S4. To root the tree, sequences from three *Rubrobacter* species were used as outgroup. The tree was constructed by MEGA 6.0 using the Kimura 2_parameter model with 1000 bootstrap replicates.

Identification of CSIs

Conserved signature indels were identified as previously described (Gupta, 2014; Zhang et al., 2016). Briefly, Blastp searches were carried out on all proteins from the genome of *A. ferrooxidans* DSM 10331 (Accession number NC_013124.1) (Clum et al., 2009) against all sequences in the GenBank non-redundant database. Multiple sequence alignments were created for homologs of all available *Acidimicrobiia* species and few other bacteria. These sequence alignments were inspected for any conserved insertions or deletions that were restricted to *Acidimicrobiia* species only and also flanked by at least 5–6 identical or conserved residues in the neighboring 30 ∼ 40 amino acids on each side. The indels, whose flanking regions were not conserved, were not further considered and removed. To assess the specificity of the identified indels, detailed BLASTp searches were carried out with a short sequence segment containing the indel and the flanking conserved regions (60–100 amino acids long) against the GenBank database. To further confirm that the identified signatures are restricted to *Acidimicrobiia* homologs, the top

TABLE 1 | Characteristic of CSIs specific to class *Acidimicrobiia* or its subclades.

Protein name	GI number[a]	Figure number	Indel size	Indel Position[b]	Specificity
DNA-directed RNA polymerase subunit beta'	1175515783	**Figure 2**	6 ∼ 8 aa ins[c]	304–348	*Acidimicrobiia*
Transcription termination factor Rho	501180362	Supplementary Figure S1	4 ∼ 6 aa ins	539–581	*Acidimicrobiia*
CCA tRNA nucleotidyltransferase	506279427	Supplementary Figure S2	1 aa del[c]	328–369	*Acidimicrobiia*
DNA-directed RNA polymerase subunit beta'	506278086	**Figure 3**	6 ∼ 7 aa ins	277–315	*Acidimicrobiia* except IMCC26256
DNA-directed RNA polymerase subunit beta'	506278086	Supplementary Figure S3	7 aa ins	99–139	*Acidimicrobiia* except IMCC26256
Mycothiol S-conjugate amidase	506279235	Supplementary Figure S4	1 aa ins	224–267	*Acidimicrobiia* except IMCC26256
Chlorite dismutase	506279459	Supplementary Figure S5	2 aa ins	117–157	*Acidimicrobiia* except IMCC26256
Polyribonucleotide nucleotidyltransferase	499277639	Supplementary Figure S6	1 aa ins	163–199	*Acidimicrobiia* except IMCC26256
Aspartate-semialdehyde dehydrogenase	502432944	**Figure 4**	3 aa del	200–236	*Acidimicrobiaceae*
Serine hydroxymethyltransferase	506279421	Supplementary Figure S7	6 ∼ 8 aa ins	200–236	*Acidimicrobiaceae*
Glutamate decarboxylase	502432855	**Figure 5**	1 aa del	253–289	*Acidimicrobium* and *Ferrimicrobium*
Pyridoxal 5′-phosphate synthase lyase subunit	506278863	Supplementary Figure S8	1 aa ins	183–222	*Acidimicrobium* and *Ferrimicrobium*
Pyridoxal phosphate-dependent aminotransferase	506279158	Supplementary Figure S9	1 aa ins	211–250	*Acidimicrobium* and *Ferrimicrobium*
Type IIA DNA topoisomerase subunit B	916327605	**Figure 6**	2 aa ins	178–210	Ilumatobacter cluster
multifunctional oxoglutarate decarboxylase	521046150	**Figure 7**	6 ∼ 7 aa ins	407–451	Microthrix cluster

[a]The GI number represents the GenBank identification number of the protein sequence from one Acidimicrobiia species that contain the specific CSI. [b]The indel region indicates the region of the protein where the described CSI is present. [c]ins, insertion; del, deletion.

500 BLAST hits with the highest similarity to the query sequence were examined for the presence or absence of these CSIs. Signature files were then created by two programs Sig_Create and Sig_Style (available from Gleans.net) (Gupta, 2014). Due to space limitation, indels containing sequence alignment in all figures and supplementary figures only include those that are found in all *Acidimicrobiia* sequences and few sequences from representative strains of other bacterial groups. It should also be noted that a number of CSIs and CSPs described here are also observed in the assembled genome of endosymbiont cyanobacterium TDX16 deposited by Hebei University of Technology (Accession number NDGV01000834.1, no publication available). We suspect that this unclassified "*Cyanobacteria*" genome assemble is not from DNA of pure culture but rather contamination from multiple bacterial strains since BLASTp searches of individual protein from this assembled genome returns top 5 hits from diverse bacteria, none of which belongs to *Cyanobacteria* and the most frequent best hits are from *Planctomycetes* species. Therefore, sequences from cyanobacterium TDX16 genome is not considered in our analysis.

Identification of CSPs

BLASTp searches were performed on individual protein from the genome of *A. ferrooxidans* DSM 10331 to identify proteins that are restricted to *Acidimicrobiia* species. These searches were executed against all sequences in the NCBI non-redundant database and the results were then examined manually for proteins with significant hits present only in *Acidimicrobiia* genomes following the same criteria as described in earlier work (Gao et al., 2006; Gao and Gupta, 2012b).

TABLE 2 | Conserved signature proteins (CSPs) that are uniquely found in the *Acidimicrobiia* and its subgroups.

Protein product	Specificity	Length	Function
WP_015799038.1	All *Acidimicrobiaceae*	226	Unknown
WP_041661805.1	All *Acidimicrobiaceae*	130	Unknown
WP_015798336.1	All *Acidimicrobiaceae*	99	Unknown
WP_015798164.1	All *Acidimicrobiaceae*	194	Unknown
WP_012226845.1	All *Acidimicrobiaceae*	185	Unknown
WP_015799164.1	*Acidimicrobiaceae*	268	Unknown
WP_015797785.1	*Acidimicrobiaceae*	71	Unknown
WP_041661722.1	*Acidimicrobiaceae*	191	Unknown
WP_041661793.1	*Acidimicrobiaceae*	351	Unknown
WP_015799101.1	*Acidimicrobiaceae*	217	Unknown
WP_015799193.1	*Acidimicrobiaceae*	138	Unknown
WP_015797967.1	*Acidimicrobiaceae*	157	Unknown
WP_015798062.1	*Acidimicrobiaceae*	124	Unknown
WP_041661604.1	*Acidimicrobium,Ferrimicrobium*	418	Unknown
WP_041661730.1	*Acidimicrobium,Ferrimicrobium*	140	Unknown
WP_041661653.1	*Acidimicrobium,Ferrimicrobium*	232	Unknown
WP_015799230.1	*Acidimicrobium,Ferrimicrobium*	139	Unknown
WP_015799176.1	*Acidimicrobium,Ferrimicrobium*	177	Unknown
WP_015799100.1	*Acidimicrobium,Ferrimicrobium*	255	Unknown
WP_015799084.1	*Acidimicrobium,Ferrimicrobium*	374	Unknown
WP_015798540.1	*Acidimicrobium,Ferrimicrobium*	145	Unknown
WP_015798531.1	*Acidimicrobium,Ferrimicrobium*	63	Unknown
WP_015798470.1	*Acidimicrobium,Ferrimicrobium*	743	Unknown
WP_015798187.1	*Acidimicrobium,Ferrimicrobium*	166	Unknown
WP_015797784.1	*Acidimicrobium,Ferrimicrobium*	200	Unknown
WP_015798639.1	*Acidimicrobium,Ferrimicrobium*	261	Unknown

RESULTS AND DISCUSSION

Phylogenetic Analysis of the Class *Acidimicrobiia* Based on Combined Protein Dataset and 16S rRNA Trees

Previous phylogenomic analyses of *Acidimicrobidae* considered two or three fully sequenced species (*A. ferrooxidans*, *M. parvicella,* and *Ilumatobacter coccineum*) and not more than six assembled genomes from metagenomic sequences (Hugerth et al., 2015; Mizuno et al., 2015). As a result, except confirming the association of these assembled genomes with *Acidimicrobiia*, no detailed evolutionary relationship among these uncultured species and known species can be concluded. In order to get a comprehensive overview of the phylogeny of class *Acidimicrobiia*, a phylogenetic tree was constructed for 7 completely sequenced species of this class and additional 13 assembled genomes from metagenomic data, whose genome information is nearly complete (Supplementary Table S1). The tree was constructed by ML analysis based on concatenation of 30 universally distributed orthologous protein sequences that are mainly involved in translation and transcription (Supplementary Table S2). To date, this tree represents the most comprehensive phylogenetic analysis of the class *Acidimicrobiia* (**Figure 1A**).

In this combined protein tree, an assembled genome "Actinobacteria bacterium IMCC26256" from freshwater sample forms the deepest branch, clearly separated from other *Acidimicrobiia* species. Four type species of the family *Acidimicrobiaceae* together with an assembled genome "Actinobacteria bacterium 21-73-9" from mine drainage metagenome form a well-defined cluster. Based on their branching pattern in the tree and their similar isolation environment, Actinobacteria bacterium 21-73-9 should be affiliated with the family *Acidimicrobiaceae*. The rest in the tree formed three distinctive clusters, and were named after the cultured type species if available found in each cluster namely the "Ilumatobacter Cluster" and "Microthrix Cluster." A third cluster comprised of assembled genomes from different marine metagenomes is named as "Marine Acidimicrobiia Cluster."

Since *Iamia*, one of the only two families within this class, do not have any genome sequenced and cannot be used as reference in the combined protein tree analysis, we constructed another phylogenetic tree based on 16S rRNA gene sequences (**Figure 1B**). In this analysis, we try to include all the named species of this class and two assembled genomes from the combined protein tree analysis since the full length 16S rRNA sequences cannot be retrieved from GenBank for the rest of assembled genomes. Consistent

with the combined protein tree, Actinobacteria bacterium IMCC26256 formed the deepest branch in the 16S rRNA gene tree of the class *Acidimicrobiia*. In addition, four clusters can be distinguished from each other with high bootstrap scores at each branch node, namely *Acidimicrobiaceae, Iamiaceae*, Microthrix Cluster, and Ilumatobacter Cluster. In both trees shown in **Figure 1**, M. parvicella branched with Actinobacteria bacterium IMCC26207 from freshwater metagenome, distinctive from *Acidimicrobiaceae* and *Iamiaceae*. A recent 16S rRNA analysis of identified *Acidimicrobiia* species and many uncultured environmental clones also indicated that strain IMCC26207 and M. parvicella form a clade clearly separated from *Acidimicrobiaceae* and *Iamiaceae* (Kim et al., 2017). In addition, although the current taxonomic outline placed *Ilumatobacter* within the *Acidimicrobiaceae*, our phylogenetic tree analysis based on both combined protein dataset and 16S rRNA sequences suggest that they are not monophyletic with *Acidimicrobiaceae* species. Hence, in view of the distinctive clustering pattern of Microthrix Cluster and Ilumatobacter Cluster from the two identified families, these two clusters may warrant assignment of novel families within this class. Certainly, this assignment requires additional molecular markers to support the monophyletic relationship of individual cluster.

Molecular Markers Specific for the Class *Acidimicrobiia*

The availability of complete and nearly complete assembled genomes from class *Acidimicrobiia* provide great resources to explore genomic characteristics that are unique to this class or subgroups within it. CSIs in genes/proteins sequences are important rare genetic changes for understanding bacterial phylogeny (Gao and Gupta, 2007; Gupta and Gao, 2009). The CSIs that serve as useful molecular markers are generally of defined size and their flanking residues are very conserved to ensure their reliability (Gupta, 2014, 2016). Because of the rarity and highly specific nature of such genetic changes, it is less likely that they could arise independently by either convergent or parallel evolution. Most likely, the genetic change responsible for a specific CSI occurred once in a common ancestor of the specific group of species and then passed on vertically to the various

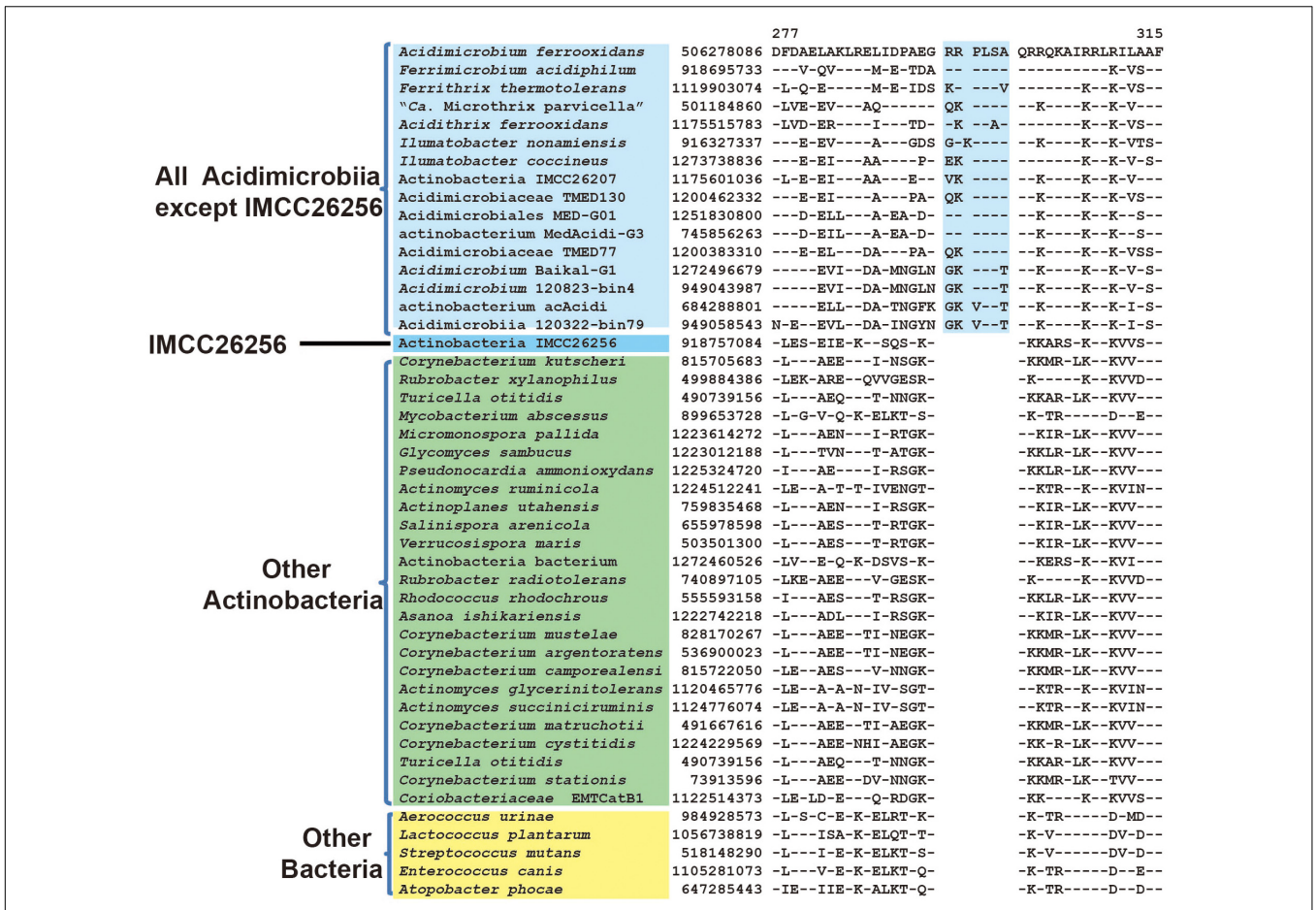

FIGURE 3 | Conserved signature indel specific to all *Acidimicrobiia* species except strain IMCC26256. Partial sequence alignment of DNA-directed RNA polymerase subunit beta' showing a 6 ~ 7 aa insertion that is specific for all *Acidimicrobiia* except Actinobacteria bacterium IMCC26256. Information for other CSIs specific for this clade are presented in **Table 1** and Supplementary Figures S3–S6.

descendants. Therefore, CSIs that are restricted to particular clade(s) have generally provided very good phylogenetic markers for evolutionary studies.

Comparative analyses of protein sequence alignment from species of class *Acidimicrobiia* and other bacterial groups led to the identification of three CSIs in different conserved proteins that are uniquely shared by all *Acidimicrobiia* species sequenced till date. As shown in **Figure 2**, a 6 ∼ 7 amino acids (aa) insertion in a highly conserved region of DNA-directed RNA polymerase subunit beta' was found to be specific to seven completely sequenced *Acidimicrobiia* species and assembled *Acidimicrobiia* genomes but not present in any other bacteria outside this class. Additionally, a 4 ∼ 6 aa insertion in transcription termination factor Rho and a 1 aa deletion in CCA tRNA nucleotidyltransferase were exclusively present in members of class *Acidimicrobiia* (Supplementary Figures S1, S2). For all these CSI containing proteins, homolog sequences of assembled genomes from metagenomic data that belong to class *Acidimicrobiia* were included in the alignment, and all including the deepest branch Actinobacteria bacterium IMCC26256, were found to contain the same CSIs. Thus, these three CSIs constituted distinctive characteristics of the class *Acidimicrobiia* and can be used as molecular markers to define and distinguish species belonging to this class.

In addition to the above CSIs, BLASTp searches of each protein from the genome of type species *A. ferrooxidans* DSM 10331 were carried out to search for CSPs that are unique to class *Acidimicrobiia*. Five proteins were found in almost all sequenced *Acidimicrobiia* genomes including the deepest branch IMCC26256 but not present in any other bacteria outside this class (**Table 2**). Similar to CSIs, these CSPs provide additional molecular markers for class *Acidimicrobiia*. These five proteins identified as hypothetical proteins with unknown function, and due to their specificity, functional studies of these proteins may reveal characteristics that are likely to be unique to members of this class.

Molecular Signatures for Some of the Subclades of *Acidimicrobiia*

As mentioned earlier, uncultivated Actinobacteria bacterium IMCC26256 formed the deepest branch in both phylogenetic trees based on combined protein dataset and 16S rRNA, which suggest that this species might be the earliest branch within known *Acidimicrobiia* species to date. In our analysis, we have identified five CSIs in four different widely distributed conserved proteins that are uniquely shared by all members of class *Acidimicrobiia* except strain IMCC26256. Although missing in IMCC26256 genome, these CSIs are not found in

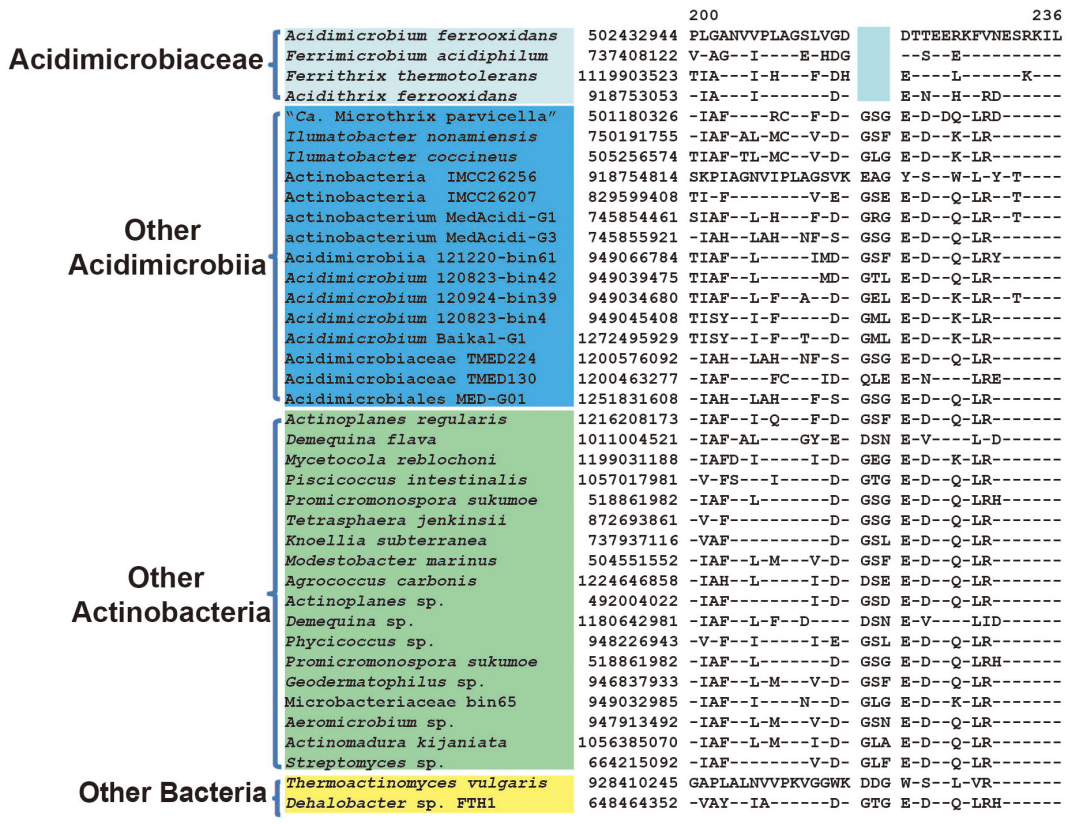

FIGURE 4 | Conserved signature indel specific to the family *Acidimicrobiaceae*. Partial alignment of the protein aspartate-semialdehyde dehydrogenase showing a 3 aa deletion that is specific for the family *Acidimicrobiaceae.* Information for other CSIs that are specific for the family *Acidimicrobiaceae* are presented in **Table 1** and Supplementary Figure S7.

any other non-*Acidimicrobiia* species. One example of these CSIs is shown in **Figure 3**. In a highly conserved region of DNA-directed RNA polymerase subunit beta', a 7 aa insert is unique to all *Acidimicrobiia* species but missing in IMCC26256 genome. Additional four CSIs showing similar specificity are presented in Supplementary Figures S3–S6. The absence of the identified CSIs in homologs of IMCC26256 genome are not due to lateral gene transfer since the best BLASTp hit of these CSIs containing proteins in IMCC26256 genome are homologous sequences of *Acidimicrobiia* species rather than other bacterial groups. There are two possible explanations for the presence of these five CSIs. First, these CSIs evolved in a common ancestor of all *Acidimicrobiia* but subsequently lost in IMCC26256 genome; second, these CSIs were introduced in the common ancestor of other *Acidimicrobiia* lineages after the branch of Actinobacteria bacterium IMCC26256. Although we cannot discriminate which of the two evolutionary scenarios account for the absence of these five CSIs in IMCC26256 genome, the unique presence of these CSIs in the rest of *Acidimicrobiia* species indicated that they constitute distinctive characteristics of this class.

Our analysis also identified two CSIs that are specifically shared by members of *Acidimicrobiaceae*, namely *A. ferrooxidans*, *F. acidiphilum*, *F. thermotolerans*, and *Acidithrix ferrooxidans*. These CSIs include a 3 aa deletion in aspartate-semialdehyde dehydrogenase (**Figure 4**) and a 6 ∼ 8 aa insertion in serine hydroxymethyltransferase (Supplementary Figure S7). They are exclusively present in the above four species belonging to the family *Acidimicrobiaceae* but not found in any other species. In addition, we also identified eight CSPs that are unique to these four species (**Table 2**). In contrast, no CSIs or CSPs were found that are uniquely shared by these species and *Ilumatobacter* species, which are currently assigned under the family *Acidimicrobiaceae*. These results suggest that most likely *Ilumatobacter* and the above four species are not monophyletic, consistent with the results from phylogenetic tree analysis. Therefore, *Ilumatobacter* should not be placed under the family *Acidimicrobiaceae*. Moreover, these two CSIs and eight CSPs provide distinctive molecular markers for the family *Acidimicrobiaceae* that can be used to define and delineate species belonging to this family.

Among members of the family *Acidimicrobiaceae*, *A. ferrooxidans*, and *F. acidiphilum* formed a clade in phylogenetic trees, thereby indicating a more closer relationship among the two than from the other species of this family (**Figure 1**). Supporting this relationship, we have identified

FIGURE 5 | Conserved signature indel specific to genera *Acidimicrobium* and *Ferrimicrobium*. A 1 aa deletion in the protein glutamate decarboxylase that is uniquely shared by *A. ferrooxidans* and *F. acidiphilum*. Information of other CSIs specific for this cluster is present in **Table 1**, and Supplementary Figures S8, S9.

three CSIs in different proteins that were present only in *A. ferrooxidans* and *F. acidiphilum*. These CSIs include: 1 aa deletion in glutamate decarboxylase (**Figure 5**), a 1 aa insertion in pyridoxal 5′-phosphate synthase lyase subunit PdxS, and a 1 aa insertion in pyridoxal phosphate-dependent aminotransferase (Supplementary Figures S8, S9). Besides, 13 CSPs were identified as unique proteins shared by both *A. ferrooxidans* and *F. acidiphilum* (**Table 2**).

As revealed by phylogenetic tree analysis, *Ilumatobacter* species branched together with assembled genome "actinobacterium acAcidi" from freshwater and "Acidimicrobium sp. Baikal-G1" from lake water. Two other phylogenetic trees based on 16S rRNA and combined protein dataset with limited *Acidimicrobiia* species also indicate that *Ilumatobacter* formed a cluster with acIV freshwater lineage (Hugerth et al., 2015; Mizuno et al., 2015). In our CSIs searches, we identified one CSI, a 2 aa insertion in type IIA DNA topoisomerase subunit B, that are uniquely shared by *Ilumatobacter* species and multiple assembled *Acidimicrobiia* genomes from freshwater samples (**Figure 6**). To further confirm the relationship of numbers of the genus *Ilumatobacter* and additional assembled freshwater *Acidimicrobiia* genomes, we constructed another phylogenetic tree based on ten ribosomal proteins for which sequences can be retrieved from the incomplete genomes of freshwater species (Supplementary Figure S10). Indeed, *Ilumatobacter* species formed a well-defined cluster with freshwater *Acidimicrobiia* species supported by high bootstrap score at the branch node. Taken together, the identified CSI and phylogenetic tree analysis suggested that members of the genus *Ilumatobacter* genus showed more close relationship with freshwater *Acidimicrobiia* lineage, and they should be assigned as an independent family different from that of the parent family *Acidimicrobiaceae*.

Microthrix parvicella was frequently retrieved from activated sludge wastewater treatment plants and had characteristic long unbranched filamentous morphology (Rossetti et al., 2005). Previous phylogenetic analyses indicated that M. parvicella belonged to *Acidimicrobiia* but formed a separate branch from

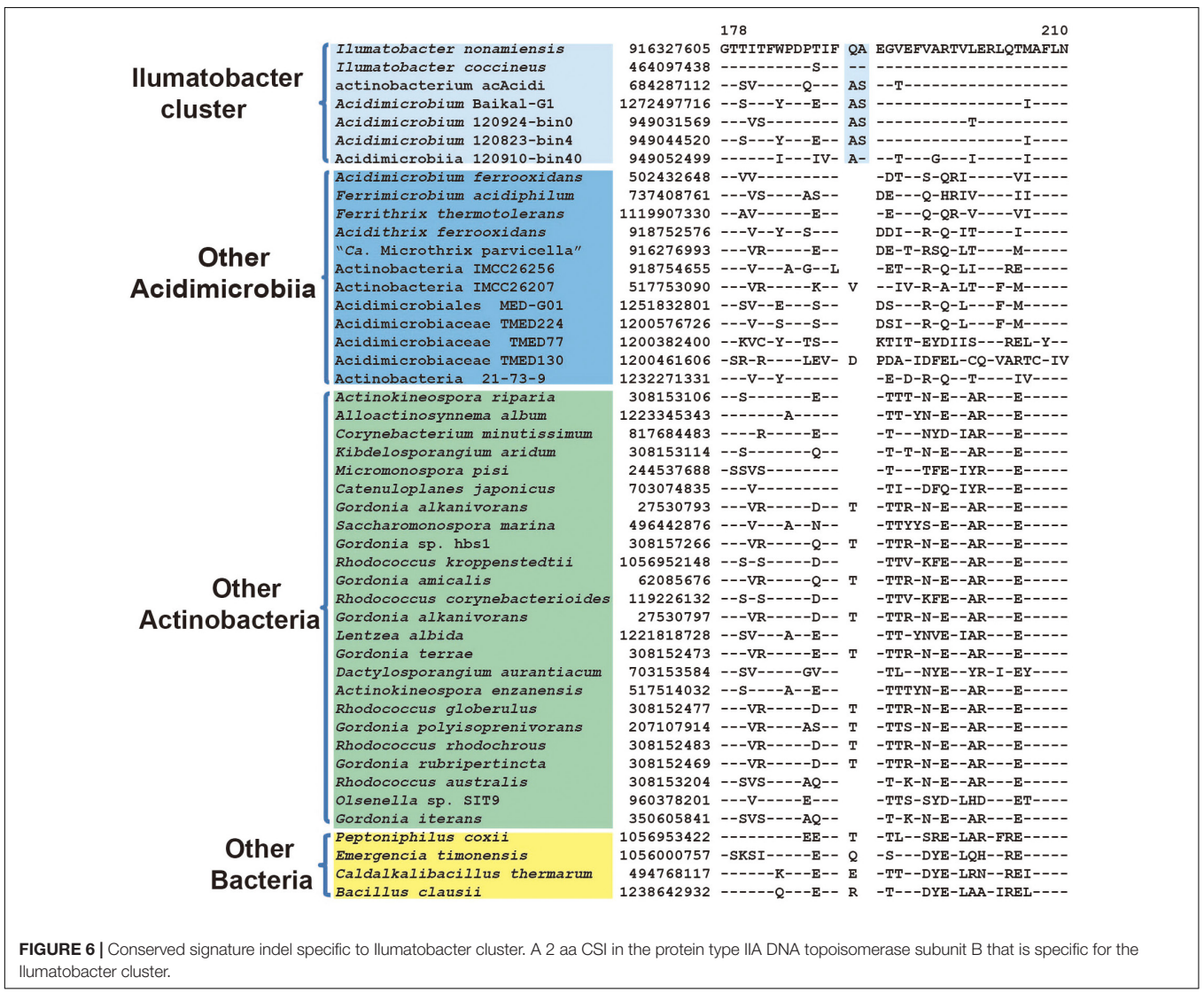

FIGURE 6 | Conserved signature indel specific to Ilumatobacter cluster. A 2 aa CSI in the protein type IIA DNA topoisomerase subunit B that is specific for the Ilumatobacter cluster.

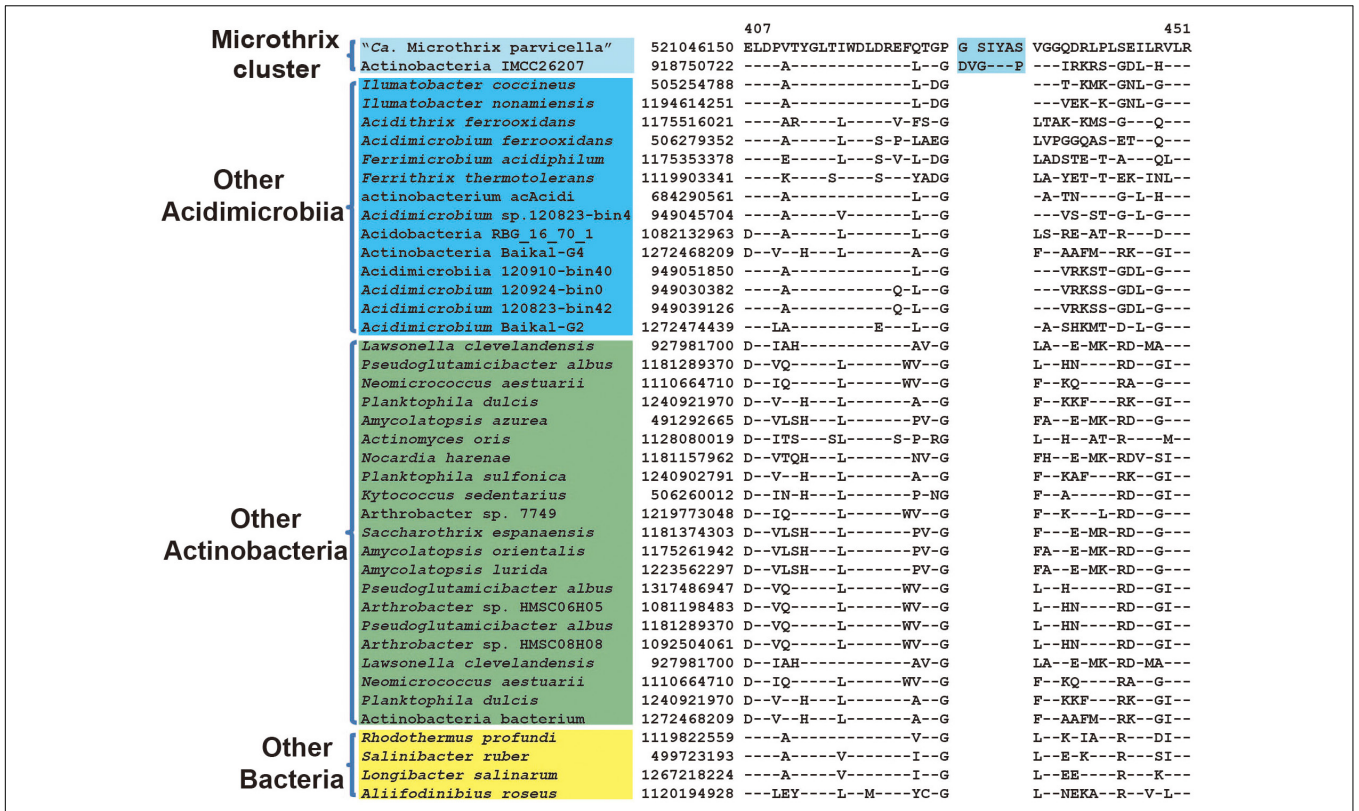

FIGURE 7 | Conserved signature indel specific to Microthrix cluster. Partial sequence alignment of multifunctional oxoglutarate decarboxylase showing a 6 ∼ 7aa insertion that is specific for the Microthrix cluster comprising of M. parvicella and strain IMCC26207.

the other type species of this class (Mizuno et al., 2015). In our phylogenetic trees based on both 16S rRNA and combined protein dataset (**Figure 1**), a recently published assembled genome of freshwater isolate, strain IMCC26207 with proposed species name "*Candidatus* Limnosphaera aquatica" (Kim et al., 2017), formed a distinctive clade with M. parvicella supported by high bootstrap score. This is the most closely related genome for M. parvicella reported to date (Kim et al., 2017). A 6 ∼ 7 aa insertion in a highly conserved region of multifunctional oxoglutarate decarboxylase was identified to be specific to M. parvicella and IMCC26207 (**Figure 7**), which provide a potential molecular marker for Microthrix cluster but awaits confirmation with more homologous sequences from closely related species.

CONCLUSION

In spite of the abundance of *Acidimicrobiia* in diverse aquatic habitats and their important role in biogeochemical cycling, presently there is limited study on the phylogeny of this deep branch class of the phylum *Actinobacteria*. The current taxonomic framework based on few cultivated species need to be updated to serve as guide map for increasing metagenomic investigation of species diversity of this class. In the present work, we have performed detailed phylogenomic analysis of sequenced *Acidimicrobiia* species and assembled genomes, which revealed three distinctive clusters namely Ilumatobacter cluster, Microthrix Cluster and Marine Acidic Cluster, in addition to the only two recognized families. More importantly, we have identified multiple CSIs in different proteins and CSPs that are specific to either class *Acidimicrobiia* or certain lineages within it. These genomic signature sequences can be used as molecular markers to define or delineate class *Acidimicrobiia* or its subgroups at higher taxonomic ranks, in addition to the current standard based on 16S rRNA alone. The class *Acidimicrobiia* currently only consists of two families-*Acidimicrobiaceae* and *Iamiaceae*, the latter of which has no genomes sequenced, and thus no CSIs/CSPs can be identified. In total, we have discovered two CSIs and eight CSPs specific for four species of the family *Acidimicrobiaceae*, but not present in species of the genera *Microthrix* and *Ilumatobacter* genera. Based on the clustering pattern of phylogenetic trees presented in **Figure 1** and the identified CSIs for Ilumatobacter Cluster and Microthrix Cluster, these two clades are not monophyletic with type species of *Acidimicrobiaceae* and should be defined as independent families. Furthermore, according to our phylogenomic analysis, *Acidimicrobiia* species from marine environments formed a cluster distinct from the other cultured type species, suggesting that these marine *Acidimicrobiia* might share unique genotypic and phenotypic characteristics. Hence, it is of much interest to identify molecular markers that are uniquely shared by marine *Acidimicrobiia* in the future.

Finally, both CSI-containing proteins and CSPs perform different functions in the bacterial cells, although the function of most of these molecular markers are unknown at present. Due to their specificity, the function of these CSIs and CSPs might be some characteristics unique to the specific taxon that contain them. For example, one *Actinobacteria*-specific CSP, ParJ (SCO1662), was functionally characterized as regulating the polymerization of ParA protein and affecting chromosome segregation and cell division during *Streptomyces* sporulation (Ditkowski et al., 2010). The *Acidimicrobiia*-specific CSIs and CSPs presented here provide novel targets for functional studies, which may reveal yet undiscovered features that are unique to species of this diverse class.

AUTHOR CONTRIBUTIONS

DH carried out comparative analyses of the *Acidimicrobiia* genomes to identify signatures reported here, DH and GC constructed the phylogenetic trees. BG and DH were responsible for the writing and editing of the manuscript. All of the work was carried out under the direction of BG.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00987/full#supplementary-material

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Revisiting the Taxonomic Status of the Biomedically and Industrially Important Genus *Amycolatopsis*, Using a Phylogenomic Approach

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Strains belonging to the genus *Amycolatopsis* are well known for the production of a number of important antimicrobials and other bioactive molecules. In this study, we have sequenced the genomes of five *Amycolatopsis* strains including *Amycolatopsis circi* DSM 45561^T, *Amycolatopsis palatopharyngis* DSM 44832^T and *Amycolatopsis thermalba* NRRL B-24845^T. The genome sequences were analyzed with 52 other publically available *Amycolatopsis* genomes, representing 34 species, and 12 representatives from related genera including *Saccharomonospora*, *Saccharopolyspora*, *Saccharothrix*, *Pseudonocardia* and *Thermobispora*. Based on the core genome phylogeny, *Amycolatopsis* strains were subdivided into four major clades and several singletons. The genus *Amycolatopsis* is homogeneous with only three strains noted to group with other genera. *Amycolatopsis halophila* YIM93223^T is quite distinct from other *Amycolatopsis* strains, both phylogenetically and taxonomically, and belongs to a distinct genus. In addition, *Amycolatopsis palatopharyngis* DSM 44832^T and *Amycolatopsis marina* CGMCC4 3568^T grouped in a clade with *Saccharomonospora* strains and showed similar taxogenomic differences to this genus as well as other *Amycolatopsis* strains. The study found a number of strains, particularly those identified as *Amycolatopsis orientalis*, whose incorrect identification could be resolved by taxogenomic analyses. Similarly, some unclassified strains could be assigned with species designations. The genome sequences of some strains that were independently sequenced by different laboratories were almost identical (99–100% average nucleotide and amino acid identities) consistent with them being the same strain, and confirming the reproducibility and robustness of genomic data. These analyses further demonstrate that whole genome sequencing can reliably resolve intra- and, inter-generic structures and should be incorporated into prokaryotic systematics.

Keywords: prokaryotic systematics, core genome, average nucleotide identity (ANI), average amino-acid identity (AAI), digital DNA-DNA hybridization, *Amycolatopsis*

INTRODUCTION

The genus *Amycolatopsis* is well known for the commercial production of multiple antibiotics, including the important broad spectrum antibiotics rifamycin and, vancomycin (Xu et al., 2014; Chen et al., 2016). These strains also have the potential to produce a number of other secondary metabolites and bioactive molecules (Adamek et al., 2018) and, can be exploited for a range of biotechnological applications (Davila Costa and Amoroso, 2014). The genus currently includes 72 validly named species (List of prokaryotic names with standing in nomenclature[1]) that may vary in their phenotypic and morphological characteristics (Tan and Goodfellow, 2015). *Amycolatopsis* strains commonly reside in arid, or hyper-arid soil and have chemo-organotrophic to facultatively autotrophic lifestyles (Tan and Goodfellow, 2015). However, some species have also been isolated from activated sludge, equine placentas and from clinical and plant material (Tan and Goodfellow, 2015). *Amycolatopsis* strains can be mesophilic or thermophilic, with a DNA GC content of 66–75 mol%. They can form branching substrate hyphae that fragment into square, or rod-shaped elements and carry aerial hyphae (Saintpierre-Bonaccio et al., 2005; Tan and Goodfellow, 2015).

A recent multilocus sequence analysis (MLSA) based on seven housekeeping genes (*atpD*, *clpB*, *gapA*, *gyrB*, *nuoD*, *pyrH* and *rpoB*) revealed the presence of four major groups of species within the genus, with some singletons (Adamek et al., 2018), whilst Sanchez-Hidalgo et al. (2018) described 11 subgroups in a 16 S rRNA analysis, and four major groupings based on an MLSA with four housekeeping genes (*atpD, dnaK, recA* and *rpoB*). The type strains of *Amycolatopsis halophila* and *Amycolatopsis marina* were found to be quite distinct from other *Amycolatopsis* strains. Although average nucleotide identities based on MUMmer (ANIm) supported the classification of these strains within the genus, the percentage of conserved proteins indicated that *A. halophila* might belong to a different genus (Adamek et al., 2018). A five gene MLSA (with *atpI*, *gyrA*, *ftsZ*, *secA* and *dnaK*) also indicated that *A. halophila* is more similar to members of the genus *Prauserella* than to *Amycolatopsis* species (Bose et al., 2016). Therefore, we have applied a more comprehensive phylogenetic and taxogenomic approach to the genus (Sangal et al., 2016) and analyzed 57 *Amycolatopsis* genome sequences belonging to 34 species, including five sequenced by us in this study. Twelve genome sequences representing other genera, including *Saccharomonospora*, *Saccharopolyspora*, *Saccharothrix*, *Pseudonocardia,* and *Thermobispora,* were included for the comparative analyses (**Supplementary Table 1**). This study highlights the subgeneric structure within the genus and supports the separation of *A. halophila* as a member of a different genus. In addition, it is clear that a number of strains have been misidentified and misclassified using traditional approaches. Some strains sequenced independently by different laboratories grouped together, suggesting that genome based approaches are more reliable and reproducible than techniques such as DNA–DNA hybridisation, which suffers from a lack of reproducibility and compatibility of results between different laboratories (Achtman and Wagner, 2008; Moore et al., 2010).

[1]http://www.bacterio.net/amycolatopsis.html

MATERIALS AND METHODS

Bacterial Strains and Genome Sequencing

Five strains, *Amycolatopsis circi* DSM 45561^T, *Amycolatopsis palatopharyngis* DSM 44832^T, *Amycolatopsis ruanii* 49.3a, *Amycolatopsis thermalba* 50.9 b and *A. thermalba* NRRL B-24845^T were grown in 5 ml Brain-Heart Infusion broth (Oxoid) at 28°C for 48 h. Genomic DNA from each strain was extracted from 1.5 ml culture using the UltraClean® Microbial DNA Isolation Kit (MoBio).

The genome sequencing was performed on an Illumina MiSeq instrument and the paired-end reads were assembled using SPAdes 3.9.0 (Bankevich et al., 2012). The draft genome sequences have been submitted to the DDBJ/EMBL/GenBank databases and are publicly available (**Supplementary Table 1**).

The genome sequences of 52 *Amycolatopsis* strains and 12 representative strains of related genera were obtained from GenBank (**Supplementary Table 1**). Two independent genome assemblies were available for seven *Amycolatopsis* strains in GenBank and both of them were included to test the reliability and reproducibility of the genomic data.

Computational Analyses

BLAST-based pairwise average nucleotide identity (ANIb) and pairwise fragment similarity scores (fragment size of 500-bp) were calculated from the nucleotide sequences using Jspecies (Richter and Rosselló-Móra, 2009) and GEGENEES (Agren et al., 2012), respectively. The genome sequences were annotated using Prokka (Seemann, 2014) and were compared using EDGAR (Blom et al., 2016) for calculation of the core- and pan- genomes and the number of genes shared within each phylogenetic cluster. Pairwise amino acid identity (AAI) was also calculated using EDGAR (Blom et al., 2016) and pairwise digital DNA–DNA hybridization values were calculated using GGDC 2.1 (Auch et al., 2010a,b). A maximum-likelihood (ML) tree was constructed from the concatenated protein sequences of the core genes after removing sites with missing data using the best-fit amino acid substitution model (LG + F + I + G4) with 100,000 SH-aLRT and 100,000 ultrafast bootstrap replicates using IQ-Tree (Nguyen et al., 2015). A Neighbor-Joining (NJ) tree was generated from the pairwise GGDC distance matrix using MEGA (Kumar et al., 2016). The tree was re-rooted on *Thermobispora bispora* DSM 43833^T. Pairwise percentage of conserved proteins (POCP) were calculated using the scripts, data_file_4.sh (Moose, 2017) and runPOCP.sh (Pantiukh and Grouzdev, 2017) that are based on the previously described approach (Qin et al., 2014).

RESULTS

Phylogenetic and Taxogenomic Groups Within the Genus

A total of 602 genes were conserved (core genome) across the 69 genomes, including the strains representing related genera (**Supplementary Table 1**). A ML tree generated from concatenated core proteins resolved *Amycolatopsis* strains, representing 34 species (including 29 type strains), into four major groups and several singletons (**Figure 1**). These groupings are consistent with both the MLSA based phylogenies albeit with minor exceptions (Adamek et al., 2018; Sanchez-Hidalgo et al., 2018). We have applied the same group designations as used by Adamek et al. (2018). Group A is the largest group with 19 isolates assigned to nine species, including two without formal species designations. Group B encompasses 16 isolates (nine species) while groups C and D are relatively smaller with eight isolates (6 species and 1 undefined) and seven isolates (four species and one undefined), respectively (**Figure 1**). *Amycolatopsis taiwanensis* DSM 45107^T, *Amycolatopsis sacchari* DSM 44468^T, *Amycolatopsis nigrescens* CSC17Ta 90^T and *Amycolatopsis xylanica* CPCC202699^T are present as singletons within the *Amycolatopsis* clade (**Figure 1**). These strains were also recovered as singletons in the MLSA analysis of Sanchez-Hidalgo et al. (2018), except for *A. xylanica* which was located at the periphery of the strains in our cluster A. Of the other singletons, *A. sacchari* DSM 44468^T is consistently associated with *A. dongchuanensis* in 16S rRNA gene trees [e.g. (Wang et al., 2018); group I in Sanchez-Hidalgo et al. (2018) and Tang et al. (2016)], whilst the *A. nigrescens* is probably closely related to *Amycolatopsis minnesotensis* (Tang et al., 2016; Sanchez-Hidalgo et al., 2018;Wang et al., 2018). Similarly, *A. taiwanensis* is consistently associated with *Amycolatopsis helveola* and *Amycolatopsis pigmentata* (Tang et al., 2016; Sanchez-Hidalgo et al., 2018; Wang et al., 2018). These associations suggest that these singletons may expand into species groups as more whole genomes become available.

Notably, *A. palatopharyngis* DSM 44832^T and *A. marina* CGMCC4 3568^T [16S and MLSA group G (Sanchez-Hidalgo et al., 2018)] formed a group that is more closely related to *Saccharomonospora* strains than to other members of *Amycolatopsis* while *A. halophila* YIM93223^T [16S group J (Sanchez-Hidalgo et al., 2018)] is quite distant to all other strains and forms a single member clade (**Figure 1**). Overall, group structure is also consistent with the NJ tree obtained from the BLAST-based genome-to-genome distances (**Supplementary Figure 1**).

The results of fragmented genome BLAST searches are consistent with the phylogenetic groupings with a mean fragmented BLAST similarity (FBS) within the *Amycolatopsis* groups varying from 35.9 ± 18.8 for Group C to 65.1 ± 17.0 for Group D (**Figure 2A**). The mean FBS score between the *Amycolatopsis* groups varied from 8.6 ± 1.8 to 11.4 ± 2.1 and between *Amycolatopsis* (excluding the three anomalous strains *A. palatopharyngis* DSM 44832^T, *A. marina* CGMCC4 3568^T, and *A. halophila* YIM93223^T) and strains of the other genera from 3.9 ± 1.6. Although the group of *A. palatopharyngis,* and *A. marina* is closely related to *Saccharomonospora* strains, the mean FBS score (5.3 ± 1.2) between these taxa was comparable to the score with the other *Amycolatopsis* strains (5.6 ± 1.1). Similarly, *A. halophila* was also equidistant from the *Amycolatopsis* strains (4.1 ± 1.1) and from the other genera (including *A. marina* and *A. palatopharyngis*; 3.5 ± 1.0).

Average nucleotide identity and AAI values also support the phylogenetic groupings (**Figures 2B,C**). Average ANIb values within and between the *Amycolatopsis* groups are >80% (85.94 ± 5.24 − 93.65 ± 3.41) and >75% (75.68 ± 0.69 − 76.97 ± 0.91), respectively. The strains of the other genera and the three anomalous strains shared lower ANIb values when compared with the *Amycolatopsis* strains (72.52 ± 1.52 and 72.77 ± 1.77, respectively). Similarly, average AAI values are >80% (86.69 ± 6.11 − 94.48 ± 3.06) within the *Amycolatopsis* groups and approximately 70%, or higher (70.99 ± 1.02 − 73.93 ± 1.98) between these groups. Average AAI values between *Amycolatopsis* strains and the three anomalous strains and the other genera are 67.54 ± 4.27 and 66.45 ± 3.70, respectively.

Pairwise dDDH values were also calculated for the dataset which varied between 36.2 ± 14.4 and 59.5 ± 19.7 within each *Amycolatopsis* group (**Figure 2D**). The average dDDH values ranged from 21.4 ± 0.5 to 22.2 ± 0.5 between the *Amycolatopsis* groups which is comparable to the values between the *Amycolatopsis* strains and the strains from the other genera (20.1 ± 0.7). This confirms that dDDH is useful in identifying strains belonging to the same species but has limited resolution at the intergeneric level.

A POCP value of 50% is often used as the genus boundary where two strains with more than 50% conserved proteins are considered to belong to the same genus (Qin et al., 2014). Consistent with this, pairwise POCP values among the *Amycolatopsis* strains (excluding anomalous strains) varied between 50.71 and 99.96% (67.38 ± 9.75; **Supplementary Table 2**). However, POCP could not resolve the status of the anomalous strains as *A. marina* and *A. palatopharyngis* type strains showed POCP values of 57.09 ± 2.95 and 58.53 ± 3.13 against the strains in the *Amycolatopsis* clade and the *Saccharomonospora* strains, respectively. POCP values between *A. halophila* YIM93223^T, and other *Amycolatopsis* strain was <50% except for some of the strains in group D, *A. marina* and *A. palatopharyngis* (**Supplementary Table 2**). All of these results suggest that 54 of the 57 *Amycolatopsis* strains belong to the same genus while *A. halophila* YIM93223^T should be assigned to a different genus. *A. palatopharyngis* DSM 44832^T and *A. marina* CGMCC4 3568^T grouped in the clade with *Saccharomonospora* strains; however, they showed comparable distances to both *Saccharomonospora* and *Amycolatopsis* strains. A larger analysis including more species from the genus *Saccharomonospora* and other representatives of the family *Pseudonocardiaceae* is required to clarify the status of these two species. However, it is notable that the recent phylogenomic study of Nouioui et al. (2018) recovered *A. marina* within the genus *Amycolatopsis*, although this study did not include *A. palatopharyngis*.

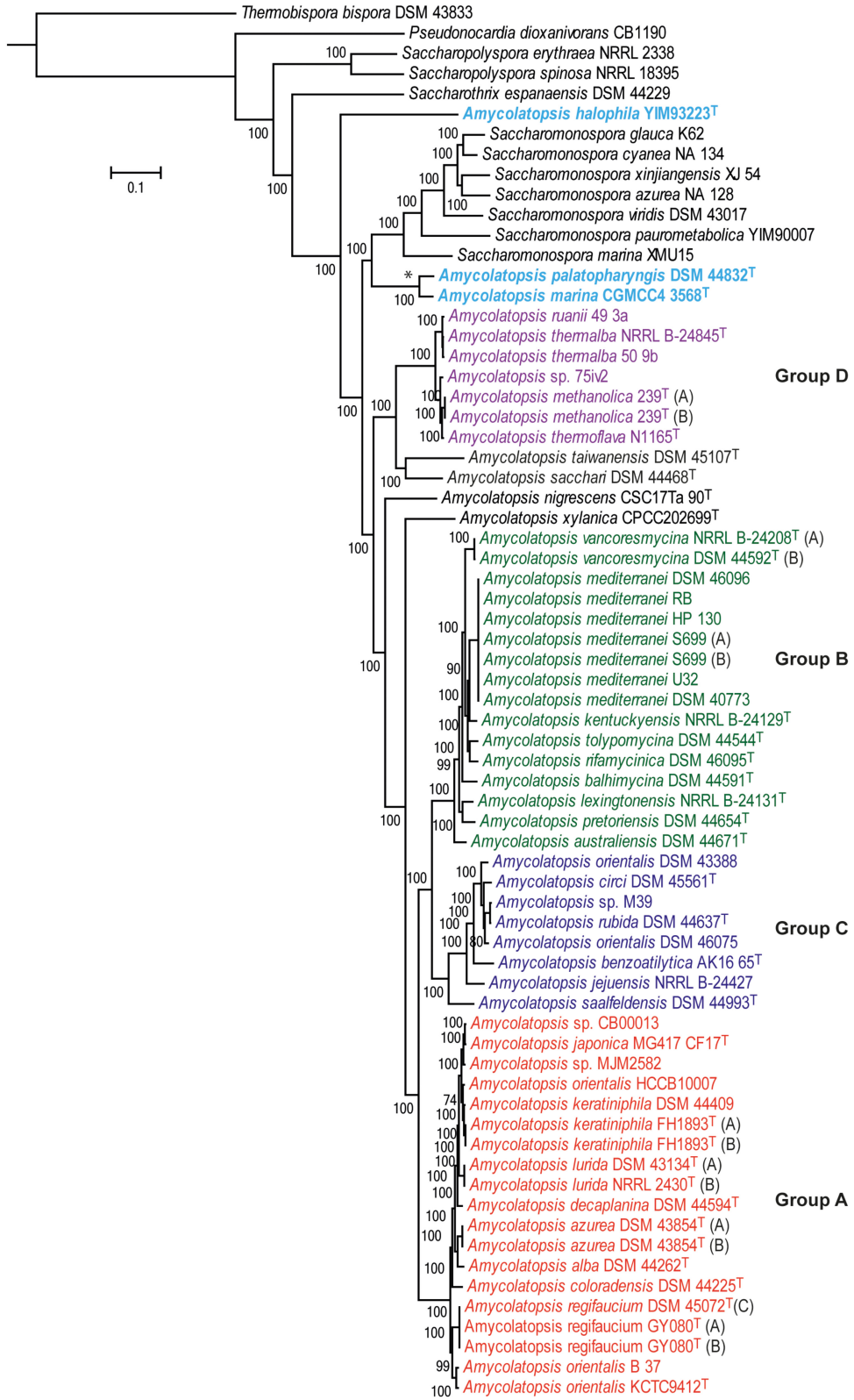

FIGURE 1 | A maximum-likelihood tree derived from concatenated core protein sequences. The scale bar represents amino acid substitutions per site. *Amycolatopsis* groups are labeled and shown in different colors. The clade with the two *Amycolatopsis* strains that are more close to *Saccharomonospora* strains is labeled with a star (*) sign.

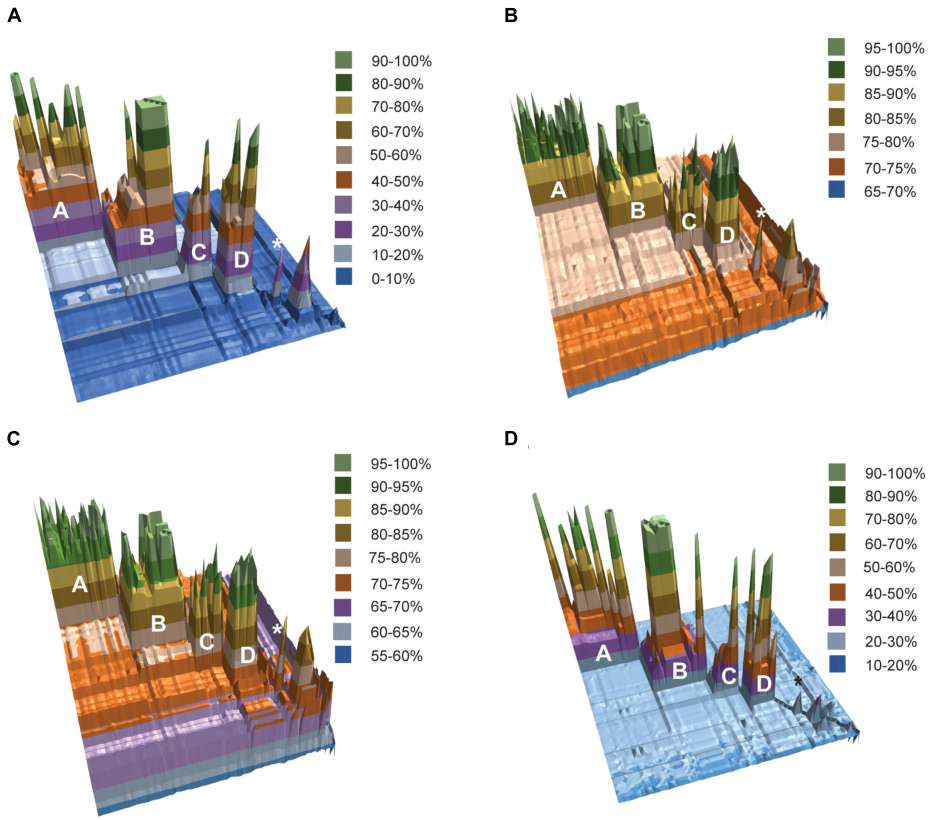

FIGURE 2 | 3D plots of pairwise matrices obtained from **(A)** fragmented BLAST searches (FBS values), **(B)** average nucleotide identities (ANIb values), **(C)** average amino-acid identities (AAI values) and **(D)** digital DNA-DNA hybridisation (dDDH values). The *Amycolatopsis* groups are labeled. The group of *A. palatopharyngis* DSM 44832^T and *A. marina* CGMCC4 3568^T is labeled with the star (*) sign.

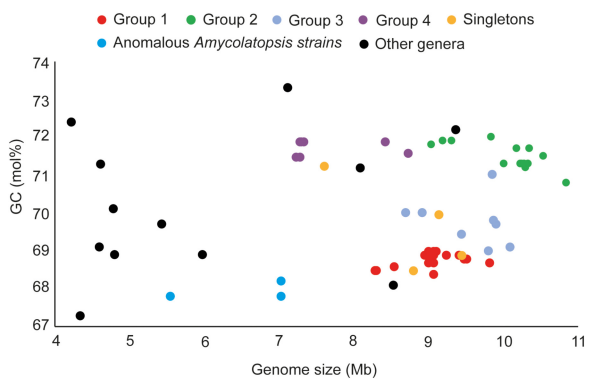

FIGURE 3 | Distribution of genome sizes and GC contents among the different phylogenetic groups.

Genomic Features of *Amycolatopsis* Strains Are Consistent With the Phylogenetic Groups

The genome sizes of the *Amycolatopsis* strains varied between 7 and 11 Mb with a GC content of 68 – 72 mol% (**Figure 3** and **Supplementary Table 1**). *A. halophila* YIM93223^T is clearly an outlier with a genome size of 5.6 Mb and a 67.8 mol% GC content. *Amycolatopsis salitolerans* consistently clusters with *A. halophila* in 16S rRNA gene trees (Guan et al., 2012; Tang et al., 2016; Sanchez-Hidalgo et al., 2018; Wang et al., 2018) and thus likely belongs to the same genus. *A. salitolerans* is reported to have a GC content of 66.4% (Guan et al., 2012); whilst this value needs confirmation from whole genome sequence data, it is similar to that for *A. halophila* YIM93223^T and is outwith the range for strains of *Amycolatopsis sensu stricto* (**Figure 3**). Cumulatively, these data are consistent with the recent proposal that *A. halophila* and *A. salitolerans* should be reclassified into the genus *Haloechinothrix* (Tang et al., 2010) as *Haloechinothrix halophila* comb. nov. and *Haloechinothrix salitolerans* comb. nov., respectively (Nouioui et al., 2018). *A. palatopharyngis* DSM 44832^T and *A. marina* CGMCC4 3568^T are also separable due to genomes sizes and GC content being at the lower end of the respective range for the *Amycolatopsis* strains (**Figure 3** and **Supplementary Table 1**). Although the range of the genome size and GC content is quite broad for *Amycolatopsis sensu stricto* strains, some patterns are visible at the group level. For instance, the strains in Group A have a genome size of 8.27 – 9.81 Mb, and a GC content of 68.5 – 69 mol%. The genomes of strains in Group B varied between 9.04 and 10.86 Mb with a GC content ranging from 70.8 to 72 mol%.

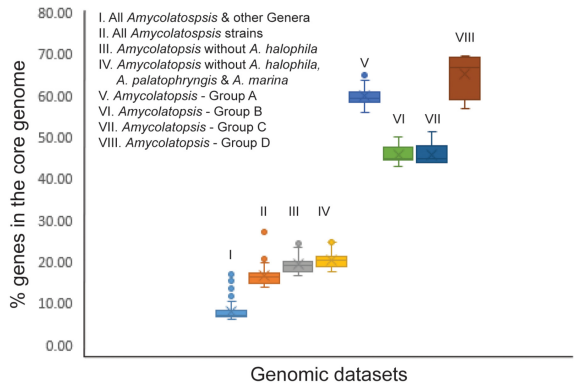

FIGURE 4 | A box plot showing the fraction of genes in the core genome for the different genomic datasets.

In order to establish whether the size of the core genome can help identify intergeneric boundaries, core genes were calculated for the entire dataset followed by a sequential removal of the other genera and the outlier strains *A. halophila*, *A. palatopharyngis* and *A. marina*. The core genome of the entire dataset contained 602 genes (6.1–16.9%). The number of annotated genes among the *Amycolatopsis* strains ranged between 5,098 and 9,890 after excluding those of other genera (**Supplementary Table 1**) with 1,382 of them (14.0 – 27.1%) shared by all of the strains, which compares well with the 1,212 core genes identified by Adamek et al. (2018). The number of core genes increased to 1,634 (17.5 – 24.7% of 6,606 – 9,890 genes) after removing the *A. halophila* strain from the dataset and only slightly to 1,739 genes (17.6 – 25%) after exclusion of the genomes of the *A. palatopharyngis* and *A. marina* strains. Therefore, the proportion of genes in the core genome may not be reliable for separating members of different genera due to overlap in values within a genus and when strain(s) from other genera are included (**Figure 4**). As expected, the number of core genes is much higher within each *Amycolatopsis* group (43.1 – 69.5%).

Species-Group A

Group A is the largest of the *Amycolatopsis* groups including nine type strains (**Figure 1** and **Supplementary Table 1**), many of which are known to produce different antibiotics and other bioactive molecules (Tan and Goodfellow, 2015). Average FBS, ANIb, and AAI values within this group are 55.9 ± 12.4, 90.76 ± 2.88, and 92.48 ± 2.56, respectively. dDDH values are consistent with the recognition of nine species, namely *Amycolatopsis alba*, *Amycolatopsis azurea*, *Amycolatopsis coloradensis*, *Amycolatopsis decaplanina*, *Amycolatopsis japonica*, *Amycolatopsis keratiniphila*, *Amycolatopsis lurida*, *Amycolatopsis orientalis,* and *Amycolatopsis regifaucium* (**Figure 1**). This analysis identified a potential case of misidentification of the industrial vancomycin producer *A. orientalis* strain HCCB10007 (Xu et al., 2014), which, as noted previously (Adamek et al., 2018; Sanchez-Hidalgo et al., 2018), is notably distant from *A. orientalis* KCTC9412^T (dDDH 38.4%; ANIb 50.8%) and shows a dDDH of 72% and ANIb of 96.4% with *A. keratiniphila* FH1893^T. Therefore, this strain should be reclassified as *A. keratiniphila*. Two strains, *Amycolatopsis* sp. CB00013 and *Amycolatopsis* sp. MJM252 could be assigned to *A. japonica* based on the dDDH cut-off value of 70%, as noted previously (Sanchez-Hidalgo et al., 2018). These assignments are also consistent with the previously suggested FBS score of >66.8% and ANIb and AAI values of ∼95% or higher (Konstantinidis and Tiedje, 2005b; Sangal et al., 2016). While *A. keratiniphila,* and *A. japonica* strains are clearly separated into two species by a dDDH value of ∼60%, other matrices suggest that they may belong to the same species.

Multiple assemblies were available in GenBank for four of the type strains in Group A (**Figure 1** and **Supplementary Table 1**) and all of them were included to check the reproducibility of the sequence data between different laboratories and the robustness of the approach. Pairwise FBS, ANIb, AAI, and dDDH values between these assembles were >99.7%, confirming the authenticity of these strains albeit with a minor exception. The dDDH value between the genome sequences of *A. keratiniphila* FH1893^T (Assembly accession numbers CA_900105855.1, and GCA_001953855.1) was 97.7%. These assemblies were submitted by two different laboratories and this variation may reflect the quality of the sequences. Other taxogenomic values are consistent with them being associated with the same strain (**Supplementary Tables 3A–D**).

Species-Group B

Group B is also quite diverse encompassing 16 of the 57 *Amycolatopsis* strains (**Figure 1** and **Supplementary Table 1**). The mean FBS (55.74 ± 21.36), ANIb (90.45 ± 4.72) and, AAI scores (91.23 ± 4.40) for this group are comparable to the values observed for Group A. All of the pairwise matrices support the presence of nine species including *Amycolatopsis australiensis*, *Amycolatopsis balhimycina*, *Amycolatopsis kentuckyensis*, *Amycolatopsis lexingtonensis*, *Amycolatopsis mediterranei*, *Amycolatopsis pretoriensis*, *Amycolatopsis rifamycinica*, *Amycolatopsis tolypomycina,* and *Amycolatopsis vancoresmycina* in the group, which is consistent with their taxonomic assignment (**Figure 1** and **Supplementary Table 1**). While most of the members of this clade are known to produce antibiotics and/or bioactive molecules (Davila Costa and Amoroso, 2014; Tan and Goodfellow, 2015), the *A. kentuckyensis*, *A. lexingtonensis,* and *A. pretoriensis* strains were isolated from lesions on equine placenta and may be pathogenic (Labeda et al., 2003). *A. vancoresmycina* DSM 44592^T has been sequenced independently by two laboratories and the high genomic similarities are consistent with them being the same strain (**Supplementary Tables 3A–D**). This group also includes seven *A. mediterranei* isolates that are grouped together with >97.7% dDDH, and 99.8–100% ANIb and AAI similarities, indicating it to be a highly homogeneous species with very limited genomic diversity.

Species-Group C

Group C shows a slightly higher diversity than was found in Groups A and B (FBS, 35.85 ± 18.83; ANIb, 85.94 ± 5.24;

AAI, 86.69 ± 6.11; **Supplementary Tables 3A–D**). Based on the taxogenomic matrices, eight strains within this group can be assigned to seven species (**Figure 1** and **Supplementary Table 1**). The assignment of five of these species, *A. circi*, *Amycolatopsis rubida*, *Amycolatopsis benzoatilytica*, *Amycolatopsis jejuensis* and *Amycolatopsis saalfeldensis* is consistent with the literature (Tan and Goodfellow, 2015). However, two strains, DSM 43388 and DSM 46075, identified as *A. orientalis*, are clearly misclassified. The type strain of *A. orientalis*, KCTC9412^T, clustered in Group A. Both these strains are quite distinct from each other as well as from other strains in Group C and belong to novel species based on the taxogenomic values. *Amycolatopsis* sp. strain M39 shares a dDDH value of 93.2, FBS value of 87.25 ± 0.17, ANIb 98.96 ± 0.10, and AAI of 99.1 with *A. rubida* DSM 44637^T suggesting that strain M39 strain should be assigned to this species, as noted previously (Sanchez-Hidalgo et al., 2018). Most of the strains in this group were isolated from diverse soil samples but with some exceptions, e.g., *A. benzoatilytica* strain AK16 65^T, was isolated from a patient with submandibular mycetoma (Tan and Goodfellow, 2015).

Species-Group D

The species group D is the smallest group with an average FBS score of 65.12 ± 16.95, an average ANIb value of 93.65 ± 3.41 and an average AAI score of 94.48 ± 3.06 (**Supplementary Tables 3A–D**). These strains were assigned to four species based on the pairwise dDDH values including *Amycolatopsis methanolica*, *A. thermalba*, *Amycolatopsis thermoflava* and a potentially novel species with *Amycolatopsis* sp. strain 75iv2 (**Supplementary Table 3D**). *A. ruanii* strain 49.3a probably should be reclassified as *A. thermalba* due to its high dDDH 93.3%, FBS 92.78 ± 0.12, ANIb 99.19 ± 0.04, and AAI value of 99.23 with the type strain of latter. Both genome sequences of the *A. methanolica* type strain show ≥99.9% ANIb and AAI similarities but their dDDH is slightly lower (98.3%). Similar to *A. keratiniphila*, this may be due to minor variations in the quality of genome sequences generated in different laboratories. Although, *Amycolatopsis* sp. strain 75iv2 and *A. thermoflava* strain N1165^T can be assigned to two different species based on the dDDH values, they share ANIb (95.08%) and AAI (96.42%) with each other that marginally overlap using the recommended cut-off for defining species (Konstantinidis and Tiedje, 2005a, 2007; Sangal et al., 2016).

DISCUSSION

Whole genome based approaches are now routinely used to resolve the structure of complex prokaryotic taxa (Kyrpides et al., 2014; Sangal et al., 2014, 2016; Schleifer et al., 2015; Sutcliffe, 2015; Mahato et al., 2017; Carro et al., 2018; Chun et al., 2018). In addition to the genome-based phylogenies, calculation of dDDH, ANI and AAI from the genomic sequences have become the gold standard for defining species with cut-off values of 70%, 95% and 95–96%, respectively (Konstantinidis and Tiedje, 2005a, 2007; Auch et al., 2010b). However, the data on separating prokaryotic genera are limited (Qin et al., 2014; Sangal et al., 2016). We have previously suggested that an ANI value of approximately 74.8% can be used to define genera and that FBS values of ∼66.8% and 6.9% can help identifying interspecific and intergeneric boundaries, respectively (Sangal et al., 2016). Using a combination of phylogenomic and taxogenomic approaches, we defined seven species groups in the genus *Rhodococcus* that were as distant from each other as from representatives of other genera (Sangal et al., 2016). In contrast, strains representing the genus *Micromonospora* were found to be monophyletic, consistent with their assignment to a single genus (Carro et al., 2018). In this study, we have extended this approach to the industrially and biomedically important genus, *Amycolatopsis*, and found that the majority of strains assigned to this taxon clustered on a single branch that was separated from the related genus *Saccharomonospora* (**Figure 1**). However, the type strains of *A. palatopharyngis* and *A. marina* clustered more closely to *Saccharomonospora* and the type strain of *A. halophila* formed a single member clade (**Figure 1**). The taxogenomic matrices are in agreement with the phylogenomic groupings (**Figure 2**) with minor exceptions. For example, the FBS and ANI values between *A. taiwanensis* strain DSM 45107^T and the other *Amycolatopsis* strains are slightly below the suggested cut-off values (**Supplementary Tables 3A,B**); however, this strains clustered close to group D within the *Amycolatopsis* clade and clearly belongs to this genus. Therefore, the suggested cut-off values should be considered a guide and used in combination with genome based phylogenies (Sangal et al., 2016).

The percentage of conserved proteins (POCP) has been used to define strains at the genus level with ≥50% proteins between a pair of strains with at least 50% alignable region and 40% sequence identity considered to indicate membership of the same genus (Qin et al., 2014). However, the status of the anomalous strains *A. marina* and *A. palatopharyngis* remains unresolved due to POCP values of > 50% both with the other *Amycolatopsis* strains as well as with the *Saccharomonospora* strains (**Supplementary Table S2**). As noted above, further analysis of a larger dataset is required not only to clarify the taxonomic status of *A. marina* and *A. palatopharyngis* but also that of the effectively named species *Amycolatopsis flava* which groups with them in 16S rRNA trees (Wei et al., 2015). In agreement with a previous study where POCP was applied to a slightly smaller set of *Amycolatopsis* genomes (Adamek et al., 2018), less than 50% proteins were conserved between *A. halophila* YIM93223^T and the majority of the *Amycolatopsis* strains but with some exceptions (**Supplementary Table S2**). These results are consistent with the classification of *A. halophila* into a different genus (Nouioui et al., 2018). We also applied a stringent approach to identify conserved proteins by calculating the core genome for the entire dataset with a sequential exclusion of genomes from members of other genera and anomalous *Amycolatopsis* strains (**Figure 4**). However, no clear correlation to core conserved proteins and intergeneric boundary could be identified from this analysis.

The majority of the *Amycolatopsis* strains clustered into four robust groups based on the phylogenomic and taxogenomic analyses (**Figures 1**, **2A–D**). The pan-genomic analyses identified

some genes that are conserved within each group but absent in other *Amycolatopsis* strains (**Supplementary Tables S4A–D**). 147 genes were specific to Group A, 114 genes to Group B, 54 genes to Group C and 244 genes to group D. A large proportion of these genes (42–59%) encode hypothetical proteins; however, some genes are annotated as transcriptional regulators, Sigma factors and some genes potentially belong to different biosynthetic gene clusters. Indeed, the biosynthetic potential to produce secondary metabolites varied between the *Amycolatopsis* groups with a strong correlation to the number of biosynthetic gene clusters (Adamek et al., 2018).

CONCLUSIONS

Amycolatopsis is a homogeneous genus where most strains conform to the phylogenomic and taxogenomic indices defined for intra-generic boundaries. In contrast, *A. palatopharyngis* DSM 44832^T and *A. marina* CGMCC4 3568^T formed a clade closer to *Saccharomonospora* strains with comparable taxogenomic distances between them and the other *Amycolatopsis* strains. We also show that genomic data are robust and reproducible between different laboratories and can help resolve cases of misclassification and misidentification. Some strains identified as *A. orientalis* should either be assigned to other species or to presumptive novel species. Genomic analyses also assigned some undefined strains to known species. These results provide further evidence that matrices derived from the whole genome sequencing data can provide a robust framework for prokaryotic systematics.

DATA ACCESSION

The genome sequence data from this study has been submitted to GenBank/DDBJ/EMBL databases and are publically available with the accession numbers given in **Supplementary Table S1**.

AUTHOR CONTRIBUTIONS

VS, MG, IS, and H-PK have designed the study. VS and GT carried out the experimental work. VS and JB analyzed the data. VS drafted the manuscript. All the authors provided intellectual inputs and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02281/full#supplementary-material

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Molecular Characterization of Plasmids Harbored by Actinomycetes Isolated From the Great Salt Plains of Oklahoma Using PFGE and Next Generation Whole Genome Sequencing

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One of the unique features of actinomycetes, especially the genus *Streptomyces*, is the presence of linear plasmids. These range in size from 12 to 600 kb, and are often termed mega-plasmids. While many of the genes involved in secondary metabolite production reside in clusters on the chromosome, several studies have identified biosynthetic clusters on large linear plasmids that produce important secondary metabolites, including antibiotics. In this study, Pulse Field Gel Electrophoresis (PFGE) was used to screen 176 actinomycete isolates for the presence of plasmids; these bacterial strains were previously isolated from the Great Salt Plains of Oklahoma. Seventy-eight of the 176 actinomycete isolates (44%) contained plasmids. Several strains contained more than one plasmid, accounting for a total of 109 plasmids. Ten isolates showed extrachromosomal DNA larger than 200 kb, thus falling into the category of mega-plasmids. A subset of plasmids from 55 isolates was treated with S1 nuclease to determine topology; all plasmids examined appeared to be linear and ranged from ∼55 to 400 kb. Eleven isolates were chosen for Whole Genome Next Generation Sequencing. From the 11 sequenced isolates, seven plasmids were partially assembled. While the majority of the genes identified on the plasmids coded for hypothetical proteins, others coded for general functions, stress response, and antibiotic and heavy metal resistance. Draft genome sequences of two mega-plasmid-bearing *Streptomyces* sp. strains, BF-3 and 4F, revealed the presence of genes involved in antibiotic production, antibiotic, and heavy metal resistance, osmoregulation, and stress response, which likely facilitate their survival in this extreme halophilic environment. To our knowledge, this is the first study to explore plasmids harbored by actinomycetes isolated from the Great Salt Plains of Oklahoma.

Keywords: actinomycetes, actinobacteria, linear plasmids, PFGE, Great Salt Plains, linear chromosome, next generation sequencing, extreme environments

INTRODUCTION

Actinomycetales, an order within the phylum Actinobacteria, represent a large and diverse group of gram-positive bacteria that are known for being soil inhabitants with a high G+C content (Nett et al., 2009). The G+C content ranges widely among free-living Actinobacteria, with streptomycetes encoding over 70% G+C, corynebacteria averaging 54%, and the pathogen *Tropheryma whipplei* containing less than 50% (Chater and Chandra, 2006). Based on sequencing, plasmids within Actinobacteria have an average G+C content of 64.5% (Shintani et al., 2015). Actinobacteria provide a rich source of natural antibiotics, secondary metabolites for industrial processes, and host-vector systems (Connell, 2001). Generally, actinomycetes are saprophytic bacteria that remain in a semi-dormant state for most of their life cycle. Growth of hyphae tends to occur only when there is a large supply of nutrients available leading to the rapid production of spores (Mayfield et al., 1972). Actinomycetes with a more complex life cycle and structure, such as *Nocardia, Actinoplanes, Micromonospora, Streptovericillium*, *Streptomyces,* and *Saccharopolyspora,* have linear chromosomes (Lin et al., 1993; Reeves et al., 1998; Redenbach et al., 2000). The complexity of the life cycle does not play a role in the presence of linear plasmids, which have been found in actinomycetes with either type of chromosome topology. While the majority of actinomycetes studied to date are found in terrestrial environments, these organisms have been identified in a wide range of extreme environments and marine locations. These bacteria occupy environments where the soil has a high salt concentration such as the Great Salt Plains of Oklahoma, Antarctica, and several marine locations (Hotta et al., 1980; Lam, 2006; Encheva-Malinova et al., 2014; Gad, 2014). Another variation seen in the growth pattern of actinomycetes is in the wide range of temperatures where they occur. This is evident by the variety of environments they inhabit, ranging from psychrophilic to mesophilic conditions, and their presence and role during the high temperatures associated with composting (Kertesz and Thai, 2018).

For many years, eukaryotic organisms were presumed to be the only organisms containing linear chromosomes and telomeres (Bendich and Drlica, 2000). However, Hinnebusch and Tilly (1993) demonstrated that the linear structure of chromosomes possessing telomere-like structures is far more prevalent in bacteria than originally assumed, and some bacterial taxa may have the ability to change structure between linear and circular chromosomal forms. Plasmids were originally thought to be restricted to a circular topology; however, the discovery of the linear plasmid pSLA2s in *Streptomyces rochei* (Hayakawa et al., 1979) was followed by the identification of linear plasmids in other actinomycetes. These linear plasmids range from 12-600 kb (Kinashi et al., 1987; Sakaguchi, 1990) and are often called mega-plasmids, while the linear chromosomes in actinomycetes range from 8 to 10 Mbp (Hopwood, 2006). Although many of the genes involved in secondary metabolite production reside in clusters on the chromosome (Hopwood, 2007), several studies have identified biosynthetic clusters on the large linear plasmids that produce important secondary metabolites (Novakova et al., 2013). Linear plasmids are generally associated with specific bacterial strains and their roles remain cryptic in the majority of prokaryotes.

There are several significant gene clusters known for producing antibiotics on large linear plasmids. These plasmids include pSLA2-L in *S. rochei* (produces lankacidin and lankamycin) (Kinashi et al., 1994; Suwa et al., 2000), SCP1 in *Streptomyces coelicolor* A3(2) (methylenomycin) (Kirby and Hopwood, 1977), and pPZG103 in *Streptomyces rimosus* (oxytetracycline) (Gravius et al., 1994; Pandza et al., 1998). *S. coelicolor* A3(2) contains the best-characterized linear plasmid in the genus. Although the majority of large linear plasmids have been isolated from *Streptomyces* spp., other actinomycetes, including *Nocardia*, *Rhodococcus*, and *Mycobacterium*, also contain large extrachromosomal material (Kalkus et al., 1993; Dabrock et al., 1994; Picardeau and Vincent, 1997), thus demonstrating the vast potential that actinomycetes may contribute to the understanding of linear genetic elements and secondary metabolite production.

With over 900 described species, *Streptomyces* is the largest and most widely-studied genus of actinomycetes due to its extensive range of secondary metabolites, which are important in medical, veterinary, industrial, and agricultural processes (Chen et al., 2002). *Streptomyces* spp. form the foundation for 60% of the antibiotics used today and play a key role in the exchange of antibiotic resistance genes among bacteria (Davies, 1994, 1997). *Streptomyces* is one of the most complex forms of actinomycetes, growing as branching hyphal filaments of mycelium and reproducing by aerial branches moving upwards in chains of spores similar to fungi (Chater, 2006). Another extensive group in the phylum Actinobacteria, the genus *Nocardiopsis* is known for its pathogenicity, environmental versatility, and production of many secondary metabolites including apoptolidin (Kim et al., 1997), lipopeptide biosurfactants (Gandhimathi et al., 2009), methylpendolmycin (Sun et al., 1991), thiopeptides (Engelhardt et al., 2010), griseusin D (Li et al., 2007), and napthospironone A (Ding et al., 2010). Based on a study of 17 *Nocardiopsis* species, it appears their wide dispersal and ability to adapt to diverse conditions is based on their dynamic genomes (Li et al., 2013).

The Great Salt Plains of Oklahoma consists of ∼11,000 acres (44.5 km^2); it originated from an inland sea that left salt deposits. During the dry season, a crust of white salt forms on the surface due as a Permian brine rises to the surface and evaporates. During heavy rainfall, the salt deposits are dissolved and form saline ponds. The Great Salt Plains is considered an extreme environment due to salinity, UV exposure, and high temperatures. In a previous study, two actinomycete genera, *Streptomyces* (recovered from both vegetated and barren regions) and *Nocardiopsis* (recovered from vegetated regions) comprised the majority of the 200 actinomycete strains isolated (Gad, 2014). Most isolates tolerated a relatively high salt concentration with a few special cases surviving at 15% salinity (Gad, 2014).

In the current study, Pulsed Field Gel Electrophoresis (PFGE) was used to screen *Streptomyces* and *Nocardiopsis* strains isolated from the Great Salt Plains of Oklahoma for the presence of plasmids. The topology of the plasmids (e.g., linear or circular) was determined by PFGE and S1 nuclease treatment.

Select plasmids were sequenced and analyzed along with their host chromosomes to identify potential secondary metabolites and stress tolerance genes that contribute to the survival of actinomycetes in the harsh environment of the Great Salt Plains.

MATERIALS AND METHODS

Plasmid Detection by PFGE

Actinomycete isolates ($n = 176$) were previously collected from the Great Salt Plains of Oklahoma (Gad, 2014) and screened for the presence of plasmids using PFGE. The isolates included 24 *Nocardiopsis* spp., 74 *Streptomyces* spp., and 78 unidentified actinomycete spp. *Salmonella* Braenderup and *Escherichia coli* strain NCTC 50192 were used as standardized markers for sizing plasmids. Biosafety procedures were adhered to during the use of *Salmonella* Branderup. The CDC PulseNet protocol was used for sample preparation of the standardized markers. A protocol for actinomycete samples was developed based on protocols for molecular subtyping of *E. coli* O157:H7 and *Staphylococcus aureus* by PFGE (Redenbach et al., 2000; Marineo et al., 2005).

Actinomycete isolates were grown on starch nitrate glycerol (SNG) agar before transferring to liquid yeast malt extract (YEME) medium for 72 h at 28°C with constant shaking. Liquid cultures were adjusted to 1.5–2.0 OD at A_{610} in TE buffer. Markers for *E. coli* and *Salmonella* were grown overnight on TSA at 37°C, and the TE cell suspensions were made by using cells directly from the plates adjusted to an OD 0.8–1.0 at A_{610}.

At least two plugs were made for each actinomycete isolate using disposable plug molds; plugs were also made for the molecular marker strains, *E. coli* NCTC 50192 and *Salmonella* Branderup. For the actinomycete samples, 400 μL of the cell suspension and 400 μL of 1% Seakem Gold Agarose were gently mixed using a pipette and heated to 50°C. Proteinase K (20 μL) was added to samples containing *E. coli* and *Salmonella*; afterwards suspensions were immediately transferred to plug molds and allowed to solidify at room temperature. Plugs were then transferred to tubes containing TE buffer and stored at 4°C until the cell lysis step.

Actinomycete agarose gel plugs were treated with 1 mg/mL of lysozyme for 2 h at 37°C with constant shaking. The lysozyme solution was then removed, and the plugs were then treated with 0.1 mg/mL of proteinase K at 54-55°C for 20–24 h. For *E. coli* strain NCTC 50192 and *S.* Braenderup, a cell lysis buffer (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% sarcosyl) was prepared according to the PulseNet protocol. After lysis, the wash step consisted of adding 10–15 mL sterile millipore water and incubating samples at 50–55°C for 30 min with constant shaking; the water was removed, and the process was repeated. Four additional washes were conducted using the same conditions in TE buffer. *E. coli* strain NCTC 50192 and *S.* Braenderup agarose plugs were

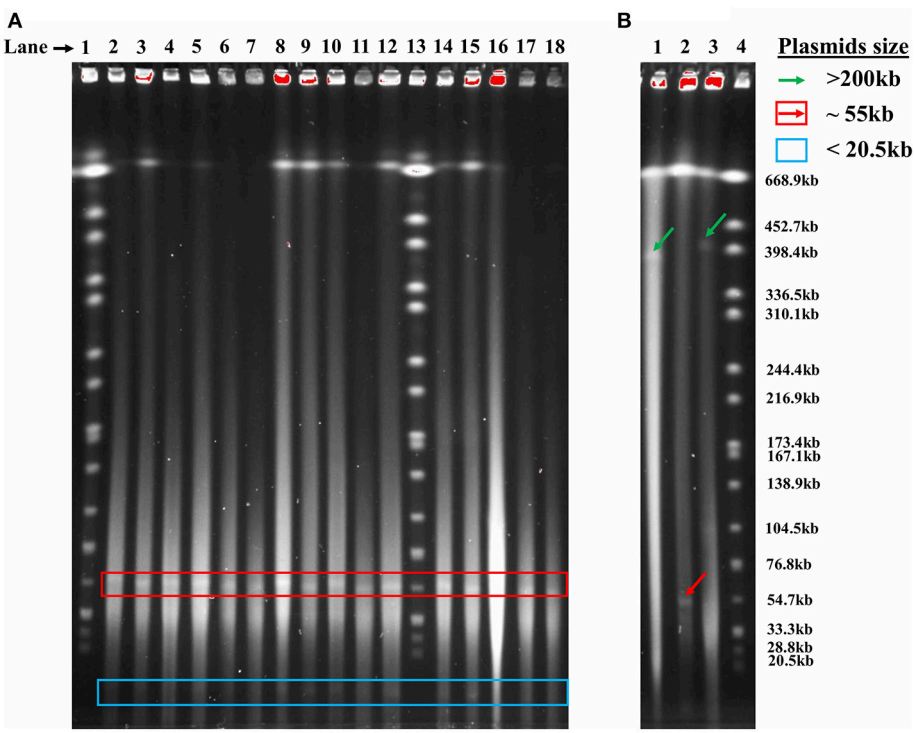

FIGURE 1 | Detection of plasmids in actinomycete isolates using PFGE. **(A)** Lanes (left to right): (1) *S.* Branderup, (2) SY-20-2-5%, (3) SY-20-3-5%, (4) SY-20-5-10%, (5) SY-20-6-10%, (6) SY-20-7-5%, (7) SY-20-8-0%, (8) SY-20-9-0%, (9) SY-21-1-0%, (10) SY-21-2-0%, (11) SY-22-1-5%, (12) SY-22-2-5%, (13) *Salmonella* Branderup, (14) SY-24-1-0%, (15) SY-25-1-0%, (16) SY-25-5-0%, (17) SY-25-9-0%, (18) SY-25-4-10%. **(B)** Lanes (left to right): (1) SW-19-2-1-0%, (2) SW-20-2-10%, (3) SW-23-2-10%, (4) *S.* Branderup.

TABLE 1 | Actinomycete isolates with plasmids with variable sizes (>200 kb shaded in gold, >70–200 Kb shaded in blue, and ≤70 kb shaded in green).

Isolates used in the study	Closest Identification/Accession no.	Plasmids and their sizes			
		>200 kb	>70–200 kb	20–70 kb	<20 kb
SGR-20-4-5%	Unidentified actinomycete isolate	405 kb	123 kb	–	–
SW-24-2-5%	Unidentified actinomycete isolate	400 kb	130 kb	–	–
SW-23-2-10%	gb|CP013142.1|*Streptomyces* sp. strain 4F	400 kb	–	–	–
SY-26-6-5%	gb|EU008821.1|*Streptomyces* sp. WBF11	398 kb	155 kb and 85 kb	–	–
SGR-27-6-0%	ref|NR_027223.1|*Streptomyces violaceolatus* strain DSM 40438	395 kb	148 kb and 136 kb	25 kb	–
SY-21-3-5%	gb|CP011522.1|*Streptomyces* sp. CFMR7	395 kb	140 kb	–	–
SGR-27-4-5%	gb|CP0131421.1|*Streptomyces* sp. strain 4F	395 kb	–	–	–
SY-27-3-5%	ref|NR_074635.1|*Nocardiopsis dassonvillei subsp. dassonvillei* DSM 43111 strain DSM 43111	380 kb	120 kb	–	–
SW-19-2-1-10%	gb|FJ982381.1|*Nocardiopsis sp.* FIRDI 009	380 kb	–	–	–
SGR-8-11-5%	gb|EU741237.1|*Streptomyces mutabilis* strain 13676F	312 kb	120 kb	–	–
SY-27-5-0%	gb|JN408756.1|*Streptomyces* sp. BF-3	–	193 kb	–	–
SGR-26-4-5%	gb|CP011522.1|*Streptomyces* sp. CFMR7	–	160 kb and 118 kb	65 kb	–
SY-27-2-10%	Unidentified actinomycete isolate	–	148 kb and 110 kb	–	–
SGR-26-1-0%	gb|GQ392058.1|*Streptomyces rochei* strain A-1	–	145 kb	–	–
SR-26-1-0%	emb|AM889494.1|*Streptomyces* sp. SHXFF-2	–	138 kb	–	–
SGR-14-4-0%	Unidentified actinomycete isolate	–	134 kb	–	–
SY-25-8-0%	gb|JX971566.1|*Streptomyces* sp. Cmuel-A718b	–	123 kb	–	–
SGR-27-4-0%	Unidentified actinomycete isolate	–	120 kb	52 kb	–
SGR-8-9-0%	gb|JN565291.1|*Streptomyces* sp. NPA1	–	120 kb	–	–
SGR-26-8-0%	ref|NR_025871.1|*Streptomyces tendae* strain ATCC 19812	–	120 kb	–	–
SGR-12-1-0%	Unidentified actinomycete isolate	–	120 kb	–	–
SGR-12-1'-0%	Unidentified actinomycete isolate	–	120 kb	–	–
SGR-20-1-0%	Unidentified actinomycete isolate	–	118 kb	–	–
SGR-19-4-0%	Unidentified actinomycete isolate	–	118 kb	–	–
SGR-20-5-0%	gb|JN565291.1|*Streptomyces* sp. NPA1	–	118 kb	–	–
SGR-24-7'-5%	Unidentified actinomycete isolate	–	116 kb	–	–
SGR-28-1'-4-0%	Unidentified actinomycete isolate	–	114 kb	–	–
SGN-19-7-10%	gb|EU741146.1|*Nocardiopsis* sp. 13647P	–	112 kb	–	–
SGR-26-2-0%	Unidentified actinomycete isolate	–	110 kb	–	<20 kb
SY-24-8-0%	gb|EU137870.1|*Streptomyces* sp. ALG5	–	108 kb	–	–
SW-25-1-5%	dbj|AB736324.1|*Nocardiopsis* sp. SI115	–	104 kb	–	–
SY-25-3-5%	gb|JX971566.1|*Streptomyces* sp. Cmuel-A718b	–	104 kb	–	–
SB-27-1-2-10%	gb|GU130105.1|*Streptomyces* sp. 0614149 clone 105T3	–	104 kb	31 kb	<20 kb
SW-20-1-5%	gb|EU410477.2|*Nocardiopsis* sp. HM7	–	102 kb	–	–
SGR-19-8-5%	Unidentified actinomycete isolate	–	102 kb	–	–
SR-14-1-0%	ref|NR_025292.1|*Streptomyces somaliensis* strain DSM 40738	–	95 kb	–	–
SR-20-1-0%	gb|DQ849079.1|*Streptomyces* sp. CPC3	–	95 kb	–	–
SGN-19-2-0%	emb|FR845719.1|*Streptomyces venezuelae* ATCC 10712	–	95 kb	–	–
SB-3-2-5%	Unidentified actinomycete isolate	–	95 kb	–	–
SB-11-14-5%	Unidentified actinomycete isolate	–	95 kb	–	–
SB-27-1-3-10%	gb|GU130105.1|*Streptomyces* sp. 0614149 clone 105T3	–	95 kb	–	–
SBa-24-1'-0%	Unidentified actinomycete isolate	–	95 kb	–	–
SGR-8-8-0%	gb|JN936839.1|*Streptomyces* sp. CPE1	–	93 kb	–	–
SGR-22-4-3-5%	gb|AF429400.1|*Streptomyces* sp. VTT E-99-1336 (B329)	–	91 kb	–	–

(Continued)

TABLE 1 | Continued

Isolates used in the study	Closest Identification/Accession no.	Plasmids and their sizes			
		>200 kb	>70–200 kb	20–70 kb	<20 kb
SGR-27-1-0%	ref|NR_042309.1|*Streptomyces violaceorubidus* strain LMG 20319	–	90 kb	60 kb	–
SY-20-1-5%	Unidentified actinomycete isolate	–	90 kb	–	–
SGR-26-6-0%	Unidentified actinomycete isolate	–	85 kb	–	–
SGR-27-7-0%	Unidentified actinomycete isolate	–	81 kb	–	–
SY-21-5-5%	gb|AF540000.2|*Nocardiopsis tangguensis*	–	80 kb	–	–
SGR-8-10-0%	gb|DQ092377.1|*Streptomyces* sp. A-37	–	78 kb	–	–
SY-24-5-5%	gb|AF540000.2|*Nocardiopsis tangguensis*	–	77 kb	–	–
SGR-30-1-0%	Unidentified actinomycete isolate	–	76 kb	–	–
SGR-25-7-0%	gb|JN627185.1|*Streptomyces variabilis* strain A4-3	–	–	70 kb and 35 kb	–
SGR-24-3-0%	gb|JN627185.1|*Streptomyces variabilis* strain A4-3	–	–	64 kb	–
SW-21-2-10%	gb|FJ267618.1| *Streptomyces* sp. 216802	–	–	52 kb	–
SW-23-1-10%		–	–	52 kb	–
SY-26-3-10%		–	–	48 kb	–
SY-27-4-5%	ref|NR_074635.1|*Nocardiopsis dassonvillei subsp. dassonvillei* DSM 43111 strain DSM 43111	–	–	44 kb	–
SY-27-5-5%		–	–	~55 kb	–
SY-20-2-5%	gb|GQ213972.1|*Streptomyces* sp. NEAU	–	–	~55 kb	–
SY-20-3-5%	gb|AY299633.1|*Nocardiopsis sp. YIM 80251*	–	–	~55 kb	–
SY-20-5-10%	ref|NR_025589.1| *Nocardiopsis aegyptia* strain SNG49	–	–	~55 kb	–
SY-20-6-10%		–	–	~55 kb	–
SY-20-7-5%	gb|EF114310.2|*Streptomyces* sp. B5W22-2	–	–	~55 kb	–
SY-20-8-0%	gb|JF727260.1|*Streptomyces* sp. LYG-1	–	–	~55 kb	–
SY-20-9-0%		–	–	~55 kb	<20 kb
SY-21-1-0%	gb|JF727260.1|*Streptomyces* sp. LYG-1	–	–	~55 kb	<20 kb
SY-21-2-0%	gb|FJ267618.1|*Streptomyces* sp. 216802	–	–	~55 kb	<20 kb
SY-22-1-5%	gb|HQ392468.1|*Streptomyces* sp. OE53	–	–	~55 kb	<20 kb
SY-22-3-5%		–	–	~55 kb	<20 kb
SY-24-1-0%		–	–	~55 kb	<20 kb
SY-25-1-0%		–	–	~55 kb	<20 kb
SY-25-9-10%	gb|AY297777.1|*Nocardiopsis* sp. 10030	–	–	~55 kb	–
SY-26-4-10%		–	–	~55 kb	–
SW-20-2-10%	gb|AF540000.2|*Nocardiopsis tangguensis*	–	–	~55 kb	<20 kb
SY-24-6-0%	gb|FJ486453.1|*Streptomyces tendae* strain HBUM174966	–	–	~55 kb	–
SGR-8-6-0%		–	–	–	<20 kb

washed as described above, except water washes were 15–20 min. Agarose plugs were stored in TE buffer at 4°C until PFGE.

S. Braenderup agarose plugs were dissected into small slices (3–4 slices per plug), and two slices were treated with 50 units of *Xba*I enzyme (Promega, Madison, WI, USA) for at least 4 h at 37°C. Similarly, *E. coli* agarose plugs were cut into smaller slices and digested with S1 nuclease at 37°C for 45–60 min (Barton et al., 1995). All plugs were dried to remove any remaining TE buffer or restriction enzymes, and then attached to the comb in a 1% TBE agarose gel with three size markers. The gel was run in 0.5X TBE buffer in a cooling module set at 14°C for 16 h using the PulseNet oxacillin-resistant *S. aureus* protocol. The gel was stained using ethidium bromide and imaged using the Bio-Rad Gel DOC^{TM} XR UV gel documentation system (BioRad, Hercules, CA, USA).

Plasmid Topology Determination by PFGE

To determine whether plasmids of different sizes were linear or circular, S1 nuclease was used to treat all plasmids and examined by PFGE to determine plasmid topology (Barton et al., 1995). The plugs, along with two *S.* Braenderup standards, a standardized molecular weight marker, and uncut control, were set on the gel comb for comparison of plasmids from 55 different isolates.

Next Generation Whole Genome DNA Sequencing and Sequence Analysis

Next generation sequencing was performed according to the Nextera® XT DNA Sample Preparation Guide (Illumina, San Diego, California, USA). Isolates were incubated in YEME broth for 72 h at 28°C with constant shaking. DNA was isolated with the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Redwood City, California, USA). Eleven isolates were sequenced based on their varying plasmid sizes.

The Qubit® dsDNA BR Assay Kit and Qubit® Fluorometer 2.0 were used to adjust DNA concentrations to 0.2 ng/μL. The manufacturer's directions were followed for the Nextera XT Library Prep and the Nextera XT Index Kits; briefly, the main steps included tagmentation of input DNA and the addition of adapter sequences for PCR. After PCR, the short library fragments were removed using AMPure XP beads and the size of the amplicon pool was adjusted to >500 bp. Lastly, equal volumes of the size-normalized libraries were pooled, diluted with hybridization buffer, and heat-denatured before sequencing.

The MiSeq® v2 Reagent Kit was used for sequencing as recommended by the manufacturer (Illumina). Raw sequence data was analyzed using the CLC Genomics Workbench (Qiagen, Redwood City, California, USA) and the Genome Finishing Module plugin at CLC genomics. Sequence data were also analyzed using BLAST to determine if specific contigs were chromosomal or plasmid DNA. After a rough assembly of contigs using CLC Genomic Workbench, the assembled sequences were submitted to RAST (Rapid Annotation using Subsystem Technology) (Aziz et al., 2008) for annotation.

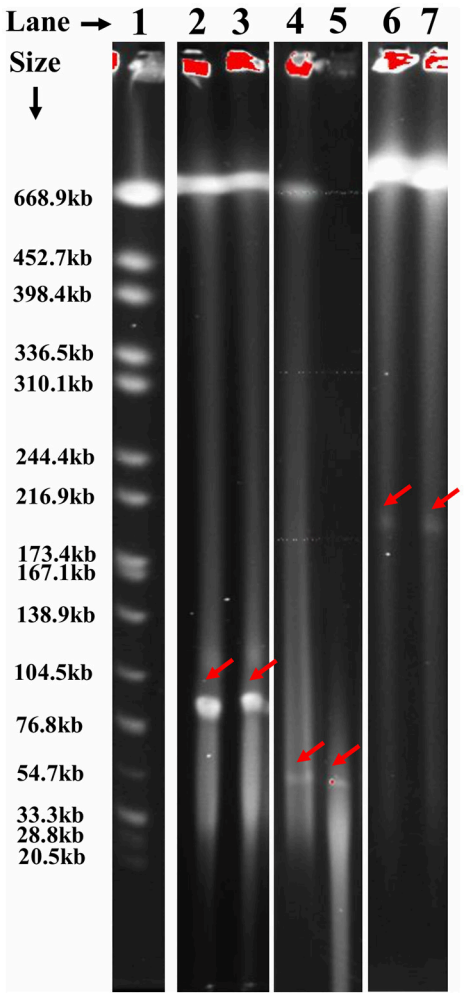

FIGURE 2 | Determination of plasmids topology in actinomycete isolates untreated and treated with S1 nuclease using PFGE. Lanes (left to right): (1) *S.* Branderup, (2) untreated SY-20-1-5%, (3) treated SY-20-1-5%, (4) untreated SW-20-2-10%, (5) treated SW-20-2-10%, (6) untreated SY-27-5-0%, (7) treated SY-27-5-0%. No difference was noticed in the migration of the tested plasmids before and after treatment with S1 nuclease which indicates that these plasmids are linear. Plasmids are indicated by red arrows.

GenBank Accession Numbers

The whole-genome shotgun sequences for *Streptomyces* sp. BF-3 (isolate SY-27-5-0%) and 4F (isolate SGR-27-4-5%) were deposited in GenBank under the accession nos. NACZ00000000 and NACY00000000, respectively. The draft genomes of the two isolates were previously reported as genome announcements (Cornell et al., 2018).

RESULTS

Screening Actinomycetes for Plasmids Using PFGE

The 176 actinomycete isolates previously collected from the Great Salt Plains of Oklahoma (Gad, 2014) were screened by PFGE, and 78 (44%) contained plasmids. A total of 109 unique plasmids were identified with the majority of isolates containing one plasmid. Isolate SGR-27-6-0% has four plasmids, whereas five isolates (SY-21-3-5%, SY-26-6-5%, SGR-26-4-5%, SGR-27-1-0%, and SB-27-1-2-10%) each contained three plasmids. PFGE revealed the presence of two large plasmids (398 kb), several 55 kb plasmids, and a smaller plasmid estimated to be <20.5 kb (**Figure 1**). As can be seen in **Figure 1**, many samples contained plasmids of similar sizes and were often collected from the same or neighboring soil sample areas, which is reflected by the first number in the name of each isolate.

The plasmids detected by PFGE screening could be grouped into three main size ranges, >200, 71–200, and ≤70 kb (**Table 1**). Several isolates were previously identified using 16S rDNA sequencing (Gad, 2014) and assigned to *Streptomyces* or *Nocardiopsis*. No association could be determined between genus and plasmid size due to the limited number of genera screened. Ten isolates (**Table 1**) had mega-plasmids larger than 200 kb; most of these were similar in size. However, an exception was SGR-8-11-5%, which contained a unique 312 kb plasmid. The majority of the plasmids ranged from 71 to 200 kb (**Table 1**), and many were ∼120 kb. Plasmids <70 kb were generally about 55 or <20 kb.

Determining the Topology of Detected Plasmids

Large plasmids (>50 Kb) in actinomycetes are often linear, while smaller plasmids tend to be circular (Kinashi et al., 1987;

Sakaguchi, 1990). To determine if the plasmids harbored by actinomycetes utilized in this study corresponded with previous findings, the topology of plasmids with a wide range of sizes (50–400 Kb) was examined. This was accomplished by S1 nuclease treatment of isolates followed by PFGE. Based on the screening of a subset of 55 isolates, all plasmids examined appeared to be linear (**Figure 2**). **Figure 2** shows representative isolates with various plasmid sizes when subjected to PFGE before and after treatment with S1 nuclease. No difference was noticed in the migration of the tested plasmids before and after treatment with S1 nuclease which indicates that these plasmids are linear.

Sequence Analysis of Plasmid-Bearing Isolates and Annotation of Selected Genes

Next generation sequencing was performed on a subset of eleven isolates (three *Nocardiopsis* spp. and eight *Streptomyces* spp.) containing plasmids of various sizes (**Table 2**). Assembled contigs were analyzed using BLASTN to determine whether sequences were plasmid-borne or chromosomal. Due to the limited data on actinomycete plasmids in GenBank, only partial plasmid sequences could be constructed for several isolates.

The partially constructed plasmid sequences were analyzed using RAST to identify genes of interest (**Table 3**). The majority of the plasmid sequences were classified as hypothetical proteins along with proteins used for general cellular functions. Some of the plasmid sequences showed the presence of genes involved in plasmid transfer, multidrug transport system, and synthesis of antibiotics (**Table 3**). Since very few genes could be defined, the draft Whole Genome Sequences of two isolates were used to construct their chromosomes to identify genes of interest. Genes of interest that were identified on these two chromosomes include those needed for survival in extreme environments, and genes involved in antibiotic biosynthesis/resistance and heavy metal resistance. The chromosome of isolate SGR-27-4-5%, *Streptomyces* sp. strain 4F, was constructed using the complete genome of *Streptomyces lividans* TK4 (GenBank accession no. CP009124). The chromosome of isolate SY-27-5-0%, *Streptomyces sp*. BF-3, was constructed using the complete *Streptomyces globisporus* C-1027 genome (accession no. CP013738). **Table 4** includes a list of notable genes encoded on the chromosome of *Streptomyces* sp. strain 4F, (isolate SGR-27-4-5%). The genes of interest on the chromosome of *Streptomyces* sp. BF-3, (isolate SY-27-5-0%) are listed in **Table 5**. Both isolates contained genes coding for universal stress proteins, resistance to cobalt-cadmium-zinc, arsenic, and tellurium, vitamin and antibiotic production, and antibiotic resistance. The draft genome sequences of *Streptomyces* sp. strains BF-3 (SY-27-5-0%) and 4F (SGR-27-4-5%) were previously reported as genome announcements and were 7,950,134 and 7,550,992 bp, respectively (Cornell et al., 2018).

DISCUSSION

Actinomycetes are ubiquitous in soils and generally occur as spores in soils until nutrients become available (Mayfield et al., 1972; Williams et al., 1984). Although prokaryotes were originally considered to contain only circular replicons, reports of linear DNA molecules are becoming more common (Hinnebusch and Tilly, 1993; Chen, 1996), particularly in actinomycetes where mega-plasmids are involved in antibiotic production (Kinashi and Shimaji, 1987; Kinashi et al., 1987). In this study, ten actinomycete isolates contained plasmids exceeding 200 kb. Previous studies indicate that plasmids in this size class are generally linear. To confirm this, 55 different isolates possessing one or more plasmids were treated with S1 nuclease and compared with an untreated isolate. The analysis showed that all plasmids $\geq$55 kb were linear. Results from next generation sequencing indicated that the plasmids and chromosomes in this study could be partially assembled with linear plasmid and chromosomal references. Thus, the results of PFGE analysis and next generation sequencing indicate a linear topology for all large plasmids (>50 Kb) and chromosomes in this study.

Microorganisms must adapt to their physiological environment to function optimally, and the ability to overcome stressful conditions is imperative. Actinomycete isolates from the Great Salt Plains possess a range of genes encoding universal stress response proteins and heat, cold and alkaline shock proteins. In general, these stress response genes are used for survival in harsh environmental conditions. In Oklahoma, temperatures can range from subzero to 37°C and higher,

TABLE 2 | Actinomycete isolates and plasmids used for next generation sequencing.

Isolate	Species	Plasmid 1 (kb)	Plasmid 2 (kb)	Plasmid 3 (kb)
SW-23-2-10%	*Streptomyces* sp.	400		
SY-21-3-5%	*Streptomyces* sp.	395	140	130
SGR-27-4-5%	*Streptomyces* sp.	395		
SW-19-2-1-10%	*Nocardiopsis* sp.	380		
SY-27-5-0%	*Streptomyces* sp.	193		
SGR-26-4-5%	*Streptomyces* sp.	160	118	65
SB-27-1-2-10%	*Streptomyces* sp.	104		
SGR-27-1-0%	*Streptomyces violaceorubidus*	90	75	60
SY-21-5-5%	*Nocardiopsis tangguensis*	80		
SY-21-2-0%	*Streptomyces* sp.	~55	<20	
SW-20-2-10%	*Nocardiopsis tangguensis*	~55	<20	

TABLE 3 | Genes present on plasmids harbored by actinomycetes sequenced in this study.

Isolate	Genes(s) Present	Gene Size (bp)*	Accession Number
SW-19-2-10%	Transcriptional regulator (2)**	681	WP_018521698.1
	Chromosome (plasmid) partitioning protein ParA and ParB	1248	WP_010064700.1
SW-20-2-10%	Transfer protein TraSA	1911	SBV06812.1
	Transcriptional regulator	249	WP_024127206.1
	Aclacinomycin oxidoreductase	1638	OCC08800.1
	Universal stress protein family	393	WP_032765329.1
	WhiB-like transcription regulator	906	WP_056704985.1
	Plasmid partitioning protein ParA	660	WP_012821767.1
SY-21-2-10%	Chromosome (plasmid) partitioning protein ParA and ParB	996	ODA70275.1
	RNA polymerase σ^{70}, ECF subfamily	660	WP_043441614.1
SY-21-5-5%	Transcriptional regulator, MerR family	399	WP_032765254.1
	RNA polymerase σ^{70}, ECF subfamily	591	EFE74770.1
	Chromosome (plasmid) partitioning protein ParA	996	ODA70275.1
SY-27-5-0%	ABC-type multidrug transport system ATPase and permease components (2)**	1746	SCF58705.1
	Transcriptional regulatory protein (7)**	489	SCF65435.1
SGR-27-1-0%	Putative integral membrane plasmid transfer protein	450	KOV81945.1
	Transcriptional regulator (3)**	741	WP_024888670.1
SGR-27-4-5%	Chromosome (plasmid) partitioning protein ParA and ParB	1155	WP_020945073.1
	Putative plasmid transfer protein, putative transfer protein SpdA, mobile element transfer protein SpdB	1356	WP_030399944.1
	Transcriptional regulator (6)**	369	WP_031169591.1
	Tellurium resistance protein TerD (2)**	576	WP_020114594.1
	Cold shock protein	204	WP_019324834.1
	Universal stress protein family (3)**	939	WP_030975022.1
	ABC-type multidrug transport system, permease component; putative drug exporters of the RND superfamily	783	SBU94049.1
SB-27-2-10%	Transcriptional regulator (3)**	831	WP_011039338.1
	Polymyxin synthetase PmxB	288	WP_005244517.1

**Gene size shown is representing the coding region.*

***Number of genes present are for genes of the same function. The size of the gene shown is for one representative gene with the corresponding accession number shown.*

which explains the importance of the temperature shock genes identified on plasmids and chromosomes in this study (**Tables 3**–**5**). The study location also exhibits extreme fluctuations in salinity, and actinomycetes are likely to survive due to the abundance of universal stress proteins and osmoregulatory proteins. Strategies for maintaining osmoregulation range from accumulating inorganic salts to controlling the concentration of compatible solutes. The chromosomes of the two actinomycetes characterized in this study contain genes encoding choline and glycine betaine aldehyde; these are precursors to glycine betaine, which protects plants from osmotic stress (**Tables 4**, **5**). The two actinomycete chromosomes also contain genes encoding ectoine biosynthetic proteins (**Tables 4**, **5**). Ectoine is an osmolyte found in many halophilic and halotolerant microorganisms including Actinobacteria (Malin and Ladpidot, 1996). Therefore, actinomycetes utilize a variety of methods to cope with the changing salt concentrations at the Great Salt Plains, such as the biosynthesis of solutes, uptake of osmoprotectants, and regulation of water transport.

Although metals play important roles in bacterial metabolism, the majority are nonessential and/or toxic. It has been hypothesized that the evolution of heavy metal resistance is ancient and was a response of prokaryotes to metal pollution. Resistance to metals is mediated through a variety of mechanisms that are borne on plasmids, transposons, and chromosomes. Bacteria have at least six metal resistance strategies, including intra- and extra-cellular sequestration, enzymatic detoxification, active transport efflux pumps, reduced sensitivity of cellular targets to metal ions, and exclusion by permeability barriers (Bruins et al., 2000). Metal resistance via plasmid-encoded genes evolved as a method to deal with toxic elements (Nies, 1992); related systems have been identified in the chromosome of *Bacillus* spp. and *E. coli* (Silver, 1996). Cadmium and zinc are chemically related, and cobalt is known to have affinity for zinc binding sites, supporting the idea that one efflux system is used to transport the cations of all three metals (Schneider-Bernlohr et al., 1988; Nies and Silver, 1989). In the current study, genes for cobalt-zinc-cadmium resistance

TABLE 4 | Chromosomally-encoded genes in *Streptomyces* sp. strain 4F (isolate SGR-27-4-5%).

Gene(s) of Interest	# of Genes Present**	Gene Size (bp)*	Accession Number
Mobile element protein (mobile element transfer protein SpdB)	11	402	WP_054100445
Universal stress protein family	4	906	WP_019327273
Osmoregulation	3	477	SBT92325
Ectoine biosynthesis and regulation	5	900	WP_052841845.1
Choline and betaine uptake and betaine biosynthesis	10	666	WP_020271580
Heat shock protein (heat shock DnaK gene cluster, extended: hypothetical radical SAM family enzyme, coproporphyrinogen III oxidase, oxygen-independent, translation termination factors, bacterial: tmRNA-binding protein SmpB, heat shock protein 60 family chaperone GroEL, heat shock protein 60 family co-chaperone GroEL, chaperone protein DnaK, chaperone protein DnaJ, heat shock protein GrpE, heat-inducible transcription repressor HrcA)	14	438	WP_043376487
Alkaline shock protein 23	3	498	WP_037909982
Cold shock protein (cold shock protein CspD, cold shock protein CspA, cold shock protein CspG)	4	204	WP_023586105
Cobalt-zinc-cadmium resistance: transcriptional regulator, MerR family (cobalt-zinc-cadmium resistance protein CzcD, DNA-binding heavy metal response regulator, probable Co/Zn/Cd efflux system membrane fusion protein)	7	1185	WP_043381403
Tellurium resistance protein (TerD, TerA)	6	456	WP_043371552
Arsenic resistance: arsenical pump-driving ATPase (arsenical-resistance protein ACR3, arsenical resistance operon repressor, arsenate reductase)	7	978	WP_043371752
Uptake of selenate and selenite: (various polyols ABC transporter, permease component 2, ATP-binding component 1)	3	783	WP_011030595
Lantibiotic ABC transporter	1	807	CCQ18686
Putative drug exporters of the RND superfamily	3	1398	WP_016327935
Salicylate and gentisate catabolism, salicylate ester degradation: salicylate hydroxylase (EC 1.14.13.1)	1	1716	WP_016327418
ABC-type multidrug transport system, permease component	1	783	WP_032757890
Multidrug resistance protein B	1	2097	WP_055419087
Tetracycline resistance, ribosome protection type, translation elongation factor G family (tetracycline resistance protein)	2	2128	WP_043381067
Vancomycin response regulator VanR	2	2808	WP_043374748
Protein involved in biosynthesis of mitomycin antibiotics/polyketide fumonisin	1	1164	WP_043377507
Putative penicillin acylase (penicillin amidase family protein)	2	2808	WP_043374748
Pyridoxin (vitamin B6) biosynthesis, thiamine biosynthesis: 1-deoxy-D-xylulose 5-phosphate synthase (pyridoxamine 5'-phosphate oxidase, pyridoxine biosynthesis glutamine amidotransferase, synthase subunit (EC 2.4.2.-), CBSS-1806.1.peg.1285, pyridoxine biosynthesis glutamine amidotransferase, glutaminase subunit (EC 2.4.2.-)	10	1929	WP_046249165
Folate biosynthesis: thymidylate synthase ThyX (EC 2.1.1.-) (dihydrofolate synthase, EC 6.3.2.12), folylpolyglutamate synthase (EC 6.3.2.17), folate biosynthesis cluster: FIG027937: secreted protein)	3	741	WP_043379203
Menaquinone via futalosine step 3 (gene SCO4494, often clustered with other genes in menaquinone via futalosine pathway, AsnC-family transcriptional regulator SCO4493 in menaquinone synthesis cluster, menaquinone via futalosine polyprenyltransferase (MenA homolog), UbiD family decarboxylase associated with menaquinone via futalosine)	5	1200	WP_043381260
Antibiotic biosynthesis monooxygenase	1	348	WP_043383968
Xylose utilization: Endo-1,4-β-xylanase A precursor (EC 3.2.1.8) (xylose utilization: α-xylosidase, xylulose kinase, xylose-responsive transcription regulator, ROK family, possible alpha-xyloside ABC transporter, ATP-binding component, possible alpha-xyloside ABC transporter, permease component)	7	1389	WP_030969144.1
Chitin and N-acetylglucosamine utilization: chitinase	1	1812	WP_030971667

*****Gene size shown is representing the coding region.*

***Number of genes present are for genes of the same function. The size of the gene shown is for one representative gene with the corresponding accession number shown.*

TABLE 5 | Chromosomally-encoded genes in Streptomyces sp. BF-3 (isolate SY-27-5-0%).

Gene(s) of Interest	# of Genes Present**	Gene Size (bp)*	Accession Number
Mobile protein element	14	414	WP_032761331
Osmoregulation	3	846	SBU96315
Ectoine biosynthesis and regulation	5	894	WP_032761401
Choline and betaine uptake and betaine biosynthesis	10	2622	WP_010070215
Universal stress protein	9	864	WP_030329972
Alkaline shock protein 23	5	543	WP_032767011
Cold shock, CspA family of proteins: cold shock proteins CspA and CspC	5	203	WP_003967102
Heat shock protein 60 family co-chaperone GroES	2	258	WP_003966899
Arsenic resistance: arsenic efflux pump protein, pump-driving ATPase, operon repressor, protein ACR3	8	1158	AGK80249
Tellurium resistance protein (TerD and TerA)	9	456	WP_006125865
Cobalt-zinc-cadmium resistance: Transcriptional regulator, MerR family, probable Co/Zn/Cd efflux system membrane fusion protein, DNA-binding heavy metal response regulator, cobalt-zinc-cadmium resistance protein CzcD	8	345	WP_032775780
Copper resistance protein, multicopper oxidase, protein D, copper-translocating P-type ATPase	5	1541	ESU48807
Tetracycline resistance protein	1	1257	WP_032766138
Phosphotransferase (aminonucleoside antibiotic resistance)	1	564	WP_032779818
Putative bicyclomycin resistance protein	1	1335	WP_032761820
Cobalamin synthesis: cobalamin synthase, threonine kinase (B12 biosynthesis), nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase, cobalt-precorrin-4 C11-methyltransferase	6	783	EGE44956
Folate biosynthesis: dihydrofolate synthase (EC 6.3.2.12), folylpolyglutamate synthase (EC 6.3.2.17), thymidylate synthase ThyX	9	1521	WP_032779143
Pyridoxin (vitamin B6) biosynthesis: predicted transcriptional regulator of pyridoxine metabolism, pyridoxamine 5'-phosphate oxidase (EC 1.4.3.5), pyridoxine biosynthesis glutamine amidotransferase, synthase subunit and glutaminase subunit	7	1182	WP_032778505
Putative toxic cation resistance protein	1	768	WP_050505692
RND multidrug efflux transporter; acriflavin resistance protein	1	3156	WP_010057790
Phosphotransferase (aminonucleoside antibiotic resistance)	1	564	WP_032779818
Drug resistance transporter, EmrB/QacA family	1	1676	WP_010059785
Penicillin amidase precursor (EC 3.5.1.11), putative penicillin-binding protein, β-lactamase class C and other penicillin binding proteins	3	2745	WP_032781722
Streptothricin resistance: streptothricin acetyltransferase, *Streptomyces lavendulae* type	1	567	WP_032776846

*****Gene size shown is representing the coding region.*

***Number of genes present are for genes of the same function. The size of the gene shown is for one representative gene with the corresponding accession number shown.*

were present and chromosomally-encoded in both actinomycete strains (**Tables 4**, **5**).

The chromosomes of the two actinomycetes studied herein also encode arsenic and tellurium resistance. Tellurium resistance genes were also identified on plasmids sequenced in this study (**Table 3**). Tellurium is toxic to most bacteria and its resistance is frequently associated with resistance to arsenic, mercury, or silver compounds (Summers and Jacoby, 1977). Arsenic is one of the most abundant toxic metals in the environment and originates from both geochemical and anthropogenic sources (Mukhopadhyay et al., 2002). Arsenate is structural analog of phosphate; it can enter the cell using the phosphate transport system and interfere with phosphorylation reactions. In the present study, the genes encoding tellurium and arsenic resistance were chromosomally-encoded in the two *Streptomyces* spp. (**Tables 4**, **5**). Genes for the uptake of selenate and selenite were also present on the linear chromosome of *Streptomyces* sp. strain 4F. The Great Salt Plains Lake is the only known area in the world where selenite crystals have been documented. This occurs when concentrated saline water combines with gypsum, thus promoting crystal growth with an hourglass shaped sand inclusion. Tellurite and selenite are chemically related to sulfate, and Scala and Williams (1963) proposed that these compounds could be potentially reduced by the sulfate pathway. Furthermore, tellurite may have the ability to replace sulfur in a number of cellular functions (Summers and Jacoby, 1977), leading to detrimental effects on bacterial cells.

Copper resistance genes were detected in *Streptomyces* sp. BF3 but not in *Streptomyces* sp. strain 4F (**Table 4**, **5**). The *cop* resistance operon has been well-characterized in the chromosome of the gram-positive bacterium *Enterococcus hirae* and in the plasmids of *Pseudomonas* (Cooksey, 1994), *Xanthomonas* (Lee et al., 1994), and *E. coli* (Brown et al., 1994, 1995). Based on a contamination study at the Salt Plains National Wildlife Refuge from 1990-2001, there were detectable levels of selenium and copper in water, sediment, and fish (Martin, 2002). Cadmium and zinc were in water samples, while other researchers found arsenic, cadmium, and zinc in sediments (Logan and Morgan, 1990; Persaud et al., 1993). The occurrence of these elements in the water and sediments of the Great Salt Plains explains the presence of the varied heavy metal resistance systems encoded by the two *Streptomyces* spp.

Many of the antibiotics produced commercially today are produced by *Streptomyces* and related bacteria, which encode genes for both antibiotic biosynthesis and resistance. With respect to antibiotic resistance, both *Streptomyces* spp. in this study had chromosomal genes conferring resistance to tetracycline, bicyclomycin, streptothricin, as well as drug exporters and multidrug transport systems. Multidrug transport system and drug exporters were also found on plasmids sequenced in this study (**Table 3**). While tetracycline and bicyclomycin are broad-spectrum antibiotics, streptothricin is not as commonly used but has the ability to inhibit prokaryotic protein biosynthesis. The vancomycin response regulator VanR, which was detected in the sequenced isolates, is part of the two-component signal transduction system, VanRS (Hutchings et al., 2006). Multi-drug resistant bacteria are increasingly common and such resistance generally occurs due to the accumulation of transferable resistance genes encoded by transposons or plasmids or is mediated by multidrug efflux pumps that transport multiple compounds (Nikadio, 2009). It is known that a major source of resistance to particular drugs, especially tetracycline, is from multidrug efflux pumps. A study by D'Costa et al. (2006) examined antibiotic resistance in *Streptomyces* spp. and relatives isolated from soil samples and showed that 60–100% of the isolates were resistant to several antibiotics.

Actinomycetes are famous for antibiotic production and are the source of over half of the natural antibiotics that are used today. In the present study, genes coding for proteins involved in the biosynthesis of mitomycin antibiotics/polyketide fumonisin, putative penicillin acylase (penicillin amidase family protein), and antibiotic biosynthesis monooxygenase were evident in *Streptomyces* sp. strain 4F (**Table 4**). Polymyxin synthetase and aclacinomycin oxidoreductase genes were found on some of the plasmids sequenced in this study (**Table 3**). *Streptomyces* spp. BF-3 possesses genes for polymyxin synthetase PmxB, antibiotic biosynthesis monooxygenase, and a penicillin amidase precursor. Penicillin amidase functions in the biosynthesis of penicillin by catalyzing the hydrolysis of amide bonds in ampicillin, penicillin G, and penicillin V. Monooxygenases generally perform the hydroxylation of intermediates in the antibiotic biosynthesis pathways (O'Keefe and Harder, 1991). The two bacterial strains characterized in this study also have the potential to produce vitamins including folate, vitamin B-12 (cobalamin), vitamin B-6 (pyridoxine), vitamin K-2 (menaquinone), and vitamin K-1 (phylloquinone) (**Tables 4**, **5**).

In conclusion, actinomycetes isolated from the Great Salt Plains of Oklahoma harbor a variety of plasmids, including mega-plasmids, which encode genes potentially involved in adaptation to this extreme environment. Further studies are underway to determine if these plasmids are similar and can be conjugally transferred to other actinomycetes. Draft genome sequences of two megaplasmid-bearing *Streptomyces* sp. strains, BF-3, and 4F, revealed the presence of genes involved in antibiotic production, antibiotic, and heavy metal resistance, osmoregulation, and the stress response, which facilitates their survival in this extreme halophilic environment. To our knowledge, this is the first study to explore plasmids harbored by actinomycetes isolated from the Great Salt Plains of Oklahoma.

AUTHOR CONTRIBUTIONS

MF prepared the research idea and design of the manuscript. CC and DM performed the experimental procedures. CC and MF prepared the manuscript.

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Transcriptomic and Ectoine Analysis of Halotolerant *Nocardiopsis gilva* YIM 90087^T Under Salt Stress

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The genus *Nocardiopsis* is an unique actinobacterial group that widely distributed in hypersaline environments. In this study, we investigated the growth conditions, transcriptome analysis, production and accumulation of ectoine by *Nocardiopsis gilva* YIM 90087^T under salt stress. The colony color of *N. gilva* YIM 90087^T changed from yellow to white under salt stress conditions. Accumulation of ectoine and hydroxyectoine in cells was an efficient way to regulate osmotic pressure. The ectoine synthesis was studied by transferring the related genes (*ectA*, *ectB*, and *ectC*) to *Escherichia coli*. Transcriptomic analysis showed that the pathways of ABC transporters (ko02010) and glycine, serine, and threonine metabolism (ko00260) played a vital role under salt stress environment. The *ectABC* from *N. gilva* YIM 90087^T was activated under the salt stress. Addition of exogenous ectoine and hydroxyectoine were helpful to protect *N. gilva* YIM 90087^T from salt stress.

Keywords: *Nocardiopsis gilva*, halotolerant, ectoine, hydroxyectoine, transcriptomic analysis, whole-cell catalysis

INTRODUCTION

The genus *Nocardiopsis* is an unique actinobacterial group which was first described by Meyer (1976). Members of this genus are widely distributed in hypersaline environments and produce very distinct secondary metabolites (Tsujibo et al., 2003; Sun et al., 2015, 2017; Sharma and Singh, 2016). In past few years, attempts to isolate novel *Nocardiopsis* strains were made and many novel species such as *N. algeriensis* (Bouras et al., 2015), *N. mangrovei* (Huang et al., 2015), *N. oceani* and *N. nanhaiensis* (Pan et al., 2015), *N. ansamitocini* (Zhang et al., 2016b), *N. sediminis* (Muangham et al., 2016), *N. rhizosphaerae* (Zhang et al., 2016c), and *N. akesuensis* (Gao et al., 2016) have been reported. At present, the genus comprises 53 species and 5 subspecies ([1]2017). A large number of

[1]http://www.bacterio.net/nocardiopsis.html

Nocardiopsis species were isolated from saline or alkaline environments and approximately two-thirds of them were halophilic or halotolerant (Bouras et al., 2015; Pan et al., 2015). To survive in hypersaline environments, *Nocardiopsis* uses various strategies such as reinforcement of cell walls and accumulation of various osmolytes (Ameur et al., 2011; Zhang et al., 2016a). Accumulation of osmolytes play a vital role during salt stress and help in providing osmotic balance without interfering with the essential cellular processes and the normal metabolism (Liu et al., 2017). One of the most abundant osmolytes present in nature is ectoine, which was first found in the halophilic bacterium *Ectothiorhodospira halochloris* (Galinski et al., 1985). Ectoines found to improve protein folding and protect biomolecules such as enzymes, nucleic acids, antibodies, and even whole cells against various stress conditions (Barth et al., 2000).

The next-generation sequencing technologies and whole genome sequencing provide a new perspective for the research (Liu et al., 2017). In the past few years, about 22 genomes of *Nocardiopsis* have been sequenced ([2]2017). Comparative genomic analysis of *Nocardiopsis* species revealed that they retain core genome [at least 2,517 genes (approximately 43% of the genome content)] and also continuously acquire genes and expand their genomes to cope with environmental pressures (Li et al., 2013). Interestingly, the genes related to ectoine biosynthesis were found in genomes of all *Nocardiopsis* species.

Though extensive research was conducted to isolate novel *Nocardiopsis* members and analyzing their genome, yet only few reports focused on understanding mechanism for their survival under salt stress.

The objectives of the present study were: (1) compare the growth status of *Nocardiopsis gilva* YIM 90087^T under different saline conditions. (2) Analyze intracellular and extracellular concentrations of ectoine and hydroxyectoine under different salt stress. (3) Evaluate the mRNA expressions of the genes related to ectoine biosynthesis by RNA-seq sequencing under different salt stress. (4) Study the effects of ectoine and hydroxyectoine on the growth of *N. gilva* YIM 90087^T in presence and absence of salt stress. (5) Investigate the effects of saline conditions on ectoine biosynthesis of *N. gilva* YIM 90087^T, by reconstructing its biosynthetic pathway and studying the process of ectoine biosynthesis in *E. coli* BW25113. (6) Identify the ectoine from recombinant *E. coli* using liquid chromatography–mass spectrometry (LC–MS).

MATERIALS AND METHODS

Strains and Media

Nocardiopsis gilva YIM 90087^T, a halotolerant actinobacterium, used in this study was isolated by one of our group members from hypersaline soil in Xinjiang Province, China (Li et al., 2006). *E. coli* DH5α, and BW25113 [F$^-$, Δ(*araD-araB*)*567*, Δ*lacZ4787*(::rrnB-3), λ$^-$, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*] were used as the host strains for the construction of recombinant plasmids and ectoine biosynthesis, respectively.

[2]https://www.ncbi.nlm.nih.gov/genome

2YT medium composed of (L^{-1}) 16 g tryptone and 10 g yeast extract. Luria-Bertani (LB) medium composed of (L^{-1}) 10 g NaCl, 10 g tryptone, and 5 g yeast extract. ZYM medium composed of (L^{-1}) 10 g tryptone, 5 g yeast extract, 5 g glycerol, 0.5 g glucose, 25 mmol Na_2HPO_4, 50 mmol NH_4Cl, 25 mmol KH_2PO_4, 5 mmol Na_2SO_4, 2 mmol $MgSO_4$, 50 mmol $FeCl_2$, 0.02 mmol $CaCl_2$, 0.01 mmol $MgCl_2$, 0.01 mmol $ZnSO_4$, 0.002 mmol $CoCl_2$, 0.002 mmol $CuCl_2$, 0.002 mmol $NiCl_2$, 0.002 mmol Na_2MoO_4, 0.002 mmol Na_2SeO_3, 0.002 mmol H_3BO_3, and 0.06 mmol HCl. EB buffer consisted of 100 mM sodium aspartate, 100 mM glutamate, 100 mM glycerol, 100 mM KCl, and 100 mM sodium phosphate buffer (pH 7.0).

Effect of Salt Concentrations on Colony Morphology, Growth, and Ectoine Biosynthesis of *N. gilva* YIM 90087^T

To investigate the effects of NaCl concentrations on the growth of *N. gilva* YIM 90087^T, 1 ml of culture was inoculated in 100 ml of 2YT medium having NaCl concentration ranging 0–15% w/v (with an increment of 5%). The flasks were incubated at 30°C for 96 h. The growth was estimated by measuring the optical density at 600 nm (OD_{600}). The variations of colony morphology under different NaCl stress were evaluated using 2YT agar at 30°C for 96 h. To study the effects of NaCl concentartions on exogenous ectoine and hydroxyectoine, 1 mg/ml of ectoine and hydroxyectoine were added to 2YT medium. *N. gilva* YIM 90087^T grew in these media under different salt concentartions at 30°C for 96 h.

Molecular Methods

Genomic DNA from *N. gilva* YIM 90087^T was extracted and purified using the Bacterial DNA Kit (Omega Bio-Tek, United States). Plasmid DNA was extracted and purified from *E. coli* DH5α using the Plasmid Mini Kit I (Omega Bio-Tek). Restriction endonucleases, T4 DNA ligase, and Q5 High-Fidelity DNA polymerase (New England Biolabs, United States) were used according to the manufacturer's instructions. Standard methods were used for transformation (Chong, 2001).

NgiectA and *NgiectBC* were amplified from the genomic DNA of *N. gilva* YIM 90087^T (NCBI Reference Sequence: NZ_ANBG01000167.1) by using PCR primers NgiectA-F/R and NgiectBC-F/R as follows: NgiectA-F: 5′-CATGCCATGGGCT CCCGTGATAATTCCTCCC-3′, NgiectA-R: 5′-CGGGATCCTC AGTTCCGGCCGCTGACCGCCGAC-3′; NgiectBC-F: 5′-CG GGATCC*AAGGAG*ATATACATGGAGATCTTCGACCGCCTC G-3′, NgiectBC-R: 5′-GGAATTCCTACTCGGCCGTGGCCAG GCTCTCC-3′ (Restriction enzyme cutting site were underlined, and ribosome bind site were in italicized). The *NgiectA* PCR product was digested with *Nco* I and *Bam*H I, while *NgiectBC* PCR product was digested with *Bam*H I and *Eco*R I. The two DNA fragments were inserted together into the sites of *Nco* I and *Eco*R I in pBAD-HisB (Invitrogen, United States) resulting in the plasmid pBNABC. The vector pBNABC was transformed into *E. coli* BW25113 (named pBNABC/BW25113).

The total RNA from YIM 90087^T (culture of mid-log growth phase) was extracted using RNAprep Pure Cell/Bacteria

Kit (TIANGEN, CN). RNA concentration was measured by UVS-99 (ActGene, United States). RNA quality was assessed by electrophoresis on a denaturing agarose gel. The samples of transcription were sequenced and analyzed by Biomarker Technologies, China.

Ectoine Biosynthesis in Recombinant *E. coli*

Ectoine biosynthesis was performed using the method of whole-cell catalysis in *E. coli* pBNABC/BW25113 (He et al., 2015). To obtain ectoine producer, *E. coli* pBNABC/BW25113 was grown in ZYM medium with ampicillin by adding 2 g/L L-arabinose as an inducer at 30°C, 200 rpm for 16 h. The induced cells were harvested by centrifugation (6000 × *g*, 20 min) and washed with 0.9% NaCl buffer twice. The cells were re-suspended with 50 ml of EB buffer ($OD_{600\ nm}$ = 10). The catalysts mixtures were shaken in a 250-ml flask at 30°C, 200 rpm for 96 h.

HPLC and LC–MS Analysis of Ectoine and Hydroxyectoine

To identify ectoine and hydroxyectoine, 1 ml of cells suspension was harvested by centrifugation and extracted according to the method of Bligh and Dyer (1959). The intracellular and extracellular concentrations of ectoine and hydroxyectoine were measured by isocratic HPLC (Agilent 1260 series, Hewlett-Packard) following the method of He et al. (2015). The ectoine from recombinant *E. coli* was identified using Agilent 1260/6460 high-performance liquid chromatography/triple quadrupole mass spectrometer (Agilent, United States) using ESI source in positive ionization (He et al., 2015).

RESULTS

Effect of Salt Concentrations on Colony Morphology and Growth of *N. gilva* YIM 90087^T

The colony color of *N. gilva* YIM 90087^T changed from yellow to white with the increase of salt concentration (**Figure 1A**). *N. gilva* YIM 90087^T had the optimum growth at 5% NaCl concentration and the growth rate declined with the increase or decrease of the NaCl concentration (**Figure 1B**).

Differential Expression Analysis

FDR < 0.01 and Fold Change ≥ 2 were used as the selection criteria to identify the differentially expressed gene (DEG). The DEGs were annotated in COG, GO, KEGG, Swissport, and NR databases. The numbers of DEGs, up-regulated and down-regulated genes, and annotated DEGs were mentioned in **Table 1**. COG classifications of the three DEG sets were presented in 25 COG groups (**Figure 2**). Amino acid transport and metabolism and carbohydrate transport and metabolism were the dominant groups in all the three DEG sets. DEGs were distributed to 34 GO groups, which constitute three domains: cellular component, molecular function, and biological process (**Figure 3**). The DEGs from the three sets (5%/0%, 10%/0%, and 15%/0%) were mapped to 77, 32, and 90 reference canonical pathways in KEGG database respectively. The DEGs number and enrichment analysis were mentioned in **Table 2**. The DEGs number and corrected *P*-values results indicated that DEGs in the pathways of ABC transporters (ko02010) and glycine, serine, and threonine metabolism (ko00260) play extremely significant roles under salt stress. Particularly in maximum-tolerated NaCl concentrations, expressions of ABC transporters genes of ectoine/hydroxyectoine and glycine betaine, and ectoine/hydroxyectoine synthesis genes increased evidently.

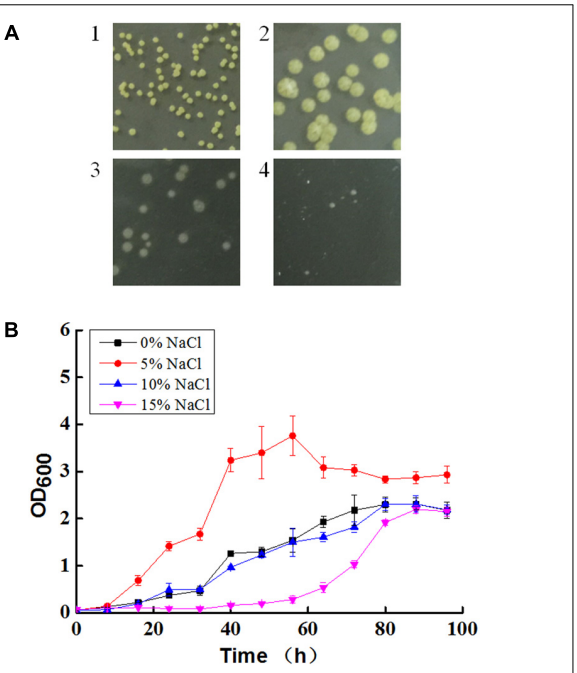

FIGURE 1 | Colony morphology **(A)** and growth curve **(B)** of *Nocardiopsis gilva* YIM 90087^T with varying concentrations of NaCl. A1: without NaCl; A2: 5% NaCl; A3: 10% NaCl; A4: 15% NaCl.

TABLE 1 | Statistics of DEGs and annotations.

	DEGs set		
	5%/0%	10%/0%	15%/0%
DEG number	1026	231	1578
Up-regulated	493	62	621
Down-regulated	533	169	957
COG	500	102	796
GO	408	75	668
KEGG	300	58	510
Swissprot	797	151	1219
NR	977	207	1470

Genetic Organization of *ect* Gene Cluster in *N. gilva* YIM 90087^T

We analyzed synthesis genes of ectoine and hydroxyectoine in *Nocardiopsis*. The *ectABCD* (synthesis genes) was found in

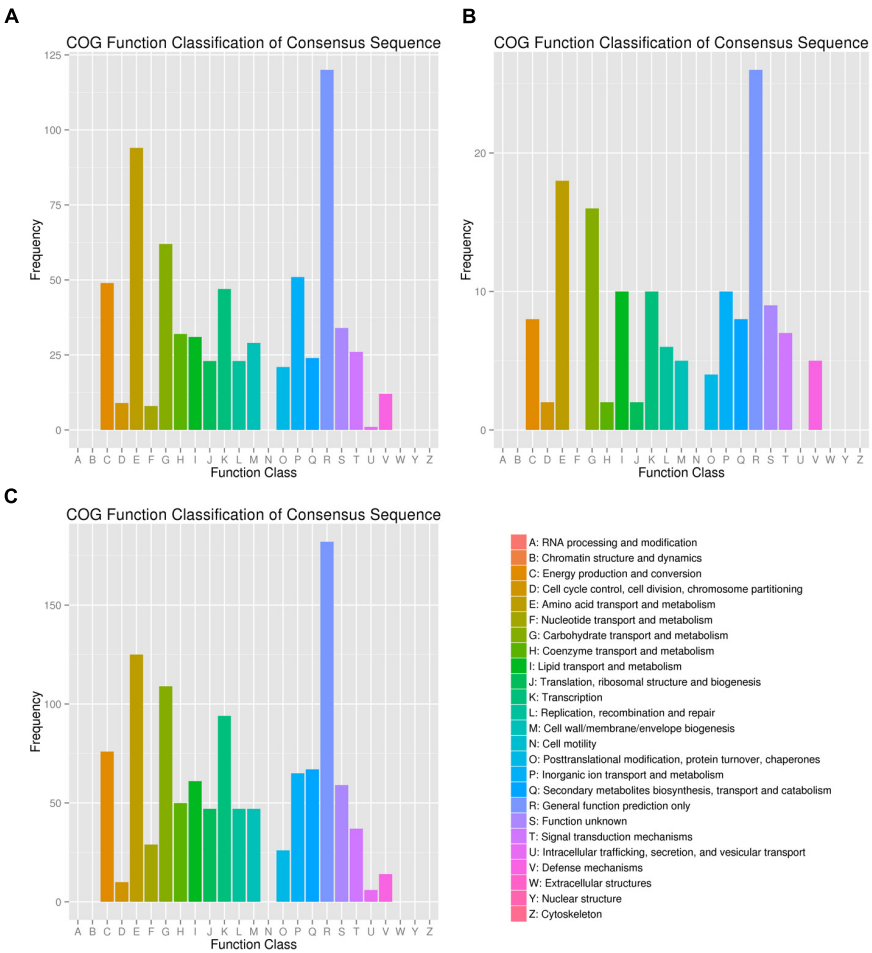

FIGURE 2 | COG classifications of the three DEG sets. The DEGs from the three sets (**A**: 5%/0%; **B**: 10%/0%; **C**: 15%/0%) had COG classifications among the 25 groups. The capital letters on the x-axis indicates the COG groups as listed on the right of the histogram. The y-axis indicates the number of DEGs.

Nocardiopsis genome. There are two common organizational types of *ectABCD* gene cluster on the 18 whole-genome sequences of *Nocardiopsis* strains (**Figure 4**). The *ectABC* were belonging to a gene cluster, and the *ectD* distributes on other locations of the genome (type A, 15 strains) or the *ectABCD* belongs to the same gene cluster (type B, 3 strains). Additionally, more than one *ectAB* or *ectD* genes were found in *N. potens* DSM 45234 and *N. prasina* DSM 43845. However, the organizational types of *ect* gene cluster from *N. gilva* YIM 90087^T was clearly different from others *Nocardiopsis* strains. The *ectA* gene completely separated from *ectBCD* gene cluster, which deviate from the commonly found genetic organization. Moreover, the *ehuABCD*, ectoine/hydroxyectoine ABC transporter gene, were situated on the downstream of *ectD* gene in genome.

Expression of Genes Involved in Synthesis and Transport of Ectoine and Hydroxyectoine

We analyzed the mRNA expression of the genes involved in ectoine/hydroxyectoine synthesis and transport at different salt concentrations (**Table 3**). The genes involved in ectoine biosynthesis process presented different levels of up-regulation with the increase of NaCl concentration, particularly in 15% NaCl. Unlike other genes, *ectD* presented two stepwise up-regulations from 5 to 10% NaCl concentration and from 10 to 15% NaCl concentration. Two potential ABC transporter genes (*ehuABCD* and *proXWV*) involved in transport of ectoine were found in *N. gilva* YIM 90087^T. The mRNA expressions of them varied with increase NaCl concentration. The expressions of *ehuABCD* were positively correlated with NaCl concentration when the NaCl concentration was above 5% in the medium. Yet, the expressions of *proXWV* had no obvious changes in optimum and mild-tolerated NaCl concentrations. Interestingly, *ehuABCD* and *proXWV* were activated strongly at maximum-tolerated NaCl concentrations.

Accumulation of Ectoine and Hydroxyectoine in *N. gilva* YIM 90087^T

Ectoine and hydroxyectoine were analyzed by HPLC. The results showed that there were two peaks with the same retention

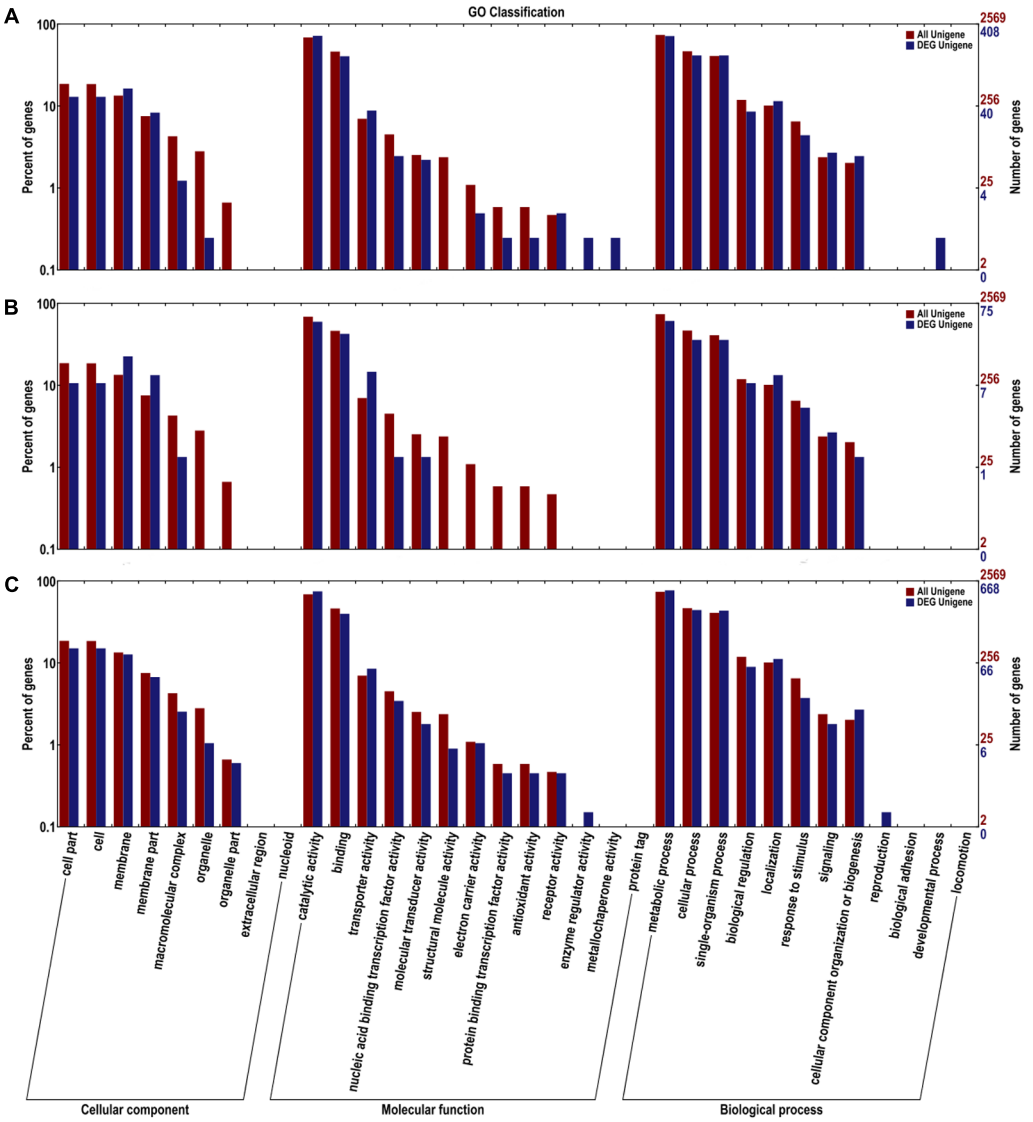

FIGURE 3 | Gene ontology (GO) terms enrichments of the DEGs from the three sets. The DEGs were divided into three clusters: cellular component, molecular function, and biological process. **(A)** 5%/0%; **(B)** 10%/0%; **(C)** 15%/0%. The right y-axis presents the number of unigenes in the clusters, while the left y-axis presents the percentage of a specific category of unigenes in that clusters.

time as the two standards [ectoine (816189, Sigma-Aldrich) and hydroxyectoine (70709, Sigma-Aldrich)] appeared from *N. gilva* YIM 90087^{T} cell. As shown in **Figures 5A,B**, the intracellular concentration changes of ectoine and hydroxyectoine were a cumulative progress. Whereas accumulation of ectoine and hydroxyectoine were influenced by NaCl concentration. They were positively correlated with increasing NaCl concentration. Noteworthy, hydroxyectoine were difficult to be synthesized in the medium with 0 or 5% NaCl. After 48 h, hydroxyectoine were detected in 2YT medium with 10 and 15% NaCl. Moreover, the amounts of ectoine and hydroxyectoine were increased with the increasing cultivation time. After 96 h, the highest level of ectoine (33.14 mg/g CDW) and hydroxyectoine (1.17 mg/g CDW) were detected in 15% NaCl concentration. The ectoine concentrations were 42.6 and 28.3 times higher than hydroxyectoine in 10 and 15% NaCl concentration, respectively. Therefore, the results indicates that ectoine was accumulated in *N. gilva* YIM 90087^{T} cells.

On the other side, we detected ectoine and hydroxyectoine outside the cells (**Figures 5C,D**). A large proportion of ectoine was released out of the cells. After 96 h, 89.20, 92.46, 91.99, and 71.09% of ectoine were released in the medium with 0, 5, 10, and 15% NaCl, respectively. About 99.31 and 99.08% of hydroxyectoine found out of the cells in medium with 10 and 15% NaCl, respectively. The transfer capacity for ectoine enhanced remarkably in the medium with 15% NaCl. Yet, the capacity for hydroxyectoine had no obvious change as concentration added. In our study, we observed the transformation from ectoine to

TABLE 2 | The DEG number, proportion, and *Q*-value in the pathway.

DEGs sets	Pathway number	Function	DEG number	Proportion (%)	Corrected *P*-value
5%/0%	ko02010	ABC transporters	37	21.14	0.02
	ko00260	Glycine, serine, and threonine metabolism	14	8.00	0.2
	ko00650	Butanoate metabolism	14	8.00	0.48
	ko00230	Purine metabolism	12	6.86	1
	ko02020	Two-component system	9	5.14	1
	ko00280	Valine, leucine, and isoleucine degradation	9	5.14	1
	ko00071	Fatty acid metabolism	9	5.14	1
	ko00362	Benzoate degradation	9	5.14	1
	ko00190	Oxidative phosphorylation	8	4.57	1
	ko00290	Valine, leucine, and isoleucine biosynthesis	8	4.57	1
	ko00500	Starch and sucrose metabolism	8	4.57	0.96
10%/0%	ko02010	ABC transporters	8	29.63	0.42
	ko00500	Starch and sucrose metabolism	3	11.11	0.42
	ko00260	Glycine, serine, and threonine metabolism	2	7.41	1
15%/0%	ko02010	ABC transporters	34	10.4	1
	ko00260	Glycine, serine, and threonine metabolism	22	6.73	0.09
	ko00230	Purine metabolism	18	5.5	1
	ko00240	Pyrimidine metabolism	17	5.2	1
	ko02020	Two-component system	16	4.89	1
	ko00010	Glycolysis/gluconeogenesis	15	4.59	1
	ko00860	Porphyrin and chlorophyll metabolism	15	4.59	1
	ko00620	Pyruvate metabolism	14	4.28	1
	ko00627	Aminobenzoate degradation	14	4.28	1
	ko00650	Butanoate metabolism	12	3.67	1
	ko00020	Citrate cycle (TCA cycle)	12	3.67	1
	ko00030	Pentose phosphate pathway	12	3.67	0.86
	ko00362	Benzoate degradation	12	3.67	1
	ko00562	Inositol phosphate metabolism	10	3.06	0.29
	ko02060	Phosphotransferase system	7	2.14	0.53

hydroxyectoine with the increase of NaCl concentration. About 50% ectoine transformed into hydroxyectoine at 15% NaCl concentration. The highest extracellular amount of ectoine and hydroxyectoine were found at 10 and 15% NaCl concentration, respectively.

Effect of Exogenous Ectoine and Hydroxyectoine on Growth of *N. gilva* YIM 90087^T

Exogenous ectoine and hydroxyectoine under salt stress increased the growth rate of *N. gilva* YIM 90087^T. The growth of *N. gilva* YIM 90087^T in absence of NaCl was basically the same, in presence or absence of exogenous ectoine and hydroxyectoine (**Figure 6A**). However, with the increase of NaCl concentrations, exogenous ectoine, and hydroxyectoine promoted the growth (**Figure 6**). The addition of ectoine and hydroxyectoine under optimum NaCl concentration of (5%) slightly increased the OD_{600} values when compared in their absence (**Figure 6B**). The addition of ectoine and hydroxyectoine at high NaCl concentration (10–15%) drastically increased the OD_{600} values when compared in their absence (**Figures 6C,D**). It was also noted that the growth with hydroxyectoine was somewhat better than ectoine at 15% NaCl concentration.

Accumulation of Ectoine in *E. coli*

Using aspartate as a substrate, glutamate as amino donor and glycerol as an acetyl-CoA donor, ectoine was produced by *E. coli* pBNABC/BW25113. The changes in the production of ectoine were shown in **Figure 7**. Ectoine was produced hardly at 15% NaCl buffer, and a few of ectoine was detected in 0% NaCl buffer. The synthetic rate of ectoine dropped significantly after 24 and 48 h at 10 and 5% NaCl buffer, respectively. The maximum values of initial synthetic rate [0.5 mg (g CDW)$^{-1}$ h^{-1}] and final output [28.85 mg (g CDW)$^{-1}$] were found in 10 and 5% NaCl buffer, respectively.

DISCUSSION

Halophilic microorganisms have the ability to adjust saline environments more quickly and efficiently (Liu et al., 2017). Survival and growth of microorganisms in saline environments require numerous morphological ecotypes and adaptations (Kralj Kuncic et al., 2010; Liu et al., 2017). Salt stress causes change in colony morphology especially, colony color hence, in the present study colony color under salt stress was evaluated. The colony color of *N. gilva* YIM 90087^T changed

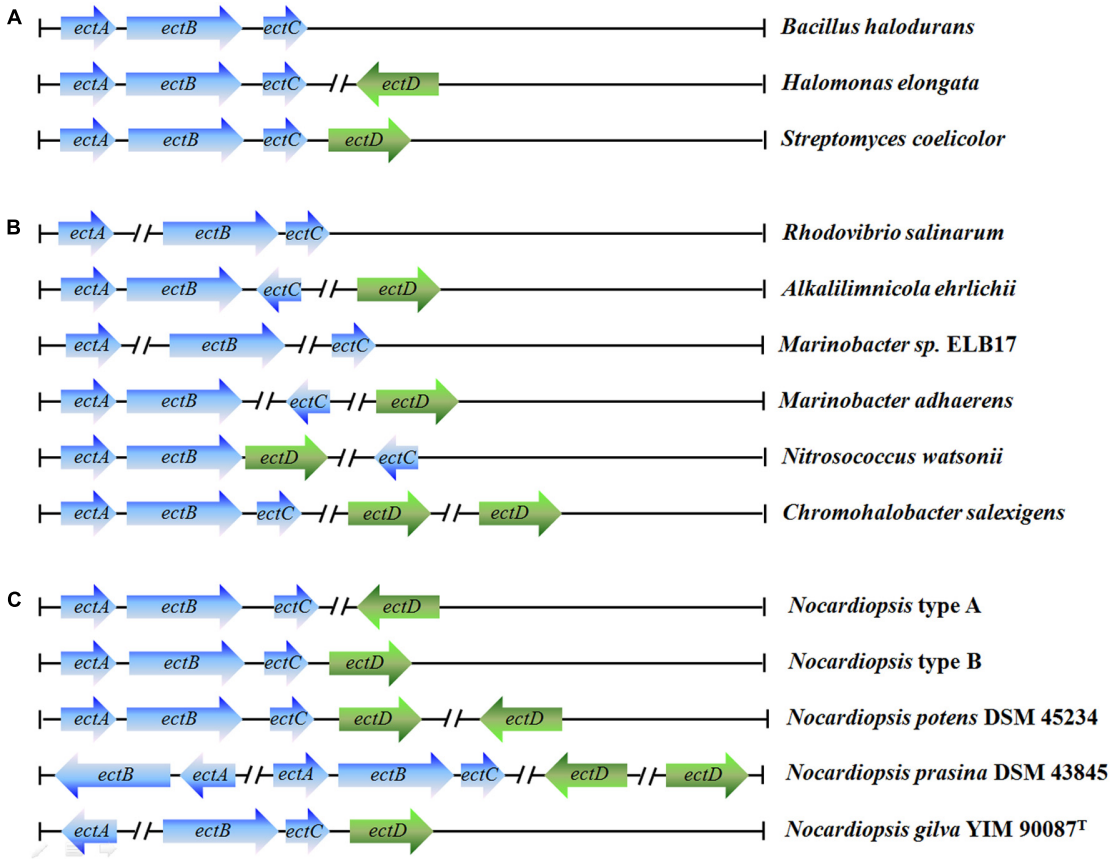

FIGURE 4 | Genetic organization of the ectoine/hydroxyectoine biosynthesis gene clusters. The different types of ectoine and hydroxyectoine biosynthesis gene clusters present in microorganisms: **(A)** most common organizational types of the ect gene clusters and representative strains; **(B)** unusual types of the ect gene clusters and representative strains; **(C)** different types of the ect gene clusters from 21 *Nocardiopsis* strains.

TABLE 3 | The fold changes of the genes involved in ectoine and hydroxyectoine synthesis and transport in response to different salt stress.

Unigene ID	Genes	Function	Fold change		
			5%/0%	10%/0%	15%/0%
GL001695	*ask*	Aspartate kinase	1.29	1.51	4.45
GL001694	*asd*	L-Aspartate-beta-semialdehyde-dehydrogenase	1.42	1.32	4.16
GL003065	*ectB*	Diaminobutyrate-2-oxoglutarate transaminase	2.16	1.84	7.35
GL001325	*ectA*	Diaminobutyrate acetyltransferase	1.33	2.23	5.26
GL003066	*ectC*	Ectoine synthase	2.58	2.22	7.59
GL003067	*ectD*	Ectoine hydroxylase	2.60	5.91	22.94
GL003068	*ehuB*	Ectoine/hydroxyectoine ABC transporter substrate-binding protein	1.17	1.73	5.85
GL003069	*ehuC*	Ectoine/hydroxyectoine ABC transporter permease subunit	0.88	2.67	10.48
GL003070	*ehuD*	Ectoine/hydroxyectoine ABC transporter permease subunit	1.19	1.95	8.48
GL003071	*ehuA*	Ectoine/hydroxyectoine ABC transporter ATP-binding protein	0.85	2.262	7.18
GL003184	*proX*	Glycine betaine/proline transport system substrate-binding protein	0.80	0.90	4.07
GL003185	*proX*	Glycine betaine/proline transport system substrate-binding protein	1.00	1.28	5.31
GL003186	*proW*	Glycine betaine/proline transport system permease protein	0.90	1.57	9.49
GL003187	*proV*	Glycine betaine/proline transport system ATP-binding protein	0.85	1.47	10.61

from yellow to white with the increase of salt concentration. Similar result was observed in many halophilic microorganisms when grown in high salt stress (Kralj Kuncic et al., 2010; Liu et al., 2017). Compatible solutes play significant roles to cope with hyperosmotic stress (Poolman and Glaasker, 1998; Ventosa et al., 1998; Roesser and Muller, 2001). These

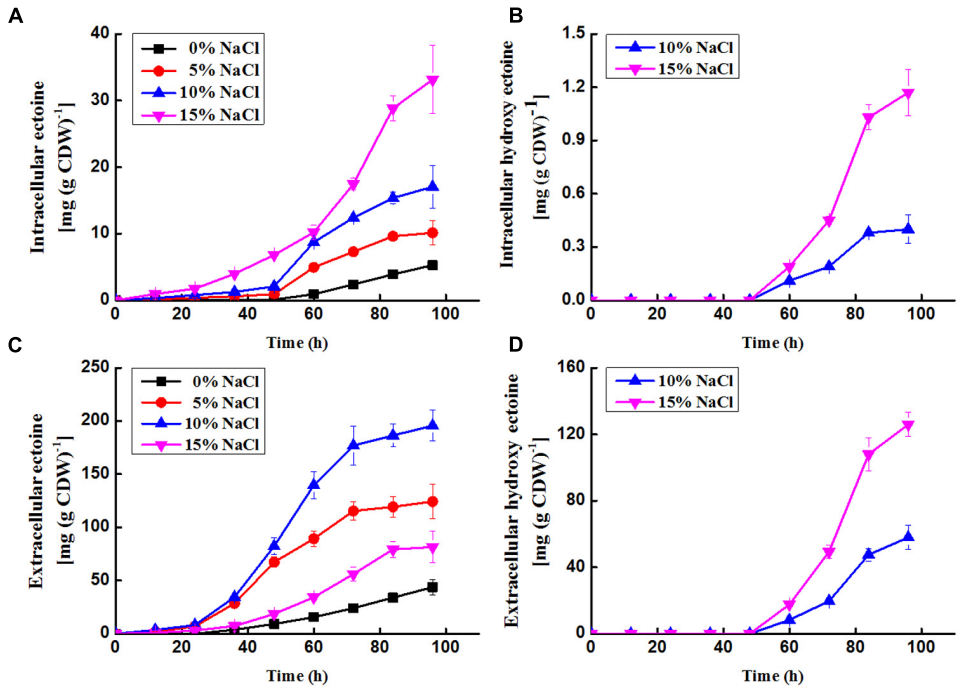

FIGURE 5 | Time profiles of production of ectoine and hydroxyectoine in *N. gilva*. **(A)** Intercellular concentration of ectoine; **(B)** intercellular concentration of hydroxyectoine; **(C)** extracellular concentration of ectoine; **(D)** extracellular concentration of hydroxyectoine. 1 ml of the cells (*N. gilva* YIM 90087^T, OD_{600} = 1) is about 0.00063 g (DCW).

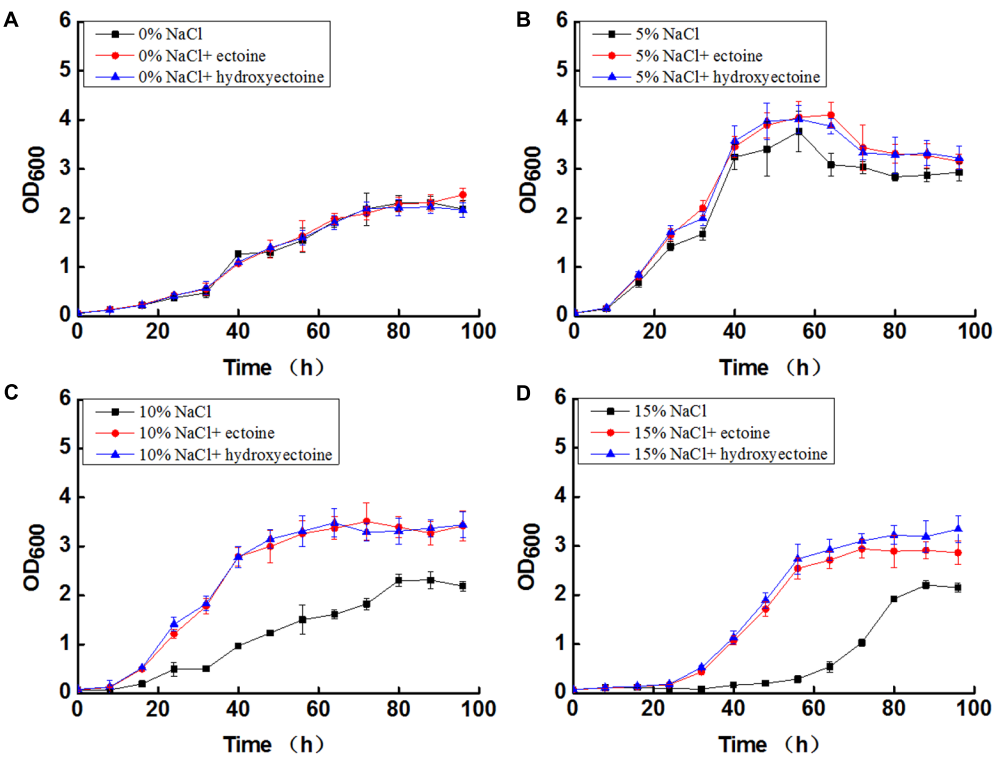

FIGURE 6 | The effect of ectoine and hydroxyectoine on Growth of *N. gilva* YIM 90087^T with varying concentrations of NaCl. **(A)** Curve of growth in the mediums with 0% NaCl; **(B)** curve of growth in the mediums with 5% NaCl; **(C)** curve of growth in the mediums with 10% NaCl; **(D)** curve of growth in the mediums with 15% NaCl.

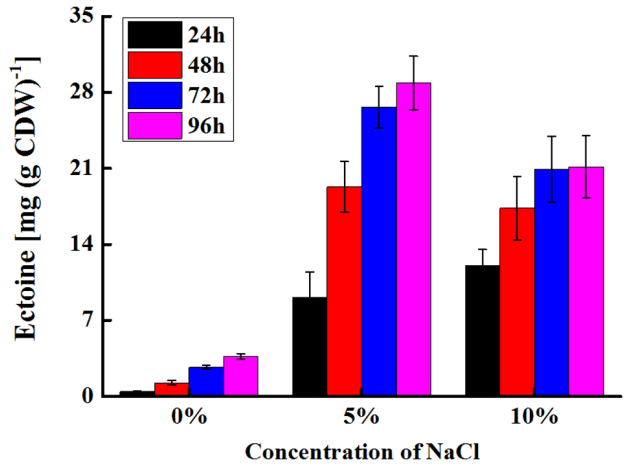

FIGURE 7 | Production changes of ectoine in *E. coli*. After induced, recombinant *E. coli* was shaken in ectoine biosynthesis buffer with different NaCl (0–15%). The ectoine productions were measured every 24 h. Ectoine did not detect in the buffer with 15% NaCl. 1 ml of the cells (*E. coli* pBNABC/BW25113, OD_{600} = 1) is about 0.00034 g (DCW).

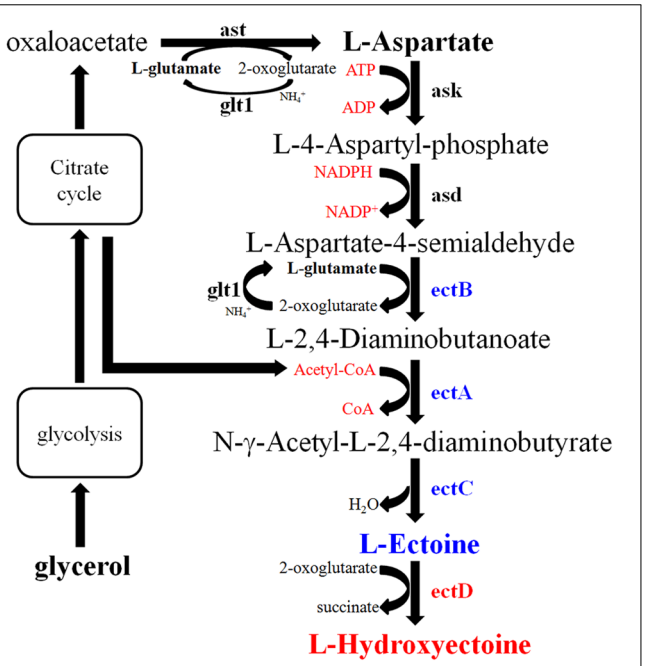

FIGURE 8 | Biosynthetic pathway of ectoine/hydroxyectoine. Ast, aspartate transaminase; Glt1, glutamate synthase; Ask, aspartate kinase; Asd, L-aspartate-beta-semialdehyde-dehydrogenase; ectB, diaminobutyrate-2-oxoglutarate transaminase; ectA, diaminobutyrate acetyltransferase; ectC, ectoine synthase; ectD, ectoine hydroxylase.

compatible solutes includes sugars (sucrose, trehalose), polyols (glycerol, sorbitol, mannitol, α-glucosylglycerol, mannosyl-glycerol, and mannosyl-glyceramide), *N*-acetylated diamino acids (*N*-acetylglutaminylglutamine amide), betaines (glycine betaine and derivatives), amino acids (proline, glutamate, glutamine, and alanine), and ectoine (hydroxyectoine) (Pastor et al., 2010). Halophilic microorganisms can synthesize different compatible solutes according to the external conditions, such as duration of the osmotic stress, level of salinity, availability of substrates, and osmolytes in the surroundings (Roberts, 2005; Aston and Peyton, 2007; Canamas et al., 2007). *Nocardiopsis* is a special halophilic microorganism, which can synthesize a variety of compatible solutes, such as ectoine, hydroxyectoine, trehalose, glutamate and β-glutamate and so on (Pastor et al., 2010). The related genes involved in compatible solutes synthesis were found in most of the *Nocardiopsis* species genomes (Li et al., 2013).

In *N. gilva* YIM 90087^T, the transcriptional regulation was influenced by NaCl stress. We found that ABC transports and glycine, serine, and threonine metabolism pathway were involved in essential biological processes in response to salinity (**Table 3**). The ProP and ProU systems (Aliases of ProXWV) involved in uptake and accumulation of ectoine, proline, and glycine betaine in *E. coli* (Jebbar et al., 1992). The EhuABCD, whose expression is induced by ectoine, specifically facilitates the movement of ectoine and hydroxyectoine across the membrane in *Sinorhizobium meliloti* (Jebbar et al., 2005). The increase of expression levels of these proteins with the increase of NaCl concentration were also found in *N. xinjiangensis* (Zhang et al., 2016a). Glycine betaine and ectoine/hydroxyectoine were two types of significant compatible solutes in glycine, serine, and threonine metabolism pathway. They offered effective protection for cells against salinity, but we found that only expressions of ectoine and hydroxyectoine synthesis genes were positively correlated with increasing of NaCl concentration in *N. gilva* YIM 90087^T.

Ectoine and hydroxyectoine are extremely vital compatible solute. Addition of ectoine and hydroxyectoine was helpful for the growth of *N. gilva* YIM 90087^T at maximum-tolerated NaCl concentrations. Ectoine and hydroxyectoine showed similar effect in *Virgibacillus pantothenticus* in response to high salinity (Kuhlmann et al., 2011). Besides this, ectoine and hydroxyectoine serve as protectants for proteins (Lippert and Galinski, 1992), nucleic acids (Malin et al., 1999), whole cell (Manzanera et al., 2002, 2004a,b), and skin (Heinrich et al., 2007).

Like many halophilic microorganisms (Kuhlmann and Bremer, 2002; Zhang et al., 2009), the synthesis and accumulation of ectoine and hydroxyectoine were response to NaCl stress in *N. gilva* YIM 90087^T. Transcriptional regulation in response to salinity was paramount. The *ectABCD* genes present different degrees of up-regulation in the range from 5 to 15% NaCl concentration, particularly at 15% NaCl concentration. Ectoine was converted into hydroxyectoine because of higher expression of *ectD*. Intracellular hydroxyectoine was far lower than ectoine in *N. gilva* YIM 90087^T. It was inclined to use ectoine as compatible solutes in cell. However, in *Prauserella alba*, a moderately halophile from saline soil (Li et al., 2003), hydroxyectoine replaced ectoine and became main compatible solute in the cell, when the salt concentration reached up to 15% outside the cell (Li et al., 2011).

The EctABC activity was also influenced by NaCl concentration. To study this effect, *E. coli* pBNABC/BW25113

was constructed. The expression level of EctABC was completely consistent in cell after induction by arabinose. An equal number of the cells were provided in the process of whole-cell catalysis. The aspartate was catalyzed by Ask (aspartate kinase) and Asd (L-aspartate-beta-semialdehyde dehydrogenase) from *E. coli*, *ectB* (diaminobutyrate-2-oxoglutarate transaminase), *ectA* (diaminobutyrate acetyltransferase), and *ectC* (ectoine synthase) from *N. gilva*, and finally transformed into ectoine (**Figure 8**). This way, *E. coli* could be used to produce ectoine under high-salt conditions, if the related enzymes were not inactive (Lin et al., 2015). Unlike the EctABC from *Halomonas elongata* (He et al., 2015), EctABC from *N. gilva* YIM 90087^T were activated under the salt stress in *E. coli*. However, the biosynthesis of ectoine was completely blocked at 15% NaCl concentration. The metabolism of host cell was inhibited in high salt environment (Metris et al., 2014). The enzymes from the host *E. coli*, such as Ask, Asd, and the enzymes involved in transamination and synthesis of acetyl-CoA, tend to keep the activity at the lower salt environments.

CONCLUSION

In this study, we analyzed various response of *N. gilva* YIM 90087^T at different NaCl concentration. Under salt stress, *N. gilva* YIM 90087^T colony color changed. The addition of ectoine and hydroxyectoine provided protection for *N. gilva* YIM 90087^T at high salt environment. Transcriptomic analysis of *N. gilva* YIM 90087^T indicated that, the synthesis and accumulation of ectoine and hydroxyectoine was an effective way to regulate osmotic pressure. Internal and external ectoine and hydroxyectoine analysis showed that synthesis of ectoine and hydroxyectoine raised with increase of NaCl concentration. Ectoine was transformed into hydroxyectoine gradually, however, ectoine was still the dominant compatible solute in the cell. To study the activity, *ectABC* from *N. gilva* YIM 90087^T transferred into *E. coli* BW25113. At the same expression level, we study the activity of *ectABC* with different NaCl concentration, and found that they were activated with a certain NaCl concentrations.

AUTHOR CONTRIBUTIONS

W-JL, YT, DA, and JH designed the research and project outline. JH, Y-GZ, and OM performed the growth and morphology observation. JH, Y-GZ, LL, and MN performed the transcriptome sample preparation and sequencing. JH, Q-XG, and W-JL provided HPLC and LC–MS analysis of ectoine and hydroxyectoine. JH, MN, MX, WH, YT, and W-JL drafted the manuscript. All authors read and approved the final manuscript.

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The Hybrid Strategy of *Thermoactinospora rubra* YIM 77501^T for Utilizing Cellulose as a Carbon Source at Different Temperatures

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Thermoactinospora rubra YIM 77501^T is an aerobic, Gram-positive, spore-forming and cellulose degrading thermophilic actinomycete isolated from a sandy soil sample of a volcano. Its growth temperature range is 28–60°C. The genomic sequence of this strain revealed that there are 27 cellulase genes belonging to six glycoside hydrolase families. To understand the strategy that this strain uses to utilize carbon sources such as cellulose at different temperatures, comparative transcriptomics analysis of *T. rubra* YIM 77501^T was performed by growing it with cellulose (CMC) and without cellulose (replaced with glucose) at 30, 40, and 50°C, respectively. Transcriptomic analyses showed four cellulase genes (*TrBG2*, *TrBG3*, *TrBG4,* and *ThrCel6B*) were up-regulated at 30, 40, and 50°C. The rate of gene expression of *TrBG2*, *TrBG3*, *TrBG4,* and *ThrCel6B* were 50°C > 30°C > 40°C. One cellulase gene (*TrBG1*) and two cellulase genes (*TrBG5* and *ThrCel6A*) were up-regulated only at 30 and 50°C, respectively. These up-regulated cellulase genes were cloned and expressed in *Escherichia coli*. The enzymatic properties of up-regulated cellulases showed a variety of responses to temperature. Special up-regulated cellulases *TrBG1* and *ThrCel6A* displayed temperature acclimation for each growth condition. These expression patterns revealed that a hybrid strategy was used by *T. rubra* to utilize carbon sources at different temperatures. This study provides genomic, transcriptomics, and experimental data useful for understanding how microorganisms respond to environmental changes and their application in enhancing cellulose hydrolysis for animal feed and bioenergy production.

Keywords: *Thermoactinospora rubra*, transcriptome, up-regulated cellulases, hybrid strategy, carbon source, different temperatures

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INTRODUCTION

Cellulose, as a main carbon source in the biosphere, is utilized by microorganisms and animals (Hungate, 1964; Batjes, 1996; Amundson, 2001). Completely digestion of cellulose involves multiple cellulases, like endo-1, 4-β-glucanases (EC 3.2.1.4), cellobiohydrolases or exo-1, 4-β-glucanases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21; Lynd et al., 2002; Gilbert et al., 2008). For microorganisms, secretion of cellulases may be effected by substances, pH, ion, and temperature (Lynd et al., 2002; López-Contreras et al., 2004; Sohail et al., 2009; Deng and Zhang, 2015; Hakkinen et al., 2015; Chen et al., 2016). Temperature is one of the most important environmental factors (Lin et al., 2016), and it may impact utilization of cellulose as a carbon source from two aspects: (1) it could affect the activity and stability of cellulases, and (2) it might be a crucial factor for inducing some cellulases under special temperatures. The different cellulases of microorganisms have diverse activity and stability at the same or different temperatures (Pardo and Forchiassin, 1999), such as enzyme, EG5C from *Paenibacillus* sp. IHB B 3084 shows optimum activity below 45°C (Dhar et al., 2015), while enzymes, ThCel6A from *Thermobifida halotolerans* YIM 90462T and CelA from *Caldicellulosiruptor bescii* show maximum activity between 50 and 60°C and higher than 80°C, respectively (Brunecky et al., 2013; Yin et al., 2015). Some cellulases are stable only at a low temperature (Dhar et al., 2015), while some remain stable at a high temperature (Brunecky et al., 2013). Under high temperatures, *Thermobifida fusca* secretes thermo stable cellulolytic enzymes to degrade cellulose (Adav et al., 2011). However, the potential strategy of carbon source acquisition (such as cellulose) at different temperatures remains unclear for microorganisms.

With the passage of time, the environment is always changing, including the microenvironment that the microorganisms inhabit. Microorganisms have developed many strategies to successfully survive, and they occupy most of the habitats on Earth after long-term evolution (Jorge-Villar and Edwards, 2013), even in extreme niches. To adapt to different environmental conditions, microorganisms can adopt three possible strategies: (1) the active strategy (special enzymes were up-regulated under different conditions), (2) the passive strategy (the same enzymes were up-regulated under different conditions), or (3) a hybrid strategy (both the same kinds of and special kinds of enzymes were up-regulated under different conditions). Microorganisms could respond to the environmental factors (such as temperature) during the process of carbon source acquisition.

Thermoactinospora rubra YIM 77501^T is an aerobic, Gram-positive, spore-forming, and thermophilic actinomycete that was isolated from a sandy soil sample collected at the Tengchong National Volcanic Geological Park, Yunnan province, south-west China (Zhou et al., 2012). The growth temperature of strain YIM 77501^T ranges from 28 to 60°C and it grows optimally at 45–55°C (Zhou et al., 2012). The result of plate testing for cellulase production using a Congo red plate assay (Teather and Wood, 1982) demonstrated that *T. rubra* has the ability to degrade cellulose (Figure S1). The crude cellulase activity test from culture supernatants of *T. rubra* in 0.2% carboxymethyl cellulose (CMC) medium to stationary phase at different temperatures showed that the cellulase activity of the culture at 30 and 50°C were higher than culture at 40°C (Figure S2). Furthermore, comparing the effect of temperature on crude enzymes (culture supernatants of *T. rubra*) activity showed that the sample from 30°C had higher activity at 30–45°C, and the sample from 50°C had higher activity at 50–70°C. These clues clearly indicate that *T. rubra* could express cellulases with different enzymatic characteristics to digest cellulose as carbon source at different temperatures.

In this study, the draft genome of *T. rubra* YIM 77501^T was sequenced. The gene predication and function annotation revealed that there were 408 glycoside-hydrolase-encoding genes. The transcriptomes of *T. rubra* under different culture conditions (different temperatures: 30, 40, and 50°C; different carbon source: CMC sodium and glucose) were profiled as well. The cellulase genes, which were significantly up-regulated in the presence of CMC under different temperatures, were cloned and expressed heterogenetically. The enzymatic properties of these cellulases were characterized. This study shed light on the strategy of strain *T. rubra* for carbon source acquisition at different temperatures.

MATERIALS AND METHODS

Growth and Genome Sequencing

T. rubra YIM 77501^T (=DSM 45614^T = CCTCC AA 2011014^T) was cultured on R_2A medium (yeast extract, 0.5 g/l; peptone, 0.5 g/l; glucose 0.5 g/l; soluble starch, 0.5 g/l; casein acid hydrolysates, 0.5 g/l; sodium pyruvate, 0.5 g/l; K_2HPO_4, 0.3 g; $MgSO_4$, 0.024 g/l) at 50°C. Genomic DNA was purified from 100 ml of mid exponential phase R_2A cultures using a MasterPure Gram-Positive DNA Purification kit (Epicentre MGP04100) following the standard DNA isolation procedure recommended by the manufacturer with modifications (Wu et al., 2009). Genomic DNA was sequenced using Illumina technology (Bennett, 2004) at the Beijing Genomics Institute (BGI Tech Solutions, Shenzhen, China). After the raw sequences were trimmed and their quality filtered ($Q > 30$), the clean reads with high quality were assembled using de Bruijn graphs in SOAP *de novo* v.1.05 (http://www.seekbio.com/soft/2754.html) with the K-mer parameter set to 41 (Li et al., 2008) and draft genomes were generated. The SOAPaligner v.2.21 alignment tool (http://soap.genomics.org.cn/soapaligner.html#down2) was used to align these reads against the *de novo* scaffolds to map reads and account for single nucleotide errors (Gu et al., 2013). Glimmer v3.0 (Chen et al., 2011) was used for gene prediction in assembled sequences of strain *T. rubra*. The sequence data described here have been deposited in JGI IMG (Submission ID: 105093) and DDBJ/ENA/GenBank (Accession number: MSZZ00000000). Carbohydrate-active enzymes (CAZymes) of *T. rubra* were determined using the CAZymes Analysis Toolkit (http://mothra.ornl.gov/cgi-bin/cat/cat.cgi; Petit et al., 2015). The results of GHs (Glycoside hydrolase families) were analyzed using the HMMER software based on the Pfam database (http://pfam.xfam.org/; Finn et al., 2011).

Transcriptome Sample Preparation and Sequencing

T. rubra YIM 77501^T was cultured in a modified form of a previously described R_2A medium containing the following (g/l): yeast extract, 0.5 g; peptone, 0.5 g; casein acid hydrolysates, 0.5 g; sodium pyruvate, 0.5 g; K_2HPO_4, 0.3 g; $MgSO_4$, 0.024 g; with 2 g/l CMC sodium as the test sample (R_2A-CMC medium) and with 2 g/l glucose as control (R_2A-glucose medium), with the pH adjusted to 7.0 using KOH. The colony of *T. rubra* was inoculated to R_2A-CMC and R_2A-glucose media. The growth curves under different conditions were determined (Figure S3). After incubation to mid exponential phase (at 30°C 180 rpm for 7 days, 40°C 180 rpm for 3.5 days, and 50°C 180 rpm for 2 days), cells were harvested by centrifugation for 5 min at 12,000 × g at 4°C. Three independent biological replicates were performed for each condition. The cell biomass was washed with phosphate buffered saline, re-suspended in 100 μl TE buffer pH 8 (EMD Chemicals) containing 2 mg/ml lysozyme (Merck, USA), and incubated at 37°C for 40 min. Total RNA was isolated using the RNeasy RNA purification kit (QIAGEN, Germany) according to the manufacturer's instructions. Contaminating DNA was removed with RNase-free DNase I (QIAGEN, Germany). Ribosome RNA in total RNA preparation was removed by using the Ribo-Zero™ Magnetic Kit for Gram-Positive Bacteria (Epicentre, USA). The quality of RNA samples was assessed on the Agilent Bioanalyzer 2100 system. Library construction and Illumina sequencing (Illumina HiSeq™ 2500) were performed at the Beijing Novogene Biological Information Technology Co., Ltd., Beijing, China (http:// www.novogene.cn/).

An RNA-seq analysis was performed according to the protocol recommended by the manufacturer (Illumina Inc.). Raw reads of fastq files were first processed through in-house Perl scripts. In this step, clean reads were utilized by removing reads containing adapter, reads containing poly-N and low quality reads from raw data. At the same time, the Q20, Q30, and GC contents of the clean data were calculated. All the following analyses were based on clean data with high quality. The reads from different conditions were mapped to the whole-genome of strain *T. rubra* using Bowtie 2.2.3 (Langmead and Salzberg, 2012). HTSeq v0.6.1 was used to count the read numbers mapped to each gene. Then, the FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene (FPKM, expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced, considers the effect of sequencing depth and gene length for the reads count at the same time and is currently the most commonly used method for estimating gene expression levels; Trapnell et al., 2009). Here, FPKM > 1 means gene expression.

Differential expression analysis of two conditions (cultures in CMC media and glucose media) at the same temperature (three biological replicates per condition) were performed using the DESeq R package (1.18.0). DESeq provided statistical methods for determining differential expression in digital gene expression data using a model based on a negative binomial distribution. The resulting *p*-values were adjusted using the Benjamini and Hochberg approach for controlling false discovery rate. Genes with an adjusted $p < 0.05$ found by DESeq were assigned as differentially expressed (Wang et al., 2010).

Gene Cloning, Expression, and Purification of Cellulases

The full-length sequences of cellulase genes were amplified from *T. rubra* genomic DNA. Chromosomal DNA of *T. rubra* was isolated using the Ezup Bacteria DNA Kit (Sangon Biotech, China) according to the manufacturer's instructions. Base on genome sequences, primers of cellulase genes (Table S1) were designed by using Primer Premier 5 (http://www.bioprocessonline.com/doc/primer-premier-5-design-program-0001). The complete ORFs of cellulase genes were amplified by PCR using the TransStar FastPfu Fly DNA Polymerase (TransGen Biotech, China). Amplification was performed for 34 cycles of 98°C for 20 s, 65°C for 20 s, and 72°C for 1 min, 72°C for 5 min with initial 2 min denaturation at 98°C. PCR products were ligated into pEASY-Blunt E1 vector (TransGen Biotech, China) and transformed into *Escherichia coli* DH5α. After sequencing verification, the entire cellulase gene was confirmed; positive recombinant vectors were transformed into *E. coli* BL21 (DE3) for cellulase gene expression.

Transformants were cultured overnight in LB culture medium with 100 μg/ml ampicillin at 37°C and 220 rpm. Then, 1 ml of the cells was added to 100 ml LB medium at 25°C and 220 rpm. During cultivation, isopropyl β-D-1-thiogalactoside (IPTG) was added to a final concentration of 1 mM at mid-exponential phase (OD600 ≈ 0.6) and followed by further incubation 8 h at 25°C with 220 rpm. Cells were harvested by centrifugation at 4,000×g and suspended in 20 ml PBS buffer (pH 8.0).

After ultrasonic cell disintegration and centrifugation at 12,000×g for 30 min at 4°C, cell-free extracts were applied to a Ni-chelating affinity column (GE, USA) because the proteins possess an N-terminal His-tag. Then, the extract was washed with five column volumes of buffer A (20 mM sodium phosphate, 0.3 M NaCl, pH 8.0), followed by 10 column volumes of buffer A with 20 mM imidazole, pH 8.0, and was eluted with buffer A with 200 mM imidazole, pH 8.0. The eluted protein was used for enzyme characterization.

Enzyme Assays and Protein Assays

The homogeneity of the purified enzyme was monitored by SDS-PAGE using 10% acrylamide gels. Proteins were visualized by Coomassie brilliant blue R-250 as described by Liu et al. (2010). Protein concentration was determined with a protein assay kit (Sangon Biotech, China) using bovine serum albumin as a standard. Activity against CMC was determined by measuring the release of reducing sugar, with 1% (w/v) CMC as substrate, by the 3,5-dinitrosalicylic acid (DNS) assay (Miller, 1959). One unit (U) of CMCase activity was defined as the amount of enzyme to release 1 μmol glucose-equivalent reducing sugars per minute. β-glucosidase activity was assayed using a 200 μl reaction mixture containing 2.5 mM p-nitrophenyl-β-D-glucopyranoside (pNPGlu) (Sigma, St. Louis, MO, USA). After 5 min of incubation at optimal temperature, the reaction was stopped by adding 0.6 ml of 1 M Na_2CO_3 (Yang et al., 2015). The p-nitrophenol was determined by monitoring the

absorbance at 405 nm (Harnpicharnchai et al., 2009). One unit of β-glucosidase activity is equivalent to 1 μmol of p-nitrophenol released from the pNPGlu in 1 min. The effect of temperature was determined at different temperatures from 20 to 90°C in optimal pH. Thermal stability of enzymes was determined by incubating equivalents of purified enzyme solutions for varied length of time intervals at 30, 40, and 50°C. The residual activity was determined by the standard method.

RESULTS

Genomic Features of *T. rubra*

The draft genome sequence of *T. rubra* YIM 77501^T consisted of 191 scaffolds, with 8,233,369 bp. The GC content was 71.7% and the genome contained 8,114 coding sequences, with an average length of 914 bp. The general genomic features of *T. rubra* are listed in Table S2. Among the predicted genes, 60% (4867 genes) have been assigned a function, and 40% (3,247 genes) have an unknown function. In addition, genes encoding one rRNA operon were found in proximity to the origin of function, and there were 54 tRNAs (Table S2).

Genes Encoding Carbohydrate-Active Enzymes

A CAZymes analysis was conducted to identify potential enzymes with plant cell-wall degradation ability. By applying this analysis, a total of 403 glycoside hydrolases (GHs) were distributed into 58 families, 226 carbohydrate-binding modules (CBMs) were distributed into 20 families, 20 polysaccharide lyases (PLs) were distributed into 7 families, 109 carbohydrate esterases (CEs) were distributed into 10 families, and 339 glycosyl transferases (GTs) were distributed into 26 families, are encoded in the genome of *T. rubra* (**Figure 1**). After analysis of GHs by using the HMMER software based on the Pfam database, 108 GH genes were distributed into 36 families (**Figure 1**). Twenty-seven cellulase genes were distributed to 6 GH families. The function of these 6 GH (GH1, GH3, GH5, GH6, GH9, and GH48) families may directly relate to cellulose digestion.

Transcriptome Sequencing and Analyses

Global gene expression profiles of *T. rubra* cultured on R_2A-glucose and R_2A-CMC media under different temperatures (30, 40, and 50°C) were examined using transcriptome sequencing. Finally, there are 406.48 million clean reads and 50.81 GB of RNA-seq data in treated and control strains after quality filtering (Error rate = 0.01%, Q20 > 98%, Q30 > 95%). More than 99% of the clean reads were mapped to the *T. rubra* YIM 77501^T genome. The GC content of clean reads is within the scope of 68.2–69.2% for all samples (Table S3). All of the Pearson's correlations between biological replicates were >0.95 and indicated a high reliability of the experiment and rationality of sample selection (Figure S4). Comparing percentage of reads mapped to the genome regions at different temperatures, more reads were mapped to intergenic region at higher temperatures. Cultures of R_2A-CMC media contained ∼8.9, 12.3, and 21% reads mapping to intergenic regions at 30, 40, and 50°C, respectively. Cultures of R_2A-glucose media contained ∼10.8, 19.9, and 22.1% reads mapping to intergenic regions at 30, 40, and 50°C, respectively (Figure S5). These results revealed that intergenic regions might play some functions in adapting to higher temperatures for strain *T. rubra*.

Total 18, 19, and 21 cellulase genes showed expression (FPKM > 1) at 30, 40, and 50°C, respectively. In these genes, seven up-regulated cellulase genes belonging to GH1, GH3, and GH6 were detected, comparing the cellulase genes expressions of CMC cultures with glucose cultures. This means that not all cellulase genes expressed during our culture conditions and the presence of CMC were no need for expression of some cellulase genes. Comparing the cellulase genes expressions of CMC cultures with glucose cultures at same temperature, four cellulase genes (*TrBG2*, *TrBG3*, *TrBG4,* and *ThrCel6B*) were up-regulated at 30, 40, and 50°C ($p < 0.01$). The rates of gene expression of *TrBG2*, *TrBG3*, *TrBG4,* and *ThrCel6B* were relatively higher at 50°C; on the contrary, they were relatively lower at 40°C. One cellulase gene (*TrBG1*) was especially up-regulated at 30°C ($p < 0.01$); two cellulase genes (*TrBG5* and *ThrCel6A*) were merely up-regulated at 50°C ($p < 0.01$; **Figure 2**). These findings suggested that culture temperature can affect cellulase gene expressions for *T. rubra*.

After data analysis, 7,084 and 7,132 genes expressed (FPKM > 1) when *T. rubra* was cultured at 30°C in R_2A-CMC and R_2A-glucose media, respectively; 7,009 and 6,839 genes were expressed (FPKM > 1) at 40°C in R_2A-CMC and R_2A-glucose media, respectively; 7,345 and 7,228 genes were expressed (FPKM > 1) at 50°C in R_2A-CMC and R_2A-glucose media, respectively, (**Figure 3**, Table S4). The expression of different genes in *T. rubra* showed high fluctuation when cultured at different temperatures on R2A-CMC and R2A-glucose media. High expression genes was observed (FPKM > 60), when *T. rubra* was cultured at 30 and 50°C as compared at 40°C. The genes 2,725 and 2,750, 2,231 and 2,543, 2,835 and 2,852 showed high expression (FPKM > 60) when *T. rubra* was cultured at 30, 40, and 50°C on R2A-CMC and R2A-glucose media, respectively (Table S4).

The genes expression profiles displayed remarkable differences when *T. rubra* was cultured at different temperatures in the same media (R_2A-CMC or R_2A-glucose). Comparing 30°C with 40°C, 4,473 genes (2,253 genes up-regulated and 2,220 genes down-regulated) were significant differentially expressed in R_2A-CMC media and 3,894 genes (2,093 genes up-regulated and 1,801 genes down-regulated) were significant differentially expressed in R_2A-glucose media. Comparing 30°C with 50°C, 4,918 genes (2,489 genes up-regulated and 2,429 genes down-regulated) were significant differentially expressed in R_2A-CMC medium and 4,988 genes (2,510 genes up-regulated and 2,478 genes down-regulated) were significant differentially expressed in R_2A-glucose medium. Comparing 40°C with 50°C, 4,293 genes (2,219 genes up-regulated and 2,074 genes down-regulated) were significant differentially expressed in R_2A-CMC medium and 3,760 genes (1,805 genes up-regulated and 1,955 genes down-regulated) were significant differentially expressed in R_2A-glucose medium (Figure S6A). The functions of these differentially expressed genes at different temperatures are mainly reflected in the biological process, cellular component, and molecular function (Figure S6B).

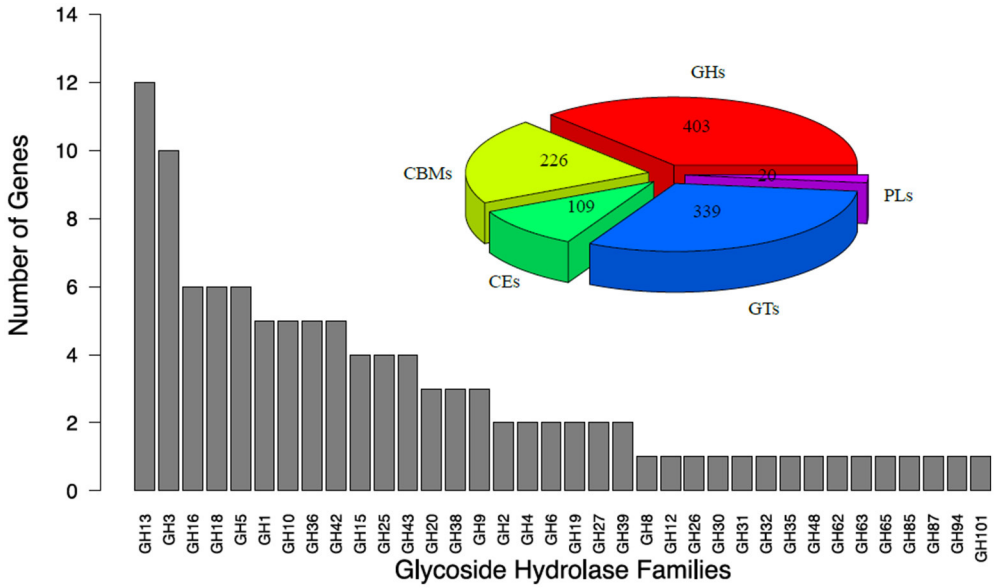

FIGURE 1 | Glycoside hydrolase (GHs) families of *T. rubra* YIM 77501^T. GHs, Glycoside Hydrolases; CBMs, Carbohydrate-Binding Modules; CEs, Carbohydrate Esterases; GTs, Glycosyl Transferases; PLs, Polysaccharide Lyases.

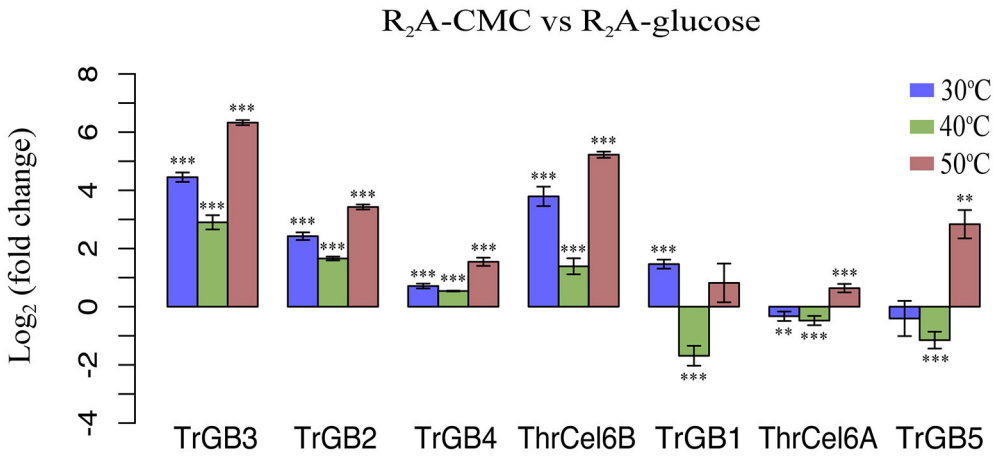

FIGURE 2 | Log2 of fold change in gene expression of *T. rubra* YIM 77501^T on R_2A-CMC and R_2A-glucose. Blue bars show the expression of R_2A-CMC cultures relative to R_2A-glucose cultures at 30°C, green bars show the expression of R_2A-CMC cultures relative to glucose (Glu) cultures at 40°C, red bars show the expression of R_2A-CMC cultures relative to R_2A-glucose cultures at 50°C. Values represent the mean of three biological replicates. Error bars show the standard deviation. ***$p < 0.01$; **$p < 0.05$.

Comparing genes expression profiles of samples from R_2A-CMC and R_2A-glucose media at different temperatures, 3,398 genes (1,706 up-regulated genes and 1,692 down-regulated genes) were found at 30°C, 3,560 genes (1,929 up-regulated genes and down-regulated 1,631 genes) were found at 40°C, and 2,869 genes (1,412 up-regulated genes and 1,457 down-regulated genes) were found at 50°C. Among these differentially expressed genes at different temperatures, 1,278 genes were up- and down- regulated at all temperatures, and 732, 504, and 577 genes were specifically expressed at 30, 40, and 50°C, respectively (**Figure 4**, Figure S7). This also showed more genes were specific expression when *T. rubra* is cultured at 30 and 50°C than at 40°C.

Cloning, Expression, and Enzyme Activity of Up-Regulated Cellulases

TrBG2, *TrBG3*, *TrBG4*, *ThrCel6B*, *TrBG1*, *TrBG5*, and *ThrCel6A* were cloned and expressed in *E. coli* BL21. TrBG1, TrBG2, TrBG3, TrBG1, TrBG5, and ThrCel6A were purified (Figure S8) and their enzyme activities were tested (Figure S9). The kinetic parameters of these enzymes are shown in Table S5. The enzyme activities of TrBG4 was tested using crude enzymes.

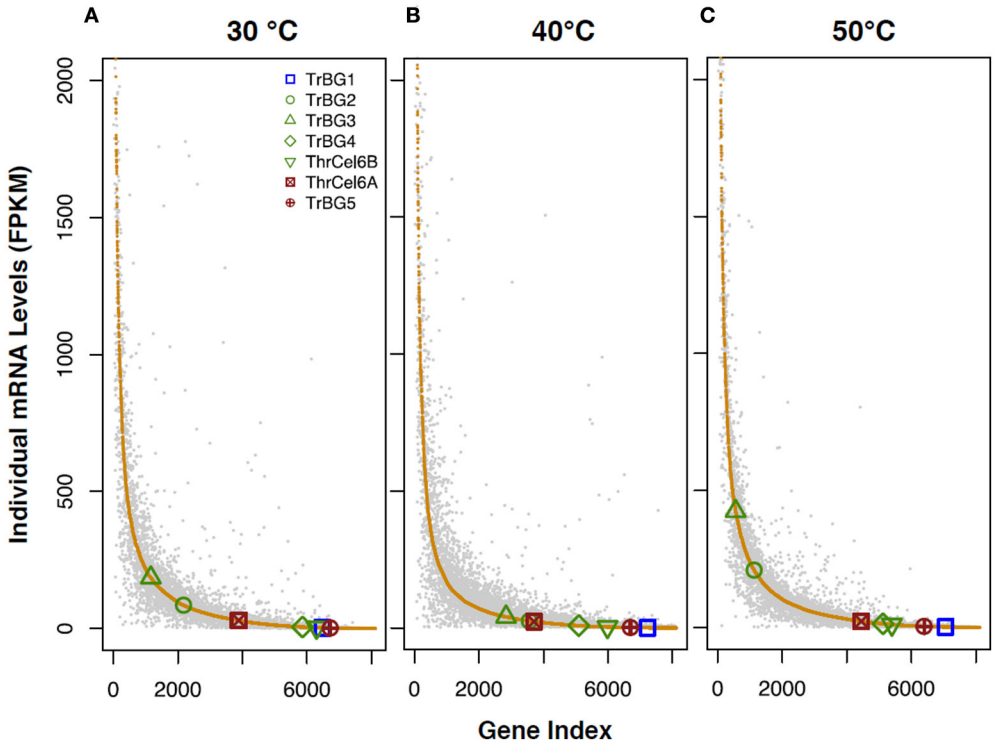

FIGURE 3 | Illustration of the variation in transcription levels of *T. rubra* YIM 77501^T cultured in R_2A-glucose (gray spots) and R_2A-CMC (orange spots) media at different temperatures. *T. rubra* YIM 77501^T was cultured at **(A)** 30°C, **(B)** 40°C, and **(C)** 50°C.

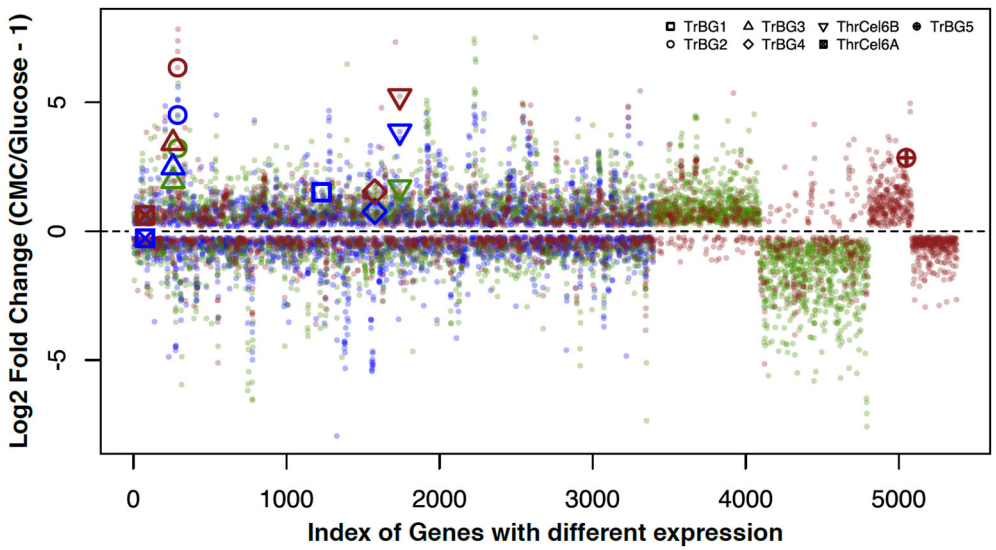

FIGURE 4 | Correlation between the $\log_2$ fold changes of genes that were differentially expressed in response to CMC and Glucose at different temperature (30, 40, and 50°C). Genes that showed $\log_2$ fold change (CMC/Glucose-1) of > 0 or < 0 in the same culture temperature were included for comparison. Blue spots show cultures at 30°C; green spots show cultures at 40°C; red spots show cultures at 50°C.

Based on experimental results, TrBG1, TrBG2, TrBG3, TrBG4, and TrBG5 showed β-glucosidase enzyme activity; ThrCel6A showed CMCase enzyme activity. The optimal temperatures of TrBG1, TrBG2, TrBG3, TrBG4, TrBG5, and ThrCel6A were 40, 60, 50, 60–70, 50–60, and 70°C, respectively. TrBG1 showed high relative enzyme activity at 30°C (∼40%). TrBG2, TrBG3, and

TrBG4 showed more than 20% relative enzyme activity at 30–50°C ThrCel6B cannot be expressed in *E. coli* (by expression vector). TrBG5 and ThrCel6A showed high relative enzyme activity at 50°C (∼100 and 78%, respectively; **Figure 5**).

Comparing effects of temperatures on enzyme stability, TrBG1, TrBG2, TrBG3, TrBG4, TrBG5, and ThrCel6A kept more than 70% relative enzyme activity after incubating at 30 and 40°C for 6 h. TrBG2 and TrBG3 lost more than 50% relative enzyme activity after incubating at 50°C for 6 h, while TrBG1 and TrBG5 lost most enzyme activity (more than 80%) after incubating at 50°C for 3 h. These results revealed TrBG1 and TrBG5 may be unstable at 50°C *in vitro*. TrBG4 and ThrCel6A were stable (kept more than 90% relative enzyme activity) after incubating at 50°C for 6 h (**Figure 6**).

DISCUSSION

Research has shown that some potential functions of microorganisms, such as degradation ability of carbon sources, implemented by special functional genes can be predicted based on genomic data analysis (Denger et al., 2006; Klippel et al., 2011; Wibberg et al., 2016). Numbers of genes encoding carbohydrate-active enzymes include more than 100 GHs were found in the genome of *T. rubra*, suggesting that this strain can convert cellulose as its own carbon source into biomass. Our results demonstrated that strain *T. rubra* has many choices of enzymes available to degrade cellulose at different temperatures.

Transcriptome analysis showed that temperature can affect the transcription of *T. rubra*. The clean reads of transcriptome sequencing displayed more reads were mapped to intergenic regions from the lower temperature to the higher temperature (30–50°C). The intergenic region may play a regulatory function in gene expression for some microorganisms (Beneke et al., 1995), e.g., *lac* operator. It demonstrated that some intergenic region sequences were increasingly transcribed with the rise of temperature, and they might regulate different gene expression to respond to temperatures. After transcriptome analysis, more genes showed high expression (FPKM > 60) when *T. rubra* was cultured both at 30 and 50°Ccompared with at 40°C. This could be attributed to 30 and 50°C being nearer the growth temperature range (28–60°C) of *T. rubra* (Zhou et al., 2012). Under the stress of lower or higher temperatures, microorganisms will overexpress some special proteins, such as cold shock proteins (CSPs) or heat shock proteins (HSPs), to protect themselves (Kondo and Inouye, 1994; Yin et al., 2012). In low temperature, secondary structures in mRNA would obstruct coupling processes of transcription and translation (El-Sharoud and Graumann, 2007). In *E. coli*, CSPs act as transcription anti-terminators or translational enhancers to destabilize RNA secondary structure (Nakaminami et al., 2006). Compared with culturing at 40 and 50°C, CSPs were up-regulated when *T. rubra* was cultured at 30°C (Figure S10A). This suggested that CSPs of *T. rubra* may function as an RNA chaperone to destabilize secondary structures and may be involved in regulating translation under lower temperatures. Heat shock proteins (HSPs) exist in all organisms and are important for stress tolerance (such as high temperature, oxidative, acid, and alkali stress) for microorganisms (Krajewski et al., 2014). When

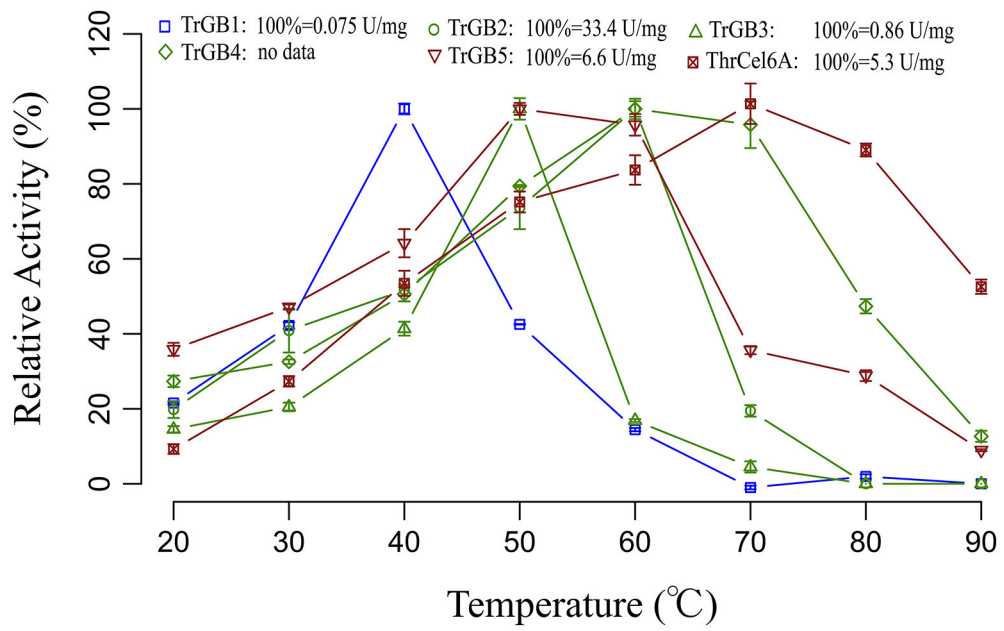

FIGURE 5 | Thermo activity profiles for cellulases. Data points are initial rates of activity at a given temperature expressed as a proportion of the highest rate. The cellulase genes were up-regulated only at 30°C (blue); the cellulase genes were up-regulated at 30, 40 and 50°C (green); the cellulase genes were up-regulated only at 50°C (red). For TrBG1, 100% = 0.075 U/mg; TrBG2, 100% = 33.4 U/mg; TrBG3, 100% = 0.86 U/mg; TrBG4, no data; TrBG5, 100% = 6.6 U/mg; ThrCel6A, 100% = 5.3 U/mg. The error bars show the standard deviations from three measurements.

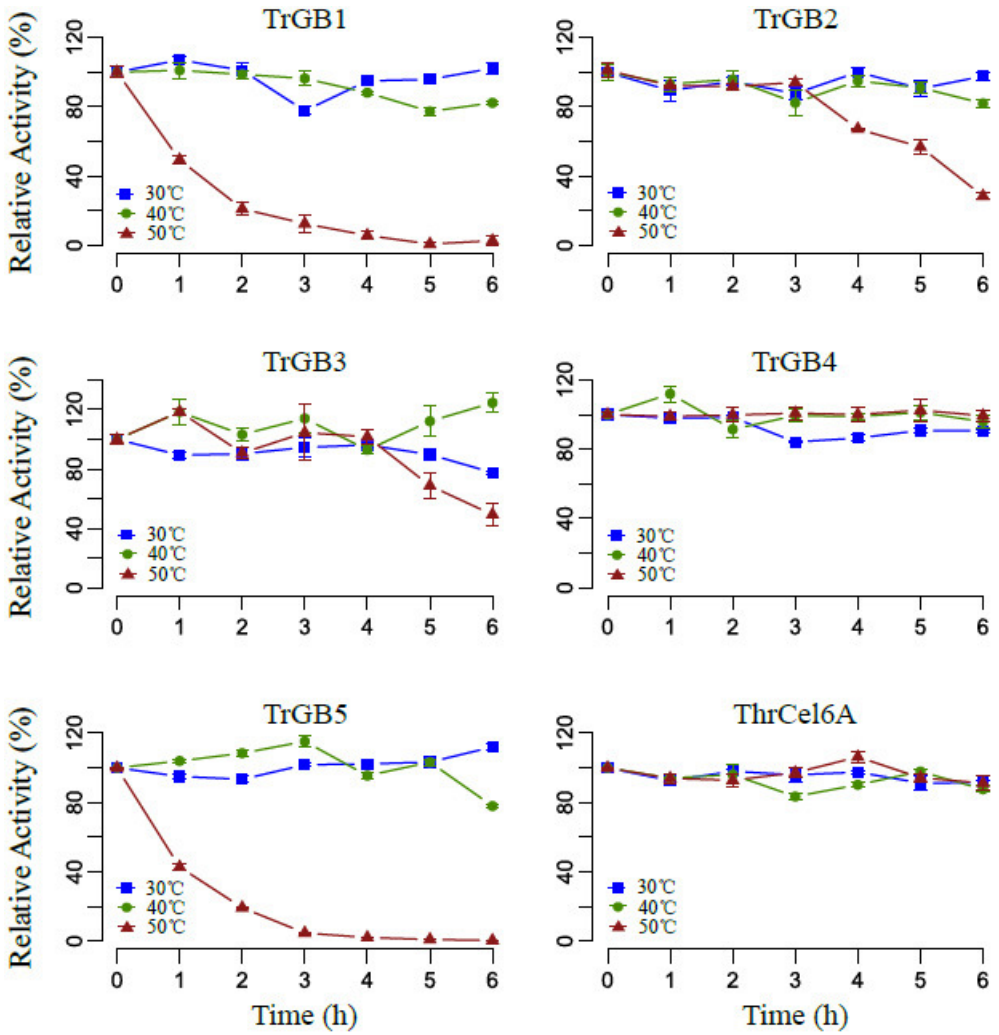

FIGURE 6 | The effect of temperature on stability. Cellulases were incubated at temperatures 30°C (blue), 40°C (green), 50°C (red) for 0–6 h, and the remaining activities were assayed at optimal conditions. For TrBG1, 100% = 0.075 U/mg; TrBG2, 100% = 33.4 U/mg; TrBG3, 100% = 0.86 U/mg; TrBG4, no data; TrBG5, 100% = 6.6 U/mg; ThrCel6A, 100% = 5.3 U/mg. The error bars show the standard deviations from three measurements.

T. rubra was cultured at 50°C, HSP (*dnaK*) was up-regulated compared with culturing at 30 and 40°C (Figure S10B).

Comparing genes expression profiles when *T. rubra* was cultured at different temperatures, more genes were up-regulated at lower temperatures than higher temperatures (30°C > 40°C, 30°C > 50°C). These results indicated that *T. rubra* may need to express more genes to live at a lower temperature. Global transcriptome analysis of *Lactococcus garvieae* strains also found more up-regulated genes at 18°C than at 37°C (Aguado-Urda et al., 2013) and demonstrated that different temperatures induced various gene expression changes in *T. rubra*.

Not all cellulase genes of *T. rubra* expressed and up regulated in presence of CMC. It revealed that expression of some cellulase genes may need some special induced conditions and there may be expression of some cellulase genes under presence of other carbon source, like glucose. For *Stachybotrys microspore*A19, carbon sources and the pH of the culture medium can direct a differential induction of various cellulases (such as endoglucanases and beta-glucosidases; Ben Hmad et al., 2014).

Temperature is an important environmental factor, and it determined the performance and stability of the enzyme used when utilizing a carbon source (Lin et al., 2016). For some microorganisms, temperature can affect the secretion of cellulases in the presence of cellulose substances, and this results in different cellulase enzyme activities at different temperatures (Sohail et al., 2009). *T. fusca*, as a thermophilic actinomycete, secretes thermo stable cellulolytic enzymes to digest cellulose at a high temperature (50°C) (Adav et al., 2011). Here, some cellulase genes were up-regulated by *T. rubra* when presented with CMC at all culture temperatures. In particular, other cellulase genes were up-regulated in just one temperature when presented with CMC. Based on the above-mentioned hypotheses, *T. rubra* used

a hybrid strategy (combining the active strategy with the passive strategy) to utilize carbon sources at different temperatures. Comparing CMC cultures with glucose cultures, the expression of cellulase genes showed different patterns and levels at different temperatures. Four cellulase genes (*TrBG2*, *TrBG3*, *TrBG4,* and *ThrCel6B*) appeared to be up-regulated at three temperature conditions significantly, and one (*TrBG1*) and two (*TrBG5* and *ThrCel6A*) cellulase genes were significantly up-regulated only at 30 and 50°C, respectively. Moreover, the expression levels of up-regulated cellulase genes (such as *TrBG2*, *TrBG3*, *TrBG4,* and *ThrCel6B*) at 30 and 50°Cwere higher than at 40°C. These results correspond with cellulase activity of culture supernatants (Figure S2) and reveal that *T. rubra* can regulate its cellulase gene expression during the process of utilizing carbon source at different temperatures.

The enzymatic properties analysis of these up-regulated cellulase genes that were cloned from *T. rubra* showed a variety of responses to temperature. *TrBG2*, *TrBG3,* and *TrBG4* were up-regulated cellulase genes at 30, 40, and 50°C and were stable with more than 40% relative enzyme activity at 40°C. However, TrBG2 and TrBG3 had low activity (<30%) at 30°C and were not stable at 50°C after 6 h. Therefore, in light of evolution, extra cellulases may appear at lower temperatures (30°C) or higher temperatures (50°C). *TrBG1*is a special up-regulated cellulase gene at 30°C, and it has high (>50%) relative enzyme activity at 30°C. *ThrCel6A* is also a special up-regulated cellulase gene at 50°C and was stable at 50°C after 6 h. TrBG5 is a special up-regulated cellulase gene at 50°C, and its optimal temperature was 50°C. However, no signal peptide was predicted by using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/, Table S5) for TrBG5, and it is not as stable as ThrCel6A at 50°C. It almost lost enzyme activity after incubating at 50°C for 3 h *in vitro* (**Figure 6**). DnaK, a main HSP in bacteria (Flaherty et al., 1991), was up-regulated in *T. rubra* at 50°C (Figure S10B), and it may protect proteins including TrBG5 at high temperatures *in vivo*. The results of these regulations ensured an adequate supply of carbon source for the growth and reproduction of *T. rubra*. Transcriptome data and up-regulated cellulases properties were consistent with the cellulase activity of culture supernatants of strain *T. rubra* (Figure S2). Compared with the culture at 40°C, there were higher levels and extra up-regulated cellulase genes at 30 and 50°C. These extra up-regulated cellulase genes showed functional adaptation for 30 or 50°C.

Both enzymatic properties and gene expression levels of cellulases displayed adaption to temperature. The reason that higher levels and more numbers of cellulase genes were up-regulated at lower or higher temperatures may be explained by the effect of temperature to enzyme. As we know, temperature can affect the activity and stability of an enzyme (Pardo and Forchiassin, 1999). Most enzymes are stable in lower temperatures but have low enzyme activity. However, at higher temperature, they have higher enzyme activity and are not stable. When the enzymes perform biological functions, microorganisms need to balance the stability and activity of enzyme. The results of these regulations show that increased enzyme gene expression and up-regulated special enzyme genes at lower or higher temperatures may maintain biological functions of enzymes economically and efficiently for the microorganism at different temperatures. In addition to temperature, microorganisms can also change the expression of cellulase genes on other environmental factors, such as substrate, pH, and ions to utilize carbon source (Lynd et al., 2002; López-Contreras et al., 2004; Deng and Zhang, 2015; Hakkinen et al., 2015; Chen et al., 2016). The expression level of different cellulase genes can be changed by various substrates (López-Contreras et al., 2004). Ambient pH can regulate expression of cellulase and hemicellulase genes by the transcriptional regulator (PACI) for *Trichoderma reesei* (Hakkinen et al., 2015). After a long period of evolution and development, microorganisms have evolved diverse strategies to overcome the changing environment.

Over billions of years of evolution, microorganisms have created special strategies for environmental change. When a microorganism uses cellulose as a carbon source, cellulose induces several metabolic pathways (Adav et al., 2011). While, further research is needed for membrane transport and signal transduction, *T. rubra* YIM 77501^T processes environmental information via different strategies to utilize carbon source at different temperatures.

CONCLUSIONS

To understand the strategies of carbon source acquisition, such as cellulose, in the environment at different temperatures for microorganisms, comparative transcriptomics of *T. rubra* grown with cellulose (CMC) and without cellulose (replace with glucose) at 30, 40, and 50°Cwere studied. The differences of the transcriptome analysis approach revealed that a hybrid strategy was used by *T. rubra* to utilize carbon sources at different temperatures. Higher levels and more numbers of cellulase genes were up-regulated at lower (30°C) or higher (50°C) temperatures than at a middle temperature (40°C). The enzymatic properties of up-regulated cellulase genes showed a variety of responses to temperature. Special up-regulated cellulases TrBG1 and ThrCel6A displayed temperature acclimation. These results may mean *T. rubra* has evolved an economical and efficient strategy to utilize carbon sources at different temperatures. This study provides genomic, transcriptomics, and experimental data useful for understanding how microorganisms respond to environmental changes and for their application in enhancing cellulose hydrolysis for animal feed and bioenergy production.

AUTHOR CONTRIBUTIONS

WL, YY, and XZ designed research and project outline. YY, ZJ, and WX performed growth and genome sequencing. XZ, EZ, and FZ performed transcriptome sample preparation and sequencing. QH, LL, and WH provided the gene cloning, expression and purification of cellulases. ZM, FZ, and YY performed enzyme assays and protein assays. WL, YY, and XZ drafted 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.2017.00942/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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