ACTINOBACTERIA: PROLIFIC PRODUCERS OF BIOACTIVE METABOLITES

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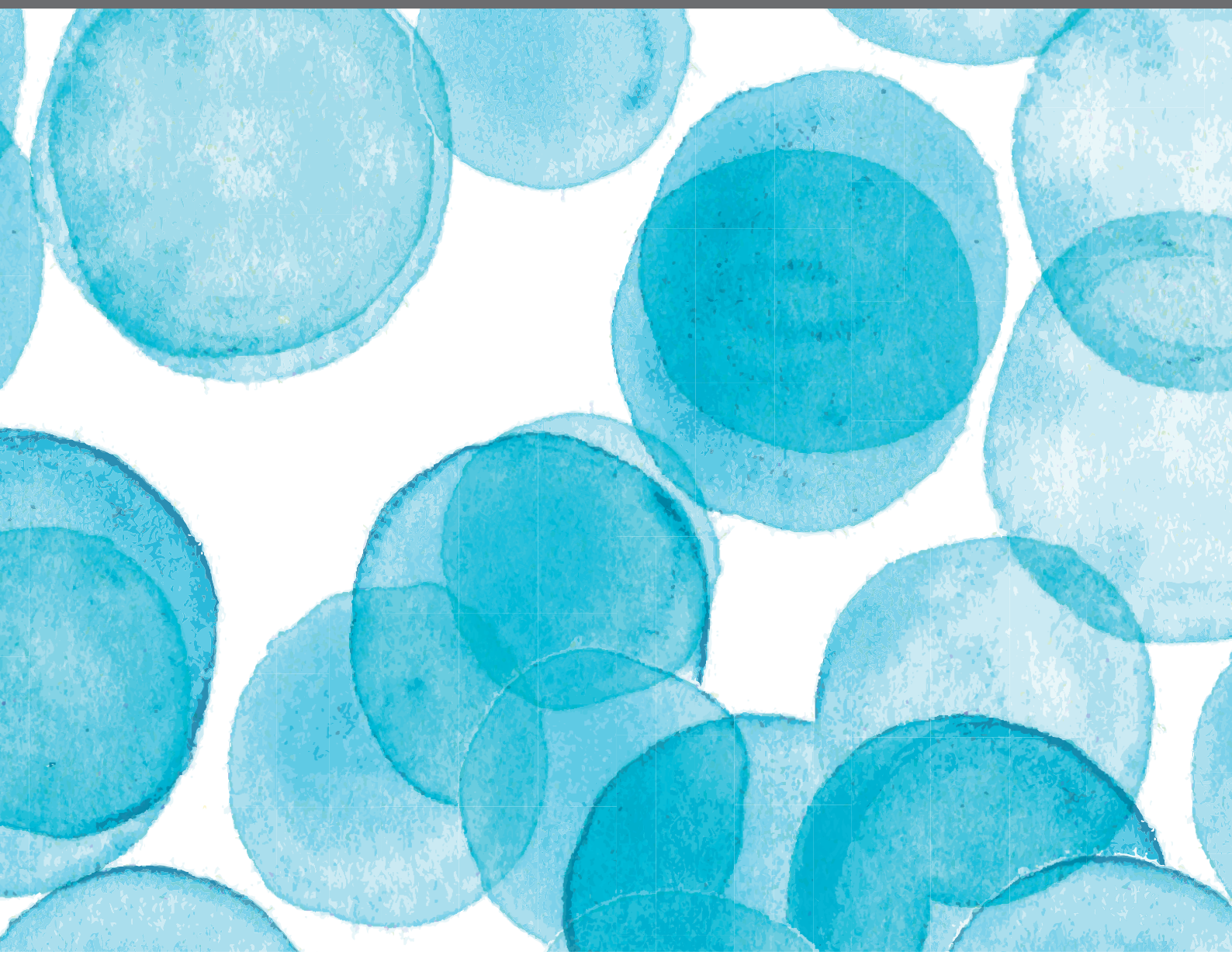

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ACTINOBACTERIA: PROLIFIC PRODUCERS OF BIOACTIVE METABOLITES

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EDITORIAL
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Editorial: Actinobacteria: Prolific Producers of Bioactive Metabolites

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Editorial on the Research Topic

Actinobacteria: Prolific Producers of Bioactive Metabolites

INTRODUCTION

For decades, scientists have conducted bioprospecting on Actinobacteria for the discovery of novel genera producing bioactive metabolites (Atalan et al., 2000; Bull et al., 2000; Goodfellow et al., 2018). Actinobacteria are the most prolific source of bioactive secondary metabolites, with diverse structural complexity (Takahashi, 2004). Actinobacteria-derived metabolites exhibit a wide spectrum of bioactivities, including antimicrobial (Umezawa et al., 1966), antifungal (Fukuda et al., 2005), anticancer (Omura et al., 1977), antiparasitic (Ōmura, 2003), and immunosuppressive activities (Barka et al., 2016). Thus, Actinobacteria continue to fuel biotechnology and medicine sectors with new biomolecules. In this Research Topic, a total of nine articles were published, illustrating wide arrays of bioactive metabolites produced by Actinobacteria derived from diverse ecosystems and the biosynthetic regulatory mechanisms of these metabolites.

STREPTOMYCES—A POWERHOUSE OF SECONDARY METABOLITES

After decades of bioprospecting, *Streptomyces* remains a priority due to its unsurpassed competency in producing a stunning multitude of diversified bioactive metabolites (Fiedler and Goodfellow, 2004; Goodfellow and Fiedler, 2010). The ability of *Streptomyces* to provide sources for new antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA) was highlighted (Kemung et al.). For instance, griseusin A, marinopyrrole A, and polyketomycin are several potent anti-MRSA compounds produced by *Streptomyces,* showing great promise for future clinical use. Balasubramanian et al. reported a compound (SKC_3) from a marine sponge-derived *Streptomyces* sp. $SBT_{34}8$ extract with antagonistic effects against growth and biofilm formation of several staphylococcal strains. Yu et al. reported two new fatty acids with nitrile group Borrelidins J and K, produced by *Streptomyces rochei* MB037, exhibiting strong activities against *S. aureus*. Besides *Streptomyces*, other genera such as *Microbacterium* within the phylum Actinobacteria derived from marine sponges also showed promising antibacterial activity against MRSA (Santos et al.).

Microbial secondary metabolites exhibited many useful applications for humankind. Many antibiotics derived from *Streptomyces* act as a defense mechanism to mediate competitive interspecies interactions (Chevrette et al., 2019). Tenconi et al. demonstrated the production of broad-spectrum molecules, the prodiginines associated with programmed cell death of the host,

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S. coelicolor. Hence, researchers suggested that the third use for antibiotics would be as molecules for self-toxicity to regulate cell proliferation other than serving as traditionally perceived tools for inter- or intra-species communication (Mccormick and Flärdh, 2012).

UNTAPPED RESERVOIR OF BIODIVERSITY FOR BIOPROSPECTING

The widespread occurrences of drug resistance in cancer and pathogens have rendered many medicines ineffective, and new strategies are therefore needed to uncover new agents (Antoraz et al., 2015). Exploring new taxa from untapped sources is an efficient strategy in searching for new drug leads/chemical scaffolds, as taxonomic diversity correlates to chemical diversity (Harvey, 2000; Sayed et al., 2020). Untapped environments like deep oceans (Abdel-Mageed et al., 2010) and mangroves (Hong et al., 2009) are proven a prolific source of bioactive Actinobacteria (Bull et al., 2005; Bull and Goodfellow, 2019). Moreover, Rangseekaew and Pathom-aree summarized that cave ecosystems harbor novel and diverse Actinobacteria, with promising bioactive metabolites, with a total of 47 species within 30 genera, including seven types of novel genera of Actinobacteria reported between 1999 and 2018. The coastal salt marsh plants represent another reservoir for diverse and novel endophytic Actinobacteria with promising biosynthetic capabilities as biocontrol agents and fibrinolytic enzymes (Chen et al.).

CO-CULTIVATION-, GENOME-, AND MODERN METABOLOMICS-BASED BIOPROSPECTING APPROACHES IN ACTINOBACTERIA

Actinobacteria have widely differing genome sizes, ranging from 1 to 12 Mb (Větrovský and Baldrian, 2013), where biologically active compounds are genetically encoded as biosynthetic gene clusters (BGCs). The advancement in Next-Generation Sequencing (NGS) technologies enhanced the understanding of secondary metabolite biosynthesis potentials of Actinobacteria (Nouioui et al., 2018). The genomic analysis revealed Actinobacteria capable of producing many more compounds than were observed in *in vitro* culture, indicating many of these BGCs are silent or weakly expressed under standard laboratory conditions.

Given that microbes commonly coexist in diverse communities in nature, microbes interact with each other *via* production of potentially useful bioactive secondary metabolites. Co-cultivation is an effective approach to simulate authentic circumstances in the environment. This approach has been shown to activate the silent genes and increase the yield of useful compounds by culturing two or more microorganisms in the same environment (Rateb et al., 2013). Herein, Yu et al. demonstrated co-culture of *Streptomyces rochei* MB037 with the gorgonian-derived fungus *Rhinocladiella similis* 35, which led to the isolation of three novel antibacterial compounds.

The ability to unravel the whole genomic sequences of *Streptomyces* strains has enabled the pleiotropic regulation or effective manipulation of regulatory genes in pathway-specific *Streptomyces* (Van Der Heul et al., 2018). Hou et al. revealed a potential role of a novel regulatory family, LmbU, to be used for yield enhancement of lincomycin from *Streptomyces*. Clearly, the enhancement of our understanding on the regulation of specialized metabolic gene clusters is the key to yielding improvement of a target compound for large-scale manufacturing in Actinobacteria. Despite the advances of bioinformatic prediction tools, new genomes are increasingly becoming available for identification in newly isolated microbial strains. Analytical chemistry techniques are indispensable in uncovering the full biosynthetic potential of microbes with use of hyphenated techniques, particularly high-resolution mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) for systematic assessment of novel molecules (Goodfellow and Fiedler, 2010). Schneider et al. identified a new analog of geninthiocin, a thiopeptide antibiotic, named as geninthiocin B, and its BGCs from a *Streptomyces* sp. derived from a lichen *Lepidostroma yunnana* sp. nov. sample via genome mining coupled with MS- and NMR-based metabolomic approaches.

CONCLUSION

In summary, this Research Topic enhances our knowledge on the immense biological potential of Actinobacteria. The fact that this bacteria can easily be found in diverse ecosystems, including caves, coastal marshes, and marine sponges, further signifies the irreplaceable role of Actinobacteria in the field of biotechnology and medicine. Furthermore, the need for the development of new and effective bioprospecting tools is important in expediting the discovery process of potentially novel compounds from these biologically active Actinobacteria. Only the combination of technologies between microbiology, molecular biology, and analytical chemistry will continue to uncover the vast hidden scaffolds for novel bioactive secondary metabolites produced by Actinobacteria.

AUTHOR CONTRIBUTIONS

L-HL, B-HG, and K-GC contributed to the literature review and writing of the project. The project was founded by L-HL. All authors contributed to the article and approved the submitted version.

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REFERENCES

Abdel-Mageed, W. M., Milne, B. F., Wagner, M., Schumacher, M., Sandor, P., Pathom-Aree, W., et al. (2010). Dermacozines, a new phenazine family from deep-sea dermacocci isolated from a Mariana Trench sediment. *Org. Biomol. Chem.* 8, 2352–2362. doi: 10.1039/c001445a

Antoraz, S., Santamaria, R. I., Diaz, M., Sanz, D., and Rodriguez, H. (2015). Toward a new focus in antibiotic and drug discovery from the Streptomyces arsenal. *Front. Microbiol.* 6:461. doi: 10.3389/fmicb.2015.00461

Atalan, E., Manfio, G. P., Ward, A. C., Kroppenstedt, R. M., and Goodfellow, M. (2000). Biosystematic studies on novel streptomycetes from soil. *Antonie Van Leeuwenhoek* 77, 337–353. doi: 10.1023/A:1002682728517

Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.-P., et al. (2016). Taxonomy, physiology, and natural products of Actinobacteria. *Microbiol. Mol. Biol. Rev.* 80, 1–43. doi: 10.1128/MMBR.00019-15

Bull, A. T., and Goodfellow, M. (2019). Dark, rare and inspirational microbial matter in the extremobiosphere: 16 000 m of bioprospecting campaigns. *Microbiology* 165, 1252–1264. doi: 10.1099/mic.0.000822

Bull, A. T., Stach, J. E., Ward, A. C., and Goodfellow, M. (2005). Marine actinobacteria: perspectives, challenges, future directions. *Antonie Van Leeuwenhoek* 87, 65–79. doi: 10.1007/s10482-004-6562-8

Bull, A. T., Ward, A. C., and Goodfellow, M. (2000). Search and discovery strategies for biotechnology: the paradigm shift. *Microbiol. Mol. Biol. Rev.* 64, 573–606. doi: 10.1128/MMBR.64.3.573-606.2000

Chevrette, M. G., Carlos-Shanley, C., Louie, K. B., Bowen, B. P., Northen, T. R., and Currie, C. R. (2019). Taxonomic and metabolic incongruence in the ancient Genus Streptomyces. *Front. Microbiol.* 10:2170. doi: 10.3389/fmicb.2019.02170

Fiedler, H.-P., and Goodfellow, M. (2004). Alkaliphilic streptomycetes as a source of novel secondary metabolites. *Microbiol. Aust.* 25, 27–29. doi: 10.1071/MA04227

Fukuda, T., Matsumoto, A., Takahashi, Y., Tomoda, H., and Omura, S. (2005). Phenatic acids A and B, new potentiators of antifungal miconazole activity produced by Streptomyces sp. K03-0132. *J. Antibiotics* 58, 252–259. doi: 10.1038/ja.2005.29

Goodfellow, M., and Fiedler, H.-P. (2010). A guide to successful bioprospecting: informed by actinobacterial systematics. *Antonie Van Leeuwenhoek* 98, 119–142. doi: 10.1007/s10482-010-9460-2

Goodfellow, M., Nouioui, I., Sanderson, R., Xie, F., and Bull, A. T. (2018). Rare taxa and dark microbial matter: novel bioactive actinobacteria abound in Atacama Desert soils. *Antonie Van Leeuwenhoek* 111, 1315–1332. doi: 10.1007/s10482-018-1088-7

Harvey, A. (2000). Strategies for discovering drugs from previously unexplored natural products. *Drug Discov Today* 5, 294–300. doi: 10.1016/S1359-6446(00)01511-7

Hong, K., Gao, A.-H., Xie, Q.-Y., Gao, H. G., Zhuang, L., Lin, H.-P., et al. (2009). Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China. *Mar. Drugs* 7, 24–44. doi: 10.3390/md7010024

Mccormick, J. R., and Flärdh, K. (2012). Signals and regulators that govern Streptomyces development. *FEMS Microbiol. Rev.* 36, 206–231. doi: 10.1111/j.1574-6976.2011.00317.x

Nouioui, I., Carro, L., García-López, M., Meier-Kolthoff, J. P., Woyke, T., Kyrpides, N. C., et al. (2018). Genome-based taxonomic classification of the phylum Actinobacteria. *Front. Microbiol.* 9:2007. doi: 10.3389/fmicb.2018.02007

Ŏmura, S. (ed.). (2003). "Mode of action of avermectin," in Macrolide Antibiotics: Chemistry, Biology and Practice (New York, NY: Academic Press), 571–776.

Omura, S., Tanaka, H., Oiwa, R., Awaya, J., Masuma, R., and Tanaka, K. (1977). New antitumor antibiotics, OS-4742 A1, A2, B1, and B2 produced by a strain of streptomyces. *J. Antibiotics* 30, 908–916. doi: 10.7164/antibiotics.30.908

Rateb, M. E., Hallyburton, I., Houssen, W. E., Bull, A. T., Goodfellow, M., Santhanam, R., et al. (2013). Induction of diverse secondary metabolites in *Aspergillus fumigatus* by microbial co-culture. *RSC Adv.* 3, 14444–14450. doi: 10.1039/c3ra42378f

Sayed, A. M., Hassan, M. H., Alhadrami, H. A., Hassan, H. M., Goodfellow, M., and Rateb, M. E. (2020). Extreme environments: microbiology leading to specialized metabolites. *J. Appl. Microbiol.* 128, 630–657. doi: 10.1111/jam.14386

Takahashi, Y. (2004). Exploitation of new microbial resources for bioactive compounds and discovery of new actinomycetes. *Actinomycetologica* 18, 54–61. doi: 10.3209/saj.18_54

Umezawa, H., Maeda, K., Takeuchi, T., and Okami, Y. (1966). New antibiotics, bleomycin A and B. *J. Antibiotics* 19, 200–209.

Van Der Heul, H. U., Bilyk, B. L., Mcdowall, K. J., Seipke, R. F., and Van Wezel, G. P. (2018). Regulation of antibiotic production in Actinobacteria: new perspectives from the post-genomic era. *Nat. Prod. Rep.* 35, 575–604. doi: 10.1039/C8NP00012C

Větrovský, T., and Baldrian, P. (2013). The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLoS ONE* 8:e57923. doi: 10.1371/journal.pone.0057923

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A New Bioactive Compound From the Marine Sponge-Derived *Streptomyces* sp. SBT348 Inhibits Staphylococcal Growth and Biofilm Formation

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Staphylococcus epidermidis, the common inhabitant of human skin and mucosal surfaces has emerged as an important pathogen in patients carrying surgical implants and medical devices. Entering the body via surgical sites and colonizing the medical devices through formation of multi-layered biofilms leads to refractory and persistent device-related infections (DRIs). Staphylococci organized in biofilms are more tolerant to antibiotics and immune responses, and thus are difficult-to-treat. The consequent morbidity and mortality, and economic losses in health care systems has strongly necessitated the need for development of new anti-bacterial and anti-biofilm-based therapeutics. In this study, we describe the biological activity of a marine sponge-derived *Streptomyces* sp. SBT348 extract in restraining staphylococcal growth and biofilm formation on polystyrene, glass, medically relevant titan metal, and silicone surfaces. A bioassay-guided fractionation was performed to isolate the active compound (SKC3) from the crude SBT348 extract. Our results demonstrated that SKC3 effectively inhibits the growth (MIC: 31.25 μg/ml) and biofilm formation (sub-MIC range: 1.95–<31.25 μg/ml) of *S. epidermidis* RP62A *in vitro*. Chemical characterization of SKC3 by heat and enzyme treatments, and mass spectrometry (HRMS) revealed its heat-stable and non-proteinaceous nature, and high molecular weight (1258.3 Da). Cytotoxicity profiling of SKC3 *in vitro* on mouse fibroblast (NIH/3T3) and macrophage (J774.1) cell lines, and *in vivo* on the greater wax moth larvae *Galleria mellonella* revealed its non-toxic nature at the effective dose. Transcriptome analysis of SKC3 treated *S. epidermidis* RP62A has further unmasked its negative effect on central metabolism such as carbon flux as well as, amino acid, lipid, and energy metabolism. Taken together, these findings suggest a potential of SKC3 as a putative drug to prevent staphylococcal DRIs.

Keywords: marine sponges, *Streptomyces*, Staphylococci, device-related infections, bioassay-guided fractionation, transcriptome

INTRODUCTION

Surgical implants and medical devices have greatly assisted in improving the survival and recovery of patients from physical ailments (Vinh and Embil, 2005). However, they also are ideal niches for colonization and biofilm formation by microorganisms from patient's own skin, healthcare workers' skin, or hospitalized settings (Percival et al., 2015). Biofilms are networks of microorganisms that are entrapped in a self-produced gluey matrix made up of polysaccharides, proteins, lipids, and eDNA (Otto, 2009; Flemming and Wingender, 2010). Microbes in biofilms exhibit 10–1000-fold increased resistance to antibiotics and host immune systems; and a number of mechanisms are supposed to contribute to this phenomenon such as the presence of biofilm matrix itself, slow growth rate and persister cell formation, efflux pumps, plasmid exchange, target mutations, and antibiotic-modifying enzymes etc. (Stewart and Costerton, 2001; Hall-Stoodley et al., 2004; Percival et al., 2011; Rajput et al., 2018). Current treatment of biofilm based device-related infections (DRIs) involves complete removal of the infected implant or device by a surgical procedure followed by prolonged antibiotic treatments (Otto, 2012). Biofilm based infections thus, lead to increased patient morbidity and mortality, and increased health care costs (Shida et al., 2013; Kleinschmidt et al., 2015; Leary et al., 2017).

The majority of the DRIs reported till date are a consequence of biofilm formation by coagulase negative (e.g., *Staphylococcus epidermidis*) and positive (e.g., *S. aureus*) staphylococci (Mack et al., 2007; Becker et al., 2014; Windolf et al., 2014). Predominantly, *S. epidermidis* an inhabitant of skin and mucosa is the leading cause of nosocomial and DRIs (Otto, 2009; Franca et al., 2012; Namvar et al., 2014; Sabate Bresco et al., 2017). The development of complications like catheter-related blood stream infections, prosthetic joint infections, early onset neonatal sepsis etc., and the rapid emergence of drug-resistant staphylococcal strains in hospital and community settings has challenged the effectiveness of current therapeutic regimes (Barros et al., 2014; WHO, 2014; Sakimura et al., 2015; Widerstrom, 2016). Therefore, it is imperative to develop novel antibacterial and anti-biofilm-based therapeutics for management of the hard-to-treat staphylococcal infections (Bjarnsholt et al., 2013).

Marine bioprospecting has gained much attention in the recent years owing to its massive chemical and biological diversity (Mayer et al., 2010; Gerwick and Moore, 2012; Martins et al., 2014; Thompson et al., 2017). A variety of bioprospecting techniques (including cultivation-dependent to independent approaches) have been described so far towards harnessing the bioactive potential of the marine realm (Abdelmohsen et al., 2015; Kodzius and Gojobori, 2015; Indraningrat et al., 2016). Particularly, marine sponges and their associated actinomycetes are abundant reserves of novel natural products with distinct biological activities of pharmaceutical importance (Thibane et al., 2010; Abdelmohsen et al., 2014a, 2015, 2017). A wide spectrum of anti-staphylococcal compounds and extracts possessing antibacterial and/or anti-biofilm activities have been reported from marine sponges and microbes (Rahman et al., 2010; Stowe et al., 2011; Beau et al., 2012; Palomo et al., 2013; Gomes et al., 2014; Balasubramanian et al., 2017).

A preliminary anti-biofilm screening (against the model isolate *S. epidermidis* RP62A) with 50 different organic extracts obtained from solid and liquid batch fermentations of 25 different marine sponge-derived actinomycetes, led to the identification of the bioactive extract from *Streptomyces* sp. SBT348. Marine sponge-derived *Streptomyces* sp. SBT348 is a Gram-positive bacterium that was previously shown to possess distinct metabolomic and rich chemistry profiles with strong biological activities (Cheng et al., 2015, 2017). In this study, bioassay-guided fractionation was performed to unravel the active component(s) in the SBT348 extract. The most active compound SKC3 was evaluated further for growth and biofilm inhibition on various *S. epidermidis*, *S. aureus,* and *Pseudomonas aeruginosa* strains. Results obtained highlighted the specific anti-biofilm nature of SKC3 with high potency and non-toxic nature. Chemical analysis revealed the heat-stable, non-proteinaceous, and high-molecular weight of SKC3 (1258.3 Da). Finally, data from transcriptome analysis revealed the regulation of expression of several genes related to carbon, amino-acid, proteins, lipids, nucleotides, and energy metabolism suggesting the possible interference of SKC3 with global metabolism of staphylococci.

MATERIALS AND METHODS

Instrumentation

Flash chromatography was done on an Interchim Puri-Flash 430 instrument (ultra performance flash purification) connected to an Interchim flash ELSD (Montlucon, France).

Semi-preparative HPLC of the active fraction was perfomed with Agilent 1100 series (Waldbronn, Germany) using Gemini-NX5u-C18-110A column (250 × 10 mm, Phenomenex, United States) and detection at 250 nm. The following gradient was applied solvent A: water and solvent B: acetonitrile. Separation method: solvent B 20% for 4 min, 40% for 11 min, 40 to 50% in 5 min, 50 to 90% in 1 min, and again to 20% in 4 min; maximum pressure of 400 bar and a flow rate of 4 ml/min.

Purity of the compound was determined with analytical HPLC system but with Gemini-NX5u-C18-110A column (250 × 4.60 mm, Phenomenex, United States). Separation method: solvent B 5% at 0 min, 5 to 100% for 25 min, 100% for 1 min, 100 to 50% in 2 min, and again to 5% in 2 min; maximum pressure of 400 bar; flow rate of 1 ml/min; wavelength of 250 nm.

Fourier transform-infrared spectroscopy (FT-IR) of SKC3 were conducted using Jasco FT/IR-6100 spectrometer with an ATR unit (Groß-Umstadt, Germany) at room temperature.

Mass spectrometry measurements were performed using normal electrospray ionization (ESI; in positive mode) in a micrOTOF-QIII mass spectrometer (Bruker Daltonics, Billerica, MA, United States) coupled to an Agilent 1100 HPLC system. ESI was operated with a capillary voltage of 4.5 KV. Nitrogen at 200°C and a flow rate of 7 l/min was used as the desolvation gas. Mass spectral data was obtained over a range of 50–3500 *m/z*.

Scanning electron microscopy (SEM) was done with JEOLJSM-7500F (Japan) with field emission gun system.

Bacterial Strains and Culture Conditions

Bacterial strains used in the work are mentioned in **Table 1**. *Streptomyces* sp. SBT348 was grown in ISP2 medium (4 g/l yeast extract, 10 g/l malt extract, and 4 g/l glucose in artificial sea water) at 30°C. All other strains in the study were cultured in Tryptic Soy Broth (TSB; Becton Dickinson) (17.0 g/l pancreatic digest of casein, 3.0 g/l peptic digest of soybean meal, 5.0 g/l sodium chloride, 2.5 g/l dipotassium hydrogen phosphate, and 2.5 g/l glucose) and incubated at 37°C.

Large Scale Fermentation and Extract Preparation

A total of 1,000 ISP2 agar plates (prepared with artificial sea water) were inoculated with a week-old liquid culture of *Streptomyces* sp. SBT348, respectively, and were incubated at 30°C for 10 d (batch fermentation). Agar with bacterial biomass was cut into small pieces and transferred into 1 l of ethyl acetate. The solutions were subjected to shaking at 175 rpm in a shaker overnight. Subsequently, the macerations were filtered, and the filtrates were evaporated *in vacuo* to obtain the dried SBT348 organic extract. Agar plates without the actinomycetes were extracted in a similar manner and this was the medium control for the bioactivity testing. Extracts were dissolved in DMSO (final concentration 3.75% v/v) and used for *in vitro* assays. Additionally, SEM was done for the *Streptomyces* sp. SBT348 10th day culture on the ISP2 agar plate. The SEM protocol has been described below.

Bioassay Guided-Fractionation for Isolation for Active Component(s)

A total of 1.2 g of the dried extract obtained was subjected to fractionation using a flash chromatography with a cyclohexane/ethyl acetate/methanol gradient eluent, yielding 10 major fractions. After biological evaluation of each major fraction *in vitro*, against the biofilm formation of *S. epidermidis* RP62A, the active fraction Fr 7 was found. Fr 7 was sub-fractionated by semi-preparative HPLC and this yielded seven sub-fractions (including the bioactive SKC1, SKC2, SKC3, SKC4, and SK7). The bioactive fraction was further purified on HPLC to yield the bioactive compound SKC3. Pure compound SKC3 was dissolved in DMSO (final concentration 3.75% on cells) or stored dry in amber colored vials at −80°C to ensure stability.

Characterization of the Active Compound SKC3

Stability of Compound to Heat and Enzyme Treatments

SKC3 at the respective effective concentrations was subjected to heat (100°C for 1°h; followed by cooling on ice) and enzymatic (proteinase K and trypsin; final concentration of 1 mg/ml, 37°C for 1 h) treatments. As controls, DMSO (final concentration of 3.75%) was subjected to similar heat and enzymatic treatments. For each of the treatments, the growth and biofilm inhibitory effects of treated and untreated SKC3 were assessed using the microtiter 96-well plate assay against *S. epidermidis* RP62A. Each data point is composed of three independent cultures performed in duplicates.

Biofilm Assay and MIC Determination

Biofilm assay was perfomed as previously described (Balasubramanian et al., 2017). Bacterial strains ($OD_{600} \sim 0.05$ in TSB) were incubated in the presence of SBT348 extract or SKC3 at different concentrations at 37°C (for *S. epidermidis* and *P. aeruginosa*) or 30°C (for *S. aureus*) for 24 h. Experimental controls included bacteria treated with ISP2 medium extract or DMSO and TSB without bacteria. MIC was determined against the various pathogenic bacterial strains in this microbroth dilution assay according to CLSI protocols. OD_{630} values were used to determine the MICs. MIC was determined as the concentration of the test substance where the lowest OD_{630} values were recorded with no visible bacterial growth. After OD_{630} measurement, the planktonic bacteria were discared by rinsing with sterile 1× PBS (sterile) and biofilm cells were heat fixed at 65°C for 1 h. Plates were then stained with 0.3% crystal violet for 5 min, washed thrice with sterile double-distilled water and air-dried briefly. Finally, OD_{492} measurements determined the extent of biofilm inhibition in test wells in comparison with control. *S. epidermidis* (ATCC12228) and *S. carnosus* TM300 were the biofilm negative strains used in the experiment.

For studying the effect on existing or pre-formed biofilms, biofilms were established shortly before the experiment with the above protocol. Formed biofilms were then treated with fresh TSB (control) or the test substance at their respective final concentrations and incubated further at 37°C or 30°C for 24 h. The extent of biofilm eradication was assessed with the crystal violet assay. $NaIO_4$ that digests the biofilm matrix (polysaccharides) was used as the positive control in the experiment.

Growth Curve Studies

The antagonistic effect of SBT348 extract and SKC3 on the growth of *S. epidermidis* RP62A was determined by growth curve measurements (Nithya et al., 2010). Briefly, SBT348 extract or SKC3 (MIC and $MBIC_{90}$) were added to tubes containing bacteria (initial OD_{600} of 0.1). Tubes were incubated at 37°C at 200 rpm. Bacterial growth was monitored for every 2 h up to 24 h by optical density and CFU measurements (every 4 h). TSB medium devoid of the bacteria was used as the negative control while medium extract or DMSO treated bacteria served as the appropriate controls in the experiment. Three independent cultures were used in this experiment to ensure reproducibility of results.

Anti-biofilm Effect on Different Surfaces

The anti-biofilm effect of SBT348 extract and the compound SKC3 was studied at their respective BICs on different surfaces; glass cover slips (diameter of 12 mm), medically relevant titan metal plates (diameter of 1.5 cm; University clinic for dental, oral and jaw diseases, Würzburg, Germany), and silicone

TABLE 1 | Strains used in this study.

Strain	Origin	Relevant characteristics	Reference and/or source
Streptomyces sp. SBT348	Marine sponge-derived actinomycetes strain[#]	Filamentous, sporulating	Cheng et al., 2015
Staphylococcus epidermidis RP62A	Reference strain isolated from intra-vascular catheter associated sepsis	+++	ATCC collection
Staphylococcus epidermidis O-47	Clinical isolate from septic arthritis	++	Heilmann et al., 1996
Staphylococcus epidermidis 1457	Clinical isolate from a patient with infected central venous catheter	+++	Mack et al., 1992
Staphylococcus epidermidis ATCC 12228	Non-infection associated strain	−−−	ATCC collection
Staphylococcus carnosus TM300	Meat starter culture	−−−	Rosenstein et al., 2009
Staphylococcus aureus Newman	MSSA isolate from osteomyelitis patient	+	Lipinski et al., 1967
Staphylococcus aureus USA300 Lac*	CA-MRSA isolate from a wrist abscess	+	McDougal et al., 2003
Staphylococcus aureus RF122	Bovine mastitis isolate	−	Fitzgerald et al., 2001
Staphylococcus aureus Mu50	Human MRSA isolate from surgical wound infections, vancomycin-resistant	−	Kuroda et al., 2001
Staphylococcus aureus COL	Human MRSA isolate	−	Dyke et al., 1966
Pseudomonas aeruginosa PAO1	Clinical isolate from wound	+++	Dr. Vinay Pawar, Braunschweig, Germany
Pseudomonas aeruginosa PA14	Clinical isolate from burn wound	+++	Dr. Vinay Pawar, Braunschweig, Germany

[#]*Isolated from Mediterranean sponge Petrosia ficiformis that was collected offshore Pollonia, Milos, Greece (N36.76612°; E24.51530°), May 2013 (GeneBank accession No. KP238417). +++ Strong biofilm former, ++ moderate biofilm former; + weak biofilm former (in comparison with S. epidermidis RP62A), – no detectable biofilms under conditions tested, −−− biofilm negative phenotype. Biofilm formation was assessed in TSB medium employing the standard crystal violet biofilm formation assay.*

tubes (length 1 cm and 0.2 cm diameter; Biotronik, Berlin, Germany). Briefly, 1 ml of *S. epidermidis* RP62A (OD_{600} of 0.05) was transferred to 24-well plates (Greiner bio-one, GmbH, Germany) containing the surfaces of interest with the test substances. Control wells containing the medium extract and DMSO were maintained in parallel. Sterile controls containing the surfaces with TSB alone were included to ensure absence of contamination. All the plates were incubated at 37°C for 24 h under static conditions. Samples were then subjected to washing with sterile PBS (2×) and subjected to SEM studies.

For SEM, samples were fixed overnight with gluteraldehyde (6.25%) and washed with Sörenson buffer (100 mM KH_2PO_4 and 100 mM Na_2HPO_4). After dehydration with a series of steps with ethanol, samples were finally coated with gold by low vacuum sputter coating, and scanned in the electron microscopy unit, University of Würzburg.

TABLE 2 | Effect of SKC3 on strains used in the study.

Strain	MIC	$BIC_{>75}$
Staphylococcus epidermidis RP62A	31.25 μg/ml	3.95 μg/ml
Staphylococcus epidermidis O-47	31.25 μg/ml	7.81 μg/ml
Staphylococcus epidermidis 1457	31.25 μg/ml	15.62 μg/ml
Staphylococcus aureus Newman	31.25 μg/ml	7.81 μg/ml
Staphylococcus aureus USA300 Lac*	15.62 μg/ml	3.95 μg/ml
Staphylococcus aureus RF122	31.25 μg/ml	ND
Staphylococcus aureus COL	15.62 μg/ml	ND
Staphylococcus aureus Mu50	15.62 μg/ml	ND
Pseudomonas aeruginosa PAO1	–	–
Pseudomonas aeruginosa PA14	–	–

MIC, minimum inhibitory concentration; $BIC_{>75}$, >75% biofilm inhibitory concentration; –, inactive; ND, not determined.

Cytotoxicity Profiling

In Vitro on Cell Lines

Cytotoxicity of the purified compound SKC3 was assessed on macrophage (J774.1) and mouse fibroblast (NIH/3T3) cell lines using alamar blue assay (Huber and Koella, 1993). RPMI 1640 (1×) + Glutamax™-1 and DMEM (1×) Glutamax™-1 (Life Technologies™, United States), supplemented with 10% FCS without antibiotics, were used for culturing J774.1 and NIH/3T3 cell lines, respectively. A total of 10^5 cells/ml were seeded on 96-well plates containing SBT348 extract (62.5–500 μg/ml) or SKC3 (3.95–500 μg/ml) and the plates were incubated at 37°C with 5% CO_2 for 24 h. A total of 20 μl of alamar blue (Thermofischer Scientific, United States) was added to each well and the plates were incubated for a further period of 24 h at 37°C with 5% CO_2. Finally, the OD_{550} values of the plates were measured and normalized to OD_{630} values. Extent of cytotoxicity was measure by comparison of extract/SKC3 treated sets with the control. MeOH (toxic to the cells) was used as the positive control in the experiment. DMSO at a final concentration of 1% was used as the control.

In Vivo on *Galleria mellonella* Larvae

Galleria mellonella larvae (at their final stage) were purchased from Mouse Live Bait (Balk, Netherlands). *In vivo* toxicity of SBT348 extract and SKC3 was assessed in *G. mellonella* using the method described previously (Gibreel and Upton, 2013; Skaf et al., 2017). Healthy larvae (clear in color without the presence of any spots or pigmentation) were used in the experiment. SBT348 extract and SKC3 at their respective test concentrations were prepared in endotoxin-free PBS (Merck, Germany) (vehicle control) and were injected in the last left pro-leg of the larvae with sterile insulin pens (BD Micro-Fine™ + Demi). A total of 10 larvae were included per group. Negative controls included

the group that underwent no injection and injection with vehicle control only, while, positive control included the group injected with pure MeOH (Roth, Germany). Larval groups were incubated at 37°C in petri dishes (devoid of light). Larval survival rates were recorded every 24 h up to 120 h. Larvae that were pigmented and did not respond to touch were scored dead and *vice versa*. Experiments were repeated three independent times to ensure the reproducibility of results.

RNA Extraction, DNase Treatment, and RNA Quality Determination

Staphylococcus epidermidis RP62A (OD_{600} of 1.0) was treated with SKC3 (62.5 μg/ml) and was statically incubated in a 6-well plate at 37°C for 20 min and 3 h. Treatment with DMSO (final concentration of 3.75% v/v on the cells) served as the appropriate control in the experiment. RNAprotect bacteria reagent (Qiagen, Germany) was added at the respective time points for protection and stabilization of RNA. Subsequently, RNA isolation was done according to the customized protocol described by Franca et al. (2012). Three independent biological replicates each from a pool of three independent wells were performed in order to reduce the variability. Isolated RNA samples were subjected to treatment with Turbo DNA-free^{TM} kit (Invitrogen, United States) following manufacturer's instructions and acid phenol:chloroform:isoamylalcohol (125:24:1) (Ambion, United States). Finally, pure RNA samples obtained, were precipitated with ethanol and checked for DNA contamination by PCR for *icaA* gene (Supplementary Figure S1).

Concentration and purity of the total RNA was evaluated spectrophotometrically using NanoDrop 2000 PEQLAB GmbH (Erlangen, Germany). The ratios A_{260}/A_{280} (mean values of all the samples was 1.97) and A_{260}/A_{230} (mean values of all the samples was 2.59) were used as indicators of protein and phenol/polysachharide contamination. Total RNA quality was also assessed with an Agilent 2100 Bioanalyzer (Agilent, CA, United States). RNA integrity numbers of all samples were ∼8.0 or more.

Ribosomal RNA Depletion, Library Preparation, and Sequencing

Extracted RNA was depleted of ribosomal RNA using the Ribo-Zero rRNA Removal Kit for bacteria (Illumina) according to the manual. Depleted RNA was fragmented for 3 min at 94°C using the NEBNext Magnesium RNA Fragmentation Module. The RNA ends were repaired with two consecutive T4 PNK incubations (±ATP) and an RppH treatment. Library preparation was performed according to the NEBNext Multiplex Small RNA Library Preparation Guide for Illumina. All adapters and primers were diluted 1:4 and 15 and 16 cycles of PCR were used, respectively. No size selection was performed at the end of the protocol. A total of 12 libraries were pooled and sequenced on a NextSeq 500 with a read length of 75 nt.

Analysis of Deep-Sequencing Data

The quality of raw reads (Phred scores, amount of duplicates and adapter) were assessed using FastQC (version-0.11.31) (Andrews, 2010). In order to assure a high sequence quality, the Illumina reads in FASTQ format were trimmed with a cut-off phred score of 20 by cutadapt (version-1.15) (Martin, 2011) that also was used to remove the adapter sequences. The following steps were performed using the subcommand "create," "align," and "coverage" of the tool READemption (Forstner et al., 2014) (version 0.4.3) with default parameters. Reads with a length below 15 nt where removed and the remaining reads were mapped to the reference genome sequences (NCBI accession no. NC_002976.3 (31 January 2014)) using segemehl (Hoffmann et al., 2009). Coverage plots in wiggle format representing the number of aligned reads per nucleotide were generated based on the aligned reads and visualized in the Integrated Genome Browser (Freese et al., 2016). Each graph was normalized to the total number of reads that could be aligned from the respective library. To restore the original data range and prevent rounding of small error to zero by genome browsers, each graph was then multiplied by the minimum number of mapped reads calculated over all libraries. The differentially expressed genes were identified using DESeq2 version 1.16.1 (Love et al., 2014). In all cases, only genes with maximum Benjamini–Hochberg corrected *p*-value (p_{adj}) of 0.05 were classified as significantly differentially expressed. The data were represented as MA plots using R.

Differentially expressed genes (cutoff of *p* adjusted $\leq$0.05 and $\log_2$FC $\geq$ 2 or ≤ -2) was used to perform Gene enrichment using the R package clusterProfiler version v3.4.4 (Yu et al., 2012). Using enrich KEGG function enrichment in KEGG pathways was analyzed. Only the pathways with Benjamini–Hochberg FDR threshold $\leq$0.05 defined as significantly enrichment terms.

The RNA-Seq data presented in this work has been deposited at the NCBI Gene Expression Omnibus (Edgar et al., 2002) and can be accessed through GEO series accession number[1] GSE109983. Samples treated with SKC3 has been referred to as C3 in the submitted files.

Statistical Analysis

All the experiments were performed three independent times with technical replicates. Data are expressed as mean ± SEM. For all the comparisons, Student's *t*-test was used. For comparing different Kaplan–Meier survival curves from *in vivo G. mellonella* experiments, log-rank (Mantel-Cox) and Gehan–Breslow–Wilcoxon test was used. *p*-value <0.05 was considered as statistically significant. GraphPad Prism® version 6.01 was used for statistical analysis of experimental data.

RESULTS

Anti-biofilm Potential of *Streptomyces* sp. SBT348

The anti-biofilm potential of *Streptomyces* sp. SBT348 was identified with a preliminary anti-biofilm screening of different actinomycetes organic extracts against the strong biofilm forming *S. epidermidis* RP62A. *Streptomyces* sp. SBT348 was characterized

[1]https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109983

by its wrinkled, rough, dry, and light-yellow mycelia on ISP2 agar medium (t = 10 d). SEM analysis revealed the filamentous nature of *Streptomyces* sp. SBT348. Branched networks with the presence of extracellular polymeric substance-like materials were identified in the scanning electron micrograph (**Figure 1A**). The ethyl acetate SBT348 extract significantly reduced the biofilm formation (at 24 h) in *S. epidermidis* RP62A ($p < 0.0001$). Extract at a concentration of 62.5 μg/ml reduced the biofilm formation by ∼90% and this was designated as the BIC_{90} (90% biofilm inhibition concentration). Notably, there were no significant differences in the effect beyond this concentration (**Figure 1B**). SBT348 extract at BIC_{90} or 2×BIC_{90} did not further alter the growth pattern of *S. epidermidis* RP62A (compared to extract from ISP2 medium control) (**Figure 1C**). SBT348 extract had no antagonistic effects on pre-formed *Staphylococcus epidermidis* RP62A biofilms at any of the tested concentrations (15.62–500 μg/ml; data not shown). Cytotoxicity profiling of the extract *in vitro* on NIH/3T3 and J774.1 cell lines (**Table 3**) and *in vivo* on *G. mellonella* larvae demonstrated the non-toxic nature of the extract (**Figure 1D**). Further, no changes in the activity of the extract was observed after heat and enzymatic (proteinase K and trypsin) treatments (data not shown). This highlighted the presence of heat-stable and non-proteinaceous active proportion(s) in the extract.

Bioassay-Guided Fractionation and Characterization of the Active Compound

The bioassay-guided fractionation approach followed to identify the active component SKC3 (MIC of 31.25 μg/ml and BIC_{90} of 3.95 μg/ml) in the extract that is shown in **Figure 2A**. SKC3 was further investigated in detail in the study. The pure compound SKC3 (Supplementary Figure S2) was obtained as yellow crystalline solid and was soluble in polar solvents like water, DMSO, and MeOH. Results obtained ESI-MS analysis revealed that SKC3 had a neutral mass of approximately 1258.3 Da (**Figure 2B**). This mass was also found in the SBT348 extract (data not shown). FT-IR spectra of SKC3 revealed some significant bands at 2,936, 3,326, 1,660, and 1,072 cm^{-1}, representing the presence of –C–H– stretches, –OH, amide carbonyl group, and an ester group (**Figure 2C**). Mass search with 1258.3 Da in databases like MarinLit® and Chemspider® did not yield any relevant hits. Further, heat and

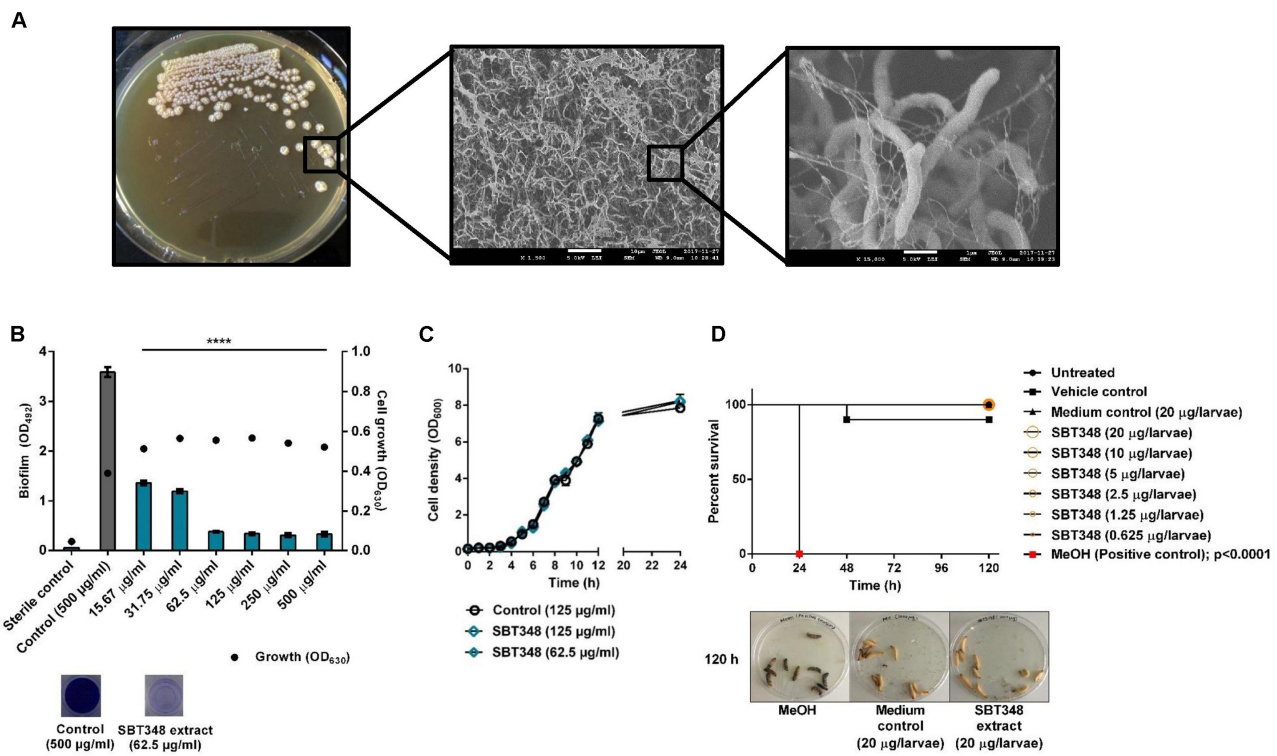

FIGURE 1 | (A) Colony morphology and scanning electron micrograph (at ×1,500 and ×15,000 magnification) of *Streptomyces* sp. SBT348 after 10 d batch fermentation on ISP2 agar plate at 30°C. Scanning electron microscopy indicates the presence of extracellular polymeric substance-like materials in *Streptomyces* sp. SBT348. Scale bar: 10 and 1 μm. **(B)** Dose-dependent inhibition of biofilm formation of *Staphylococcus epidermidis* RP62A by ethyl acetate extract of *Streptomyces* sp. SBT348. **(C)** Influence of SBT348 extract on the growth of *S. epidermidis* RP62A at BIC_{90} (62.5 μg/ml) and 2×BIC_{90} (125 μg/ml). **(D)** Kaplan–Meier survival curve of *Galleria mellonella* larvae treated with 16×BIC_{90}−0.5×BIC_{90} of SBT348 extract (0.625–20 μg/larvae). Pure MeOH (20 μl) that was toxic to larvae was used as the positive control. Control in the experiments **(B–D)** consisted of ethyl acetate extract (at the respective highest concentration) from sterile ISP2 medium which was used as growth medium for *Streptomyces* sp. SBT348. Graphs represent the mean ± SEM from three independent repetitions of experiment done with multiple technical replicates. ns, not significant; ****$p < 0.0001$.

TABLE 3 | *In vitro* cytotoxicity of SBT348 extract and SKC3 on cell lines.

Cell line	Percentage reduction in cell viability			
	500 μg/ml	250 μg/ml	125 μg/ml	3.9–125 μg/ml
NIH/3T3	41.07 ± 1.37****	NC	NC	NC
J774.1	62.91 ± 0.83****	63.90 ± 1.84****	32.78 ± 7.00***	NC

*Each data point is comprised of three independent trials done in quadruplicate. Mean ± SEM are reported. Differences in the mean were compared to the control and considered statistically significant when p (***$p < 0.001$, ****$p < 0.0001$) calculated by Student's t-test. SBT348 extract (62.5–500 μg/ml) exhibited no significant toxicity on both the cell lines tested. Positive control, MeOH reduced the cell viability of NIH/3T3 by 66.73 ± 0.59**** and J774.1 by 72.10 ± 2.16****. NC, no cytotoxicity.*

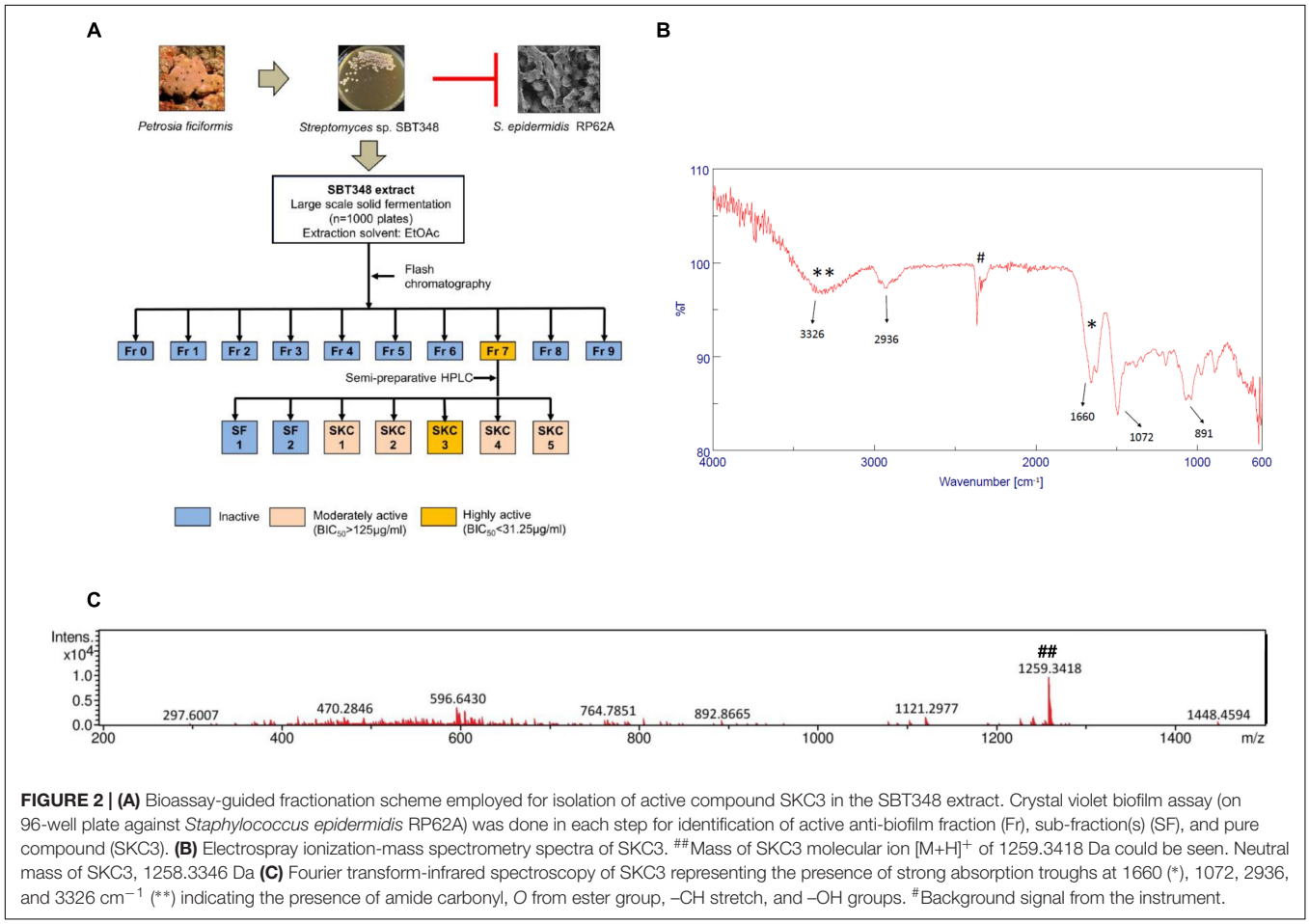

FIGURE 2 | (A) Bioassay-guided fractionation scheme employed for isolation of active compound SKC3 in the SBT348 extract. Crystal violet biofilm assay (on 96-well plate against *Staphylococcus epidermidis* RP62A) was done in each step for identification of active anti-biofilm fraction (Fr), sub-fraction(s) (SF), and pure compound (SKC3). **(B)** Electrospray ionization-mass spectrometry spectra of SKC3. ##Mass of SKC3 molecular ion $[M+H]^+$ of 1259.3418 Da could be seen. Neutral mass of SKC3, 1258.3346 Da **(C)** Fourier transform-infrared spectroscopy of SKC3 representing the presence of strong absorption troughs at 1660 (*), 1072, 2936, and 3326 cm^{-1} (**) indicating the presence of amide carbonyl, *O* from ester group, –CH stretch, and –OH groups. #Background signal from the instrument.

enzymatic treatments did not significantly alter the biological activity of SKC3 (Supplementary Figure S3). This was in line with the results obtained from the stability studies of the extract. The absence of relevant hits with the existing mass and spectral data indicates that SKC3 is likely to be a new compound. The structure elucidation of SKC3 is currently under investigation.

Antagonistic Activities of SKC3 Against Staphylococci

SKC3 displayed an MIC of 31.25 μg/ml on *S. epidermidis* RP62A and the sub-MIC concentrations (1.95–<31.25 μg/ml) effectively inhibited the biofilm formation in the crystal violet biofilm assay (**Figure 3A**). BIC_{90} value of SKC3 was 3.95 μg/ml. Interference of SKC3 (MIC) with the growth of *S. epidermidis* RP62A was further confirmed with the growth curve analysis (**Figure 3B**). Thus, presence of SKC3 at MIC, effectively inhibited bacterial growth (approximately 100-fold reduction in CFUs/ml; data not shown) while SKC3 at BIC_{90} had no significant influence. Further, SKC3 (at the highest tested concentration: 500 μg/ml) had no clearing effect on existing biofilms of *S. epidermidis* RP62A (**Figure 3C**). Complete biofilm dispersal by $NaIO_4$ (40 mM) was used a positive control in this experiment. SKC3 was also effective in inhibiting the growth and biofilm formation of other strains used in the study (**Table 2**). Noteworthy, SKC3 was more effective against MSSA, MRSA, and VRSA strains used in the study, but was ineffective against the tested Gram negative *P. aeruginosa* strains.

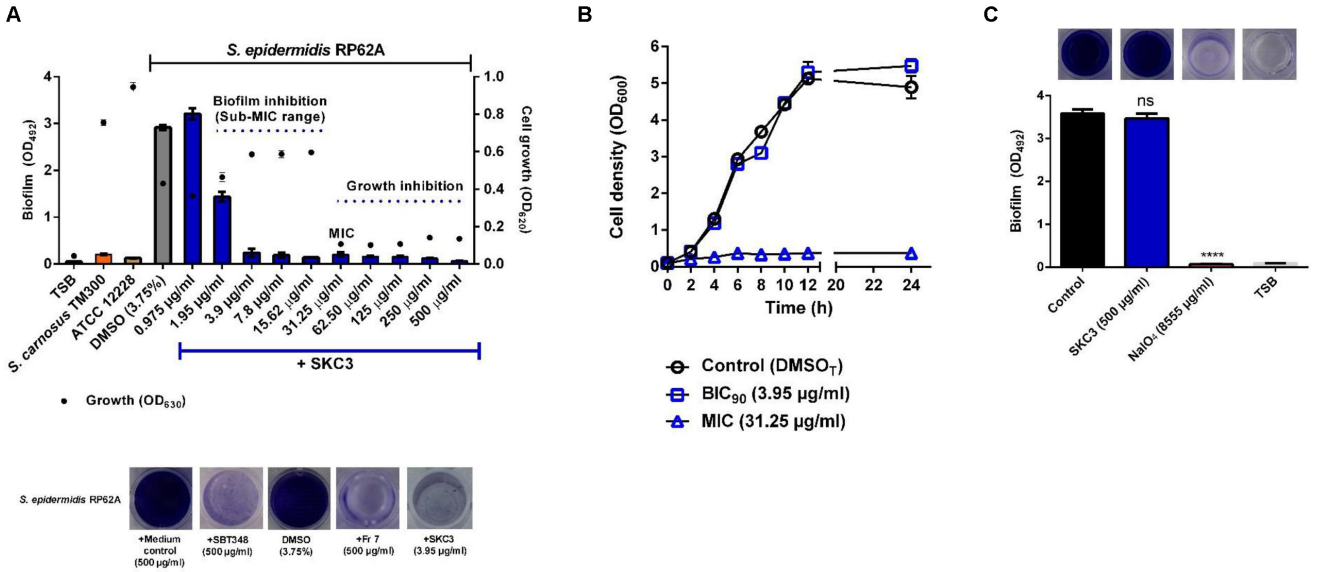

FIGURE 3 | (A) Inhibition of growth (MIC: 31.25 μg/ml) and biofilm formation (sub-MIC range: <31.25 μg/ml) of *Staphylococcus epidermidis* RP62A. Controls consisted of biofilm negative strains *S. epidermidis* ATCC 12228, *S. carnosus* TM300, DMSO treated *S. epidermidis* RP62A, and sterile TSB without bacteria. **(B)** Effect of SKC3 on growth (OD_{600}) at MIC (31.25 μg/ml) and BIC_{90} (3.95 μg/ml), respectively. DMSO treated *S. epidermidis* RP62A was the appropriate control in the growth curve analysis. **(C)** Influence of SKC3 on pre-formed (existing) biofilms of *S. epidermidis* RP62A. $NaIO_4$ that digests the polysaccharide biofilms was used as the positive control. All the experiments were repeated at least three times in multiple technical replicates. ns, not significant; ****$p < 0.0001$.

SEM Analysis

Investigation of the anti-biofilm efficacy of SKC3 at sub-MICs were further evaluated with SEM of *S. epidermidis* RP62A biofilms grown on glass, titan metal, and silicone tube surfaces. From the scanning electron micrographs, clear differences in appearance were observed in the three sterile surfaces under study. In the control sets of the surfaces (treated with DMSO; 3.75%), three-dimensional dense biofilm structures were observed. Treatment with SKC3 (BIC_{90} and $2\times BIC_{90}$) significantly reduced the biofilm formation on these surfaces and this further confirmed the results obtained from crystal violet biofilm assay (**Figure 4**). Particularly, the three-dimensional networks were absent, and the surfaces were clearly seen (between sporadic microcolonies or single cells) in the SKC3-treated sets. A closer look on the SEM images at higher magnification revealed no alterations in the cell morphology of staphylococci. These findings further point towards the anti-biofilm potential of the isolated compound SKC3.

In Vitro and *in Vivo* Toxicity of SKC3

In vitro toxicity assessment of SKC3 was done on mouse macrophage (J774.1) and fibroblast cell lines (NIH/3T3) using the alamar blue assay. Results from the cytotoxicity analysis demonstrated the non-toxic nature of SKC3 at effective concentrations (**Table 3**).

Toxicity of SKC3 was additionally assessed *in vivo* in the greater wax moth larvae, *G. mellonella*. In recent years, *G. mellonella* larvae have emerged as an interesting model system for evaluating the toxicity and efficacy of novel compounds and for studying various microbial infections (Gibreel and Upton, 2013; Aparecida Procopio Gomes et al., 2016; Skaf et al., 2017). The ease of handling, low maintenance costs, absence of ethical concerns, survival at human physiological temperatures are some of the advantages of using *G. mellonella* larvae for pre-screening of toxicity (Tsai et al., 2016). Survival rates of larvae treated with SKC3 (BIC_{90}–$200\times BIC_{90}$) are shown in **Figure 5**. None of the tested concentrations lead to death of the larvae, whereas, the positive control MeOH lead to 90% reduction in the larval survival rates. Thus, SKC3 was completely non-toxic to the larvae at the tested concentration.

Transcriptome Analyses of SKC3-Treated *S. epidermidis* RP62A

Total RNA sequencing was done for *S. epidermidis* RP62A treated with SKC3 (62.5 μg/ml) after 20 min and 3 h points. Global transcriptome analysis with the obtained RNA sequencing results revealed the existence of several differentially expressed genes upon SKC3 treatment. The differentially expressed genes were identified by setting the threshold of $\log_2$foldchange ≥ 2 or ≤ -2 with an adjusted *p*-value of <0.05 for statistical significance. From the MA plots (**Figures 6A,B**), it is evident that higher number of genes were differentially expressed (upon SKC3 treatment) after 3 h than 20 min. This was additionally confirmed in the PCA plot and a well-distributed grouping of the different biological replicates were observed (Supplementary Figure S4). According to the set threshold, a total of 31 genes representing 1.1% of the transcriptome were significantly altered in response to SKC3 after 20 min and a total of 509 genes

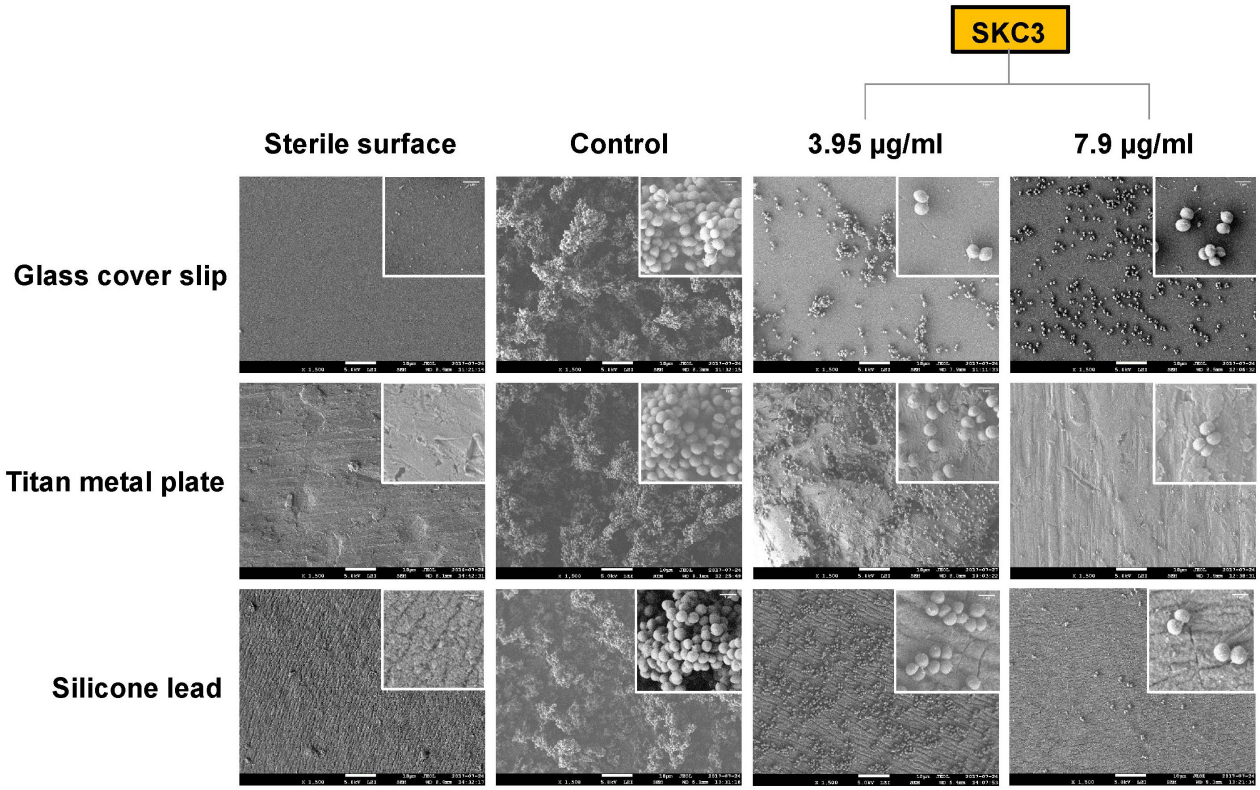

FIGURE 4 | Scanning electron micrographs of *Staphylococcus epidermidis* RP62A (24 h) biofilms on glass cover slips, titan metal plate and silicone tubes at ×1,500 magnification (scale bar: 10 µm). Treatment with SKC3 at BIC_{90} and 2×BIC_{90} significantly reduced the staphylococcal biofilm formation on these surfaces. No apparent changes in the morphology of RP62A (zoom in image) was observed at higher magnification (×10,000; scale bar: 1 µm).

representing 19.5% of the transcriptome were significantly altered in response to SKC3 after 3 h. Among these genes, 29 genes were upregulated, and 2 genes were downregulated after 20 min (Supplementary Table S1), whereas, 265 genes were upregulated, and 244 genes were downregulated after 3 h (Supplementary Table S2). After data filtering and searches in PubMed and UniProtKB, the differentially expressed genes at the two-time points were manually sorted in several categories based on their biological functions of the products they encode. Majority of the differentially regulated genes in the entire data set encoded for hypothetical proteins. Transcriptome analysis revealed that after 20 min, several of the differentially expressed genes were attributed to signal transduction mechanism, transporters, transcription, and antibiotic response-related functions (**Figure 6C**). This suggests that *S. epidermidis* RP62A responds to SKC3 by signal transduction mechanisms and by expressing several transcription, transporters, and antibiotic-stress related genes. Transcriptome analysis further revealed that after 3 h, several of the metabolic processes (pertaining to carbon, amino acid, protein, lipid, nucleotide, and energy metabolism) and transport processes were strongly affected (**Figure 6D**). Functional enrichment analysis also yielded similar antagonistic effects of SKC3 on metabolism (Supplementary Figure S5). A list of all differentially regulated metabolism-related genes and virulence genes upon SKC3 treatment after 3 h are further detailed in **Table 4**. Overall, the results from transcriptome analysis suggest that SKC3 possibly works by interference with the overall metabolism of staphylococci.

DISCUSSION

The increased use of implanted medical devices, the subsequent risk of biofilm formation on these devices and the emergence of drug-resistant strains has altogether imposed a heavy burden on patient and health care systems (Becker et al., 2014; WHO, 2014; Casillo et al., 2017). About, 5,027 anti-biofilm agents against Gram-positive and negative bacteria, and fungi have been reported between 1988 and 2017 (Rajput et al., 2018). However, up to our knowledge none of them have been successfully translated to the market for clinical and medical applications. Our research aimed at harnessing the potential of marine sponge-derived actinomycetes for discovery of novel antibacterial and anti-biofilm compounds (Abdelmohsen et al., 2014a,b; Dashti et al., 2014). Actinomycetes from marine sponges represent an untapped reservoir of a wide range of unforeseen biological compounds (Xi et al., 2012; Abdelmohsen et al., 2015; Sun et al., 2015). Previous results have demonstrated the anti-biofilm efficacy of an organic extract from *Streptomyces*

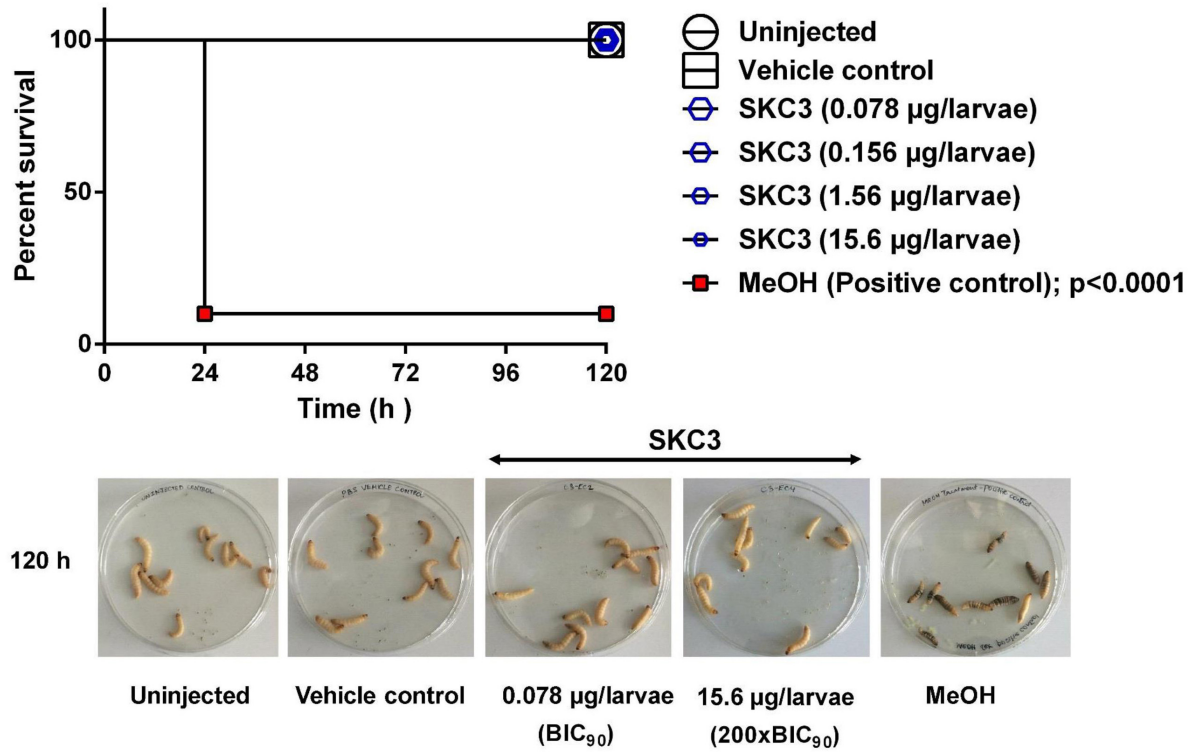

FIGURE 5 | *In vivo* toxicity evaluation of SKC3 on *Galleria mellonella* larvae. No death was observed in the larval groups treated with vehicle control (1× PBS; endotoxin-free) and SKC3 (BIC_{90} −200×BIC_{90}). MeOH treatment lead to 90% death of the larvae.

sp. SBT343 isolated from marine sponge *Petrosia ficiformis* (Balasubramanian et al., 2017). In this study, we describe the anti-staphylococcal activity of another strain *Streptomyces* sp. SBT348 isolated from the same sponge. We applied a bioassay-guided fractionation strategy to identify, isolate, and purify the active compound responsible for this activity.

Streptomyces sp. SBT348 is a filamentous Gram-positive bacterium that was previously shown to possess distinct metabolomic and rich chemistry profiles with strong biological activities (Cheng et al., 2015, 2017). SEM of the 10 d old *Streptomyces* sp. SBT348 culture used for extraction and isolation of the bioactive SKC3 indicated the presence of biofilm-like networks (**Figure 1A**). This extends the possibility of SKC3 to be a compound produced in the biofilm networks that is antagonistic to other bacteria. However, more experiments are needed to confirm the same.

Leary et al. (2017) proposed a combination of autoclave and chlorhexidine treatment for complete removal of biofilms from orthopedic materials. Alternative, coating-based strategies have been proposed to prevent this phenomenon (Windolf et al., 2014). The isolated compound SKC3 effectively inhibited the growth and biofilm formation of different staphylococcal strains (**Figure 3** and **Table 2**). Kinetics of staphylococcal biofilm formation in the presence of SKC3 revealed its action during early steps of biofilm formation between 3 and 4 h (Supplementary Figure S6). Further, the inefficacy of SKC3 against dispersing pre-formed biofilms highlights its usage in prevention of staphylococcal infections. This is advantageous, since, targeting the disassembly could lead to increased inflammatory response and severity of a disease (Franca et al., 2016). SKC3 was also shown to inhibit the staphylococcal biofilm formation on different medically relevant surfaces (glass, titan metal, and silicone tubes). The non-toxic nature of SKC3 *in vitro* (cell lines) and *in vivo* (*G. mellonella* larvae) explains its applicability as antimicrobial and anti-biofilm agents on medical devices. As a step forward, the potential of SKC3 to protect *G. mellonella* from *S. aureus* USA300 Lac* was also assessed in an independent experiment. Results obtained indicated that SKC3 could not protect the larvae from staphylococcal infection (data not shown). The exact reason behind this failure remains unclear. However, further investigations are needed to evaluate the toxicity and *in vivo* antimicrobial efficacy of SKC3 on higher *in vivo* model systems to support its usage. The huge mass (1258.3 Da), stability towards heat and enzymatic treatments, and the absence of relevant hits in several databases point towards a complex structure of SKC3. Thus, SKC3 is expected to be a new compound and further NMR spectrometric investigations to elucidate its complete structure are currently in progress.

Transcriptomics have been increasingly used for understanding the responses of staphylococci to antimicrobial agents and for obtaining insights into the antimicrobial mode of action (Sianglum et al., 2012; Chung et al., 2013; Qin et al., 2014; Wang et al., 2018). In our study, the global gene expression pattern of SKC3 treated *S. epidermidis* RP62A was studied by

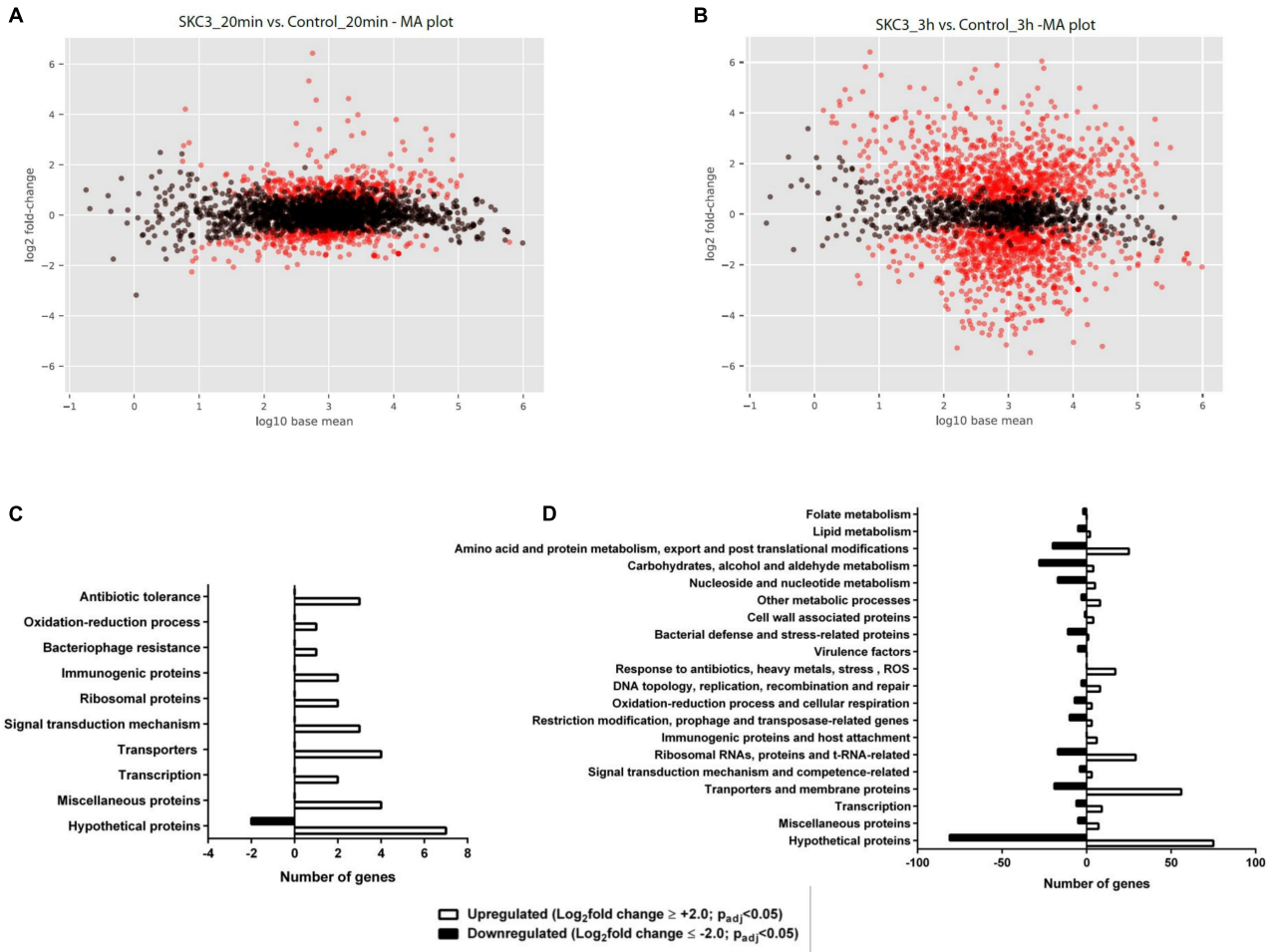

FIGURE 6 | MA plots representing the significant gene expression changes (*red dots*; p_{adj} value <0.05) in SKC3 (62.5 μg/ml) treated-*S. epidermidis* RP62A after **(A)** 20 min and **(B)** 3 h time points in comparison with control (DMSO treated-*S. epidermidis* RP62A). *Black dots* represent the insignificant gene expression changes (p_{adj} value >0.05). Histogram of differentially expressed genes in the presence of SKC3 (62.5 μg/ml) after 20 min **(C)** and 3 h **(D)**. Results are summarized based on biological process. X-axis indicates the number of differentially expressed genes in a category. Positive and negative axes represent the numbers of up- and down-regulated genes, respectively. Absolute value of $\log_2$foldchange ≥ 2 or ≤ −2 and p_{adj} value <0.05 was used as the threshold to screen the differentially expressed genes. UniProtKB was used to search for the biological function of differentially expressed genes.

RNA sequencing and transcriptome analysis (after 20 min and 3 h post treatment). Functional classification of all the genes regulated by SKC3 could be seen in **Figures 6C,D**.

Transcriptome data from early time point (20 min) indicated that genes encoding a two-component system (sensor histidine kinase and response regulator), several proteins involved in transport of macromolecules, such as ATP-binding cassette (ABC) transporters and quaternary ammonium compound efflux pumps (SugE) were significantly upregulated. ABC transporters are often involved in multi-drug resistance by serving as efflux pumps for transport of anti-infectives (Lage, 2003). SugE, a drug efflux pump belonging to the small multi-drug resistance family (SMR) was shown to be involved in resistance to a narrow range of quaternary ammonium compounds in *Escherichia coli* (Chung and Saier, 2002). However, these ABC transporters and *sugE* regulated by SKC3 have not been documented to be involved in resistance to antimicrobial compounds in *S. epidermidis* till date. Further studies are needed to understand the exact roles of these transporters and efflux pump in this organism. Thus, it could be presumed that after 20 min *S. epidermidis* RP62A recognizes SKC3 by a yet unknown two-component system and reacts by expressing a variety of transporters.

Transcriptome data from the late time point (3 h) indicated that genes encoding for hypothetical proteins were the most differentially regulated (representing 30.64% of the total differentially expressed genes after 3 h). Major fraction of the known differentially expressed genes after 3 h included the genes encoding proteins involved in global metabolism (representing 23.37% of the total differentially expressed genes), and transporters and membrane proteins (representing 14.73% of the total differentially expressed genes). In addition, bacterial stress and defense related proteins were strongly downregulated indicating the sensitivity of bacterial cells at this time point. Like the 20 min transcriptome data, several ABC

TABLE 4 | List of metabolism-related genes affected in response to SKC3 after 3 h.

Metabolic process and genes	Function	Log_2fold change	p_{adj} value
Carbon metabolism			
glmU	UDP-*N*-acetylglucosamine pyrophosphorylase	−2.1757	3.88E-44
SERP0257	Alcohol dehydrogenase zinc-containing	−2.9328	7.33E-24
fruK	1-phosphofructokinase	−2.0069	7.77E-11
hprK	HPr kinase/phosphatase	−2.3083	3.43E-37
Pgk	Phosphoglycerate kinase	−2.0399	3.02E-17
tpiA	Triosephosphate isomerase	−2.2812	2.56E-15
Pgi	Glucose-6-phosphate isomerase	−3.4822	1.38E-49
pdhA	Pyruvate dehydrogenase complex E1 component alpha subunit	−3.1290	1.06E-26
pdhB	Pyruvate dehydrogenase complex E1 component beta subunit	−3.3469	7.02E-40
pdhC	Pyruvate dehydrogenase complex E2 component dihydrolipoamide acetyltransferase	−3.5036	1.37E-27
pdhD	Pyruvate dehydrogenase complex E3 component lipoamide dehydrogenase	−2.4225	1.55E-16
Pyc	Pyruvate	−2.7047	3.23E-51
trxA	Thioredoxin	−2.3877	4.66E-11
Tkt	Transketolase	−2.3499	1.62E-104
SERP0974	Acylphosphatase	−3.7088	5.81E-45
malA	Alpha-glucosidase	−2.4798	3.93E-27
Gnd	6-phosphogluconate dehydrogenase decarboxylating	−2.3314	5.38E-91
pfkA	6-phosphofructokinase	−2.5866	9.06E-34
SERP1290	PTS system IIBC components	2.6442	1.92E-32
Tal	Transaldolase	−2.1920	4.46E-19
sceD	SceD protein	4.8221	7.26E-69
lacR	Lactose phosphotransferase system repressor	2.0861	7.09E-16
sdhA	*L*-serine dehydratase iron-sulfur-dependent alpha subunit	−2.1059	3.48E-44
SERP2112	Alcohol dehydrogenase zinc-containing	−2.1470	2.46E-14
SERP2114	PTS system IIABC components	−3.3405	5.78E-34
budA	Alpha-acetolactate decarboxylase	−2.4764	2.38E-37
budB	Acetolactate synthase catabolic	−3.4875	2.65E-55
ldh	*L*-lactate dehydrogenase	−3.3363	1.49E-48
SERP2345	Dihydroxyacetone kinase family protein	−2.0835	3.63E-14
gldA	Glycerol dehydrogenase	−2.4748	1.96E-33
SERP2354	Tributyrin esterase EstA putative	2.9073	1.37E-14
pflB	Formate acetyltransferase	−3.6275	2.36E-49
Amino acid and protein metabolism			
SERP0033	Cyclase putative	2.1197	1.70E-08
cysK	Cysteine synthase	−2.5176	1.47E-38
cysE	Serine acetyltransferase	−3.4603	1.64E-52
cysS	Cysteinyl-tRNA synthetase	−2.7266	4.95E-54
ilvE	Branched-chain amino acid aminotransferase	−2.5610	6.43E-11
SERP0349	Deoxyribodipyrimidine photolyase putative	2.1987	2.22E-18
prfB	Peptide chain release factor 2	−2.5844	5.22E-23
lgt	Prolipoprotein diacylglyceryl transferase	−2.1091	7.12E-33
gcvH	Glycine cleavage system H protein	−2.4191	2.29E-23
def	Peptide deformylase	−2.1348	2.13E-24
def-2	Polypeptide deformylase	2.1031	4.64E-59
fmt	Methionyl-tRNA formyltransferase	2.1719	7.90E-22
glnR	Glutamine synthetase repressor	−2.7030	7.01E-27
trpD	Anthranilate phosphoribosyltransferase	4.0312	1.23E-14
trpC	Indole-3-glycerol phosphate synthase	2.2689	1.50E-08
trpB	Tryptophan synthase beta subunit	2.0434	6.37E-06
argB	Acetylglutamate kinase	2.3225	6.68E-10
glyS	Glycyl-tRNA synthetase	−2.9083	1.29E-22
SERP1176	Peptidase U32 family	2.3811	1.83E-24

(Continued)

TABLE 4 | Continued

Metabolic process and genes	Function	Log_2fold change	p_{adj} value
SERP1177	Peptidase U32 family	2.6006	3.16E-30
infC	Translation initiation factor IF-3	2.3684	4.36E-17
ald	Alanine dehydrogenase	−2.7547	2.67E-09
SERP1292	Serine protease HtrA putative	−2.7124	1.23E-30
SERP1310	Dipeptidase family protein	−3.0286	8.51E-63
SERP1376	Protein export protein PrsA putative	−2.6829	7.19E-41
SERP1549	Death-on-curing family protein	2.3238	1.91E-56
leuB	3-isopropylmalate dehydrogenase	2.6290	7.14E-09
glyA	Serine hydroxymethyltransferase	−2.3637	4.36E-33
secY	Preprotein translocase SecY subunit	2.5474	1.38E-20
SERP2034	Amino acid permease family protein	3.3899	3.46E-17
SERP2043	Peptidase M42 family	−3.4218	1.54E-47
cysJ	Sulfite reductase (NADPH) flavoprotein alpha-component	−2.1931	2.60E-23
cysH	Phosophoadenylyl-sulfate reductase	−2.5134	3.48E-28
arcA	Arginine deiminase	−2.3875	1.56E-39
sepA	Extracellular elastase precursor	2.3264	4.49E-17
SERP2272	Peptide methionine sulfoxide reductase putative	2.6559	5.30E-18
SERP2276	SecA family protein	2.2325	1.96E-31
hisH	Amidotransferase HisH	2.5488	6.82E-14
hisB	Imidazoleglycerol-phosphate dehydratase	3.4466	1.62E-17
hisD	Histidinol dehydrogenase	2.6795	3.23E-18
hisG	ATP phosphoribosyltransferase	2.8203	5.58E-10
SERP2338	Peptide synthetase	2.4122	9.72E-67
SERP2364	Peptidase M20/M25/M40 family	2.6489	1.23E-16
SERP2375	Diaminopimelate epimerase family protein	2.6285	3.49E-18
serS	Seryl-tRNA synthetase	−2.7533	3.76E-36
Lipid metabolism			
SERP0309	Lipase/esterase putative	2.2785	1.41E-11
fabH	3-oxoacyl-(acyl-carrier-protein) synthase III	−2.1260	4.25E-10
plsX	Fatty acid/phospholipid synthesis protein PlsX	−2.1079	1.80E-60
acpP	Acyl carrier protein	−2.8822	7.31E-15
SERP1001	DegV family protein	−2.0926	2.95E-41
SERP2337	4-phosphopantetheinyl transferase family protein	2.9365	5.06E-42
SERP2523	Glycerophosphoryl diester phosphodiesterase UgpQ putative	−2.4642	8.93E-19
Nucleotide and energy metabolism			
prsA	Ribose-phosphate pyrophosphokinase	−2.2578	1.40E-57
SERP0371	ExsD protein	6.0454	4.40E-29
SERP0372	6-pyruvoyl tetrahydrobiopterin synthase putative	5.8808	9.47E-31
SERP0373	exsB protein	5.7653	9.79E-77
folD	Methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase	−2.5692	3.02E-88
purE	Phosphoribosylaminoimidazole carboxylase catalytic subunit	−4.1495	5.44E-70
purK	Phosphoribosylaminoimidazole carboxylase ATPase subunit	−3.9826	4.21E-85
purC	Phosphoribosylaminoimidazole-succinocarboxamide synthase	−4.4226	2.61E-60
purS	Phosphoribosylformylglycinamidine synthase PurS protein	−4.5392	7.95E-95
purQ	Phosphoribosylformylglycinamidine synthase I	−4.5973	2.90E-78
purL	Phosphoribosylformylglycinamidine synthase II	−4.2042	6.09E-107
purF	Amidophosphoribosyltransferase	−4.2181	3.25E-136
purM	Phosphoribosylformylglycinamidine cyclo-ligase	−4.3477	2.30E-69
purN	Phosphoribosylglycinamide formyltransferase	−3.8846	2.36E-80
purH	Phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	−3.7319	6.33E-113
purD	Phosphoribosylamine – glycine ligase	−2.6846	2.00E-23
purB	Adenylosuccinate lyase	−2.9759	1.15E-62
cdd	Cytidine deaminase	−2.0209	1.32E-49

(Continued)

TABLE 4 | Continued

Metabolic process and genes	Function	Log_2fold change	p_{adj} value
thiI	Thiamine biosynthesis protein ThiI	2.6751	1.97E-48
Fhs	Formate – tetrahydrofolate ligase	−3.4566	2.24E-39
upp	Uracil phosphoribosyltransferase	−2.2580	1.20E-35
adk	Adenylate kinase	2.5524	1.03E-10
SERP1865	Inosine-uridine preferring nucleoside hydrolase family protein	−2.0061	2.04E-09
rbsK	Ribokinase	−2.2333	3.77E-29
Other Metabolic processes			
SERP0250	Acetyltransferase GNAT family	−2.4617	3.97E-40
SERP0461	Glyoxalase family protein	2.8462	1.43E-08
SERP0556	Fumarylacetoacetate hydrolase family protein	−2.1019	4.39E-07
SERP0561	Hydrolase haloacid dehalogenase-like family	−2.2982	1.85E-41
SERP1178	*O*-methyltransferase family protein	2.3694	4.74E-47
SERP1280	Aminotransferase class V	2.7479	3.78E-43
SERP1918	Amidohydrolase family protein	2.1722	5.93E-10
SERP1996	Acetyltransferase GNAT family	2.0172	4.71E-05
SERP2054	Glycosyl transferase group 1 family protein	2.5257	1.06E-23
SERP2299	*N*-acetyltransferase family protein	2.6212	2.47E-16
SERP2547	YjeF-related protein	3.2688	4.13E-16

Only genes with a log_2fold change ≥ 2 or ≤ −2 and a p_{adj} value <0.05 were included.

transporters, ion transporters, drug transporters, and efflux pump were influenced in the presence of SKC3 after 3 h. These are speculated to be the typical responses of *S. epidermidis* to toxic agents (Putman et al., 2000; Cecil et al., 2011). However, the specific effects of SKC3 on metabolism are much stronger. Interference with metabolism involved differential regulation of genes involved in carbon metabolism (down regulation of genes related to processes of glycolysis, gluconeogenesis, pentose phosphate pathway, glycerol, fructose, and lactose metabolism), lipid metabolism (repression of genes related to fatty acid biosynthesis and phospholipid metabolism), nucleotide and energy metabolism (repression of several genes related to purine biosynthetic process from *de novo* and salvage pathways), and amino acid, and protein metabolism (repression in biosynthesis of cysteine, isoleucine, leucine, valine, glycine, glutamine, and lipoproteins; repression of alanine and arginine catabolism; up regulation in biosynthesis of tryptophan, arginine, and histidine).

Particularly, the *purEKCSQLFMNHD* operon, *purA*, *purB,* and *purR* genes responsible for *de novo* purine biosynthesis were the heavily downregulated genes in SKC3-treated *S. epidermidis* RP62A (Supplementary Figure S7). Purine biosynthesis is vital for various cellular processes and bacterial growth. The purine biosynthetic process involves conversion of 5′-phosporibosyl-α-pyrophosphate (PRPP) to inositol monophosphate (IMP). IMP is then converted to adenosine monophosphate (AMP) and guanosine monophosphate (GMP) in independent steps. Purine biosynthesis is costly to the cell involving the consumption of ATPs in multiple steps. The repression of purine biosynthesis by SKC3 could lead to reduced energy production, amino acid biosynthesis, and DNA synthesis in staphylococci. Thus, the staphylococcal cells could get metabolically stressed in the presence of SKC3. Further, defects in purine biosynthesis are known to negatively affect the biofilm formation. Mutations in purine biosynthetic genes of *Photorhabdus temperata* (*purL*), *Streptococcus sanguinis* (*purB* and *purL*), *Burkholderia* sp. (*purL*, *purM*, and *purT*), and *Pseudomonas fluorescens* Pf0-1 (*purD*, *purH*, *purL*, *purC*, *purM*, *purF*, *purK*, and *purE*) led to a defect in the biofilm formation of these bacteria (Ge et al., 2008; Ruisheng and Grewal, 2011; Kim et al., 2014a,b; Yoshioka and Newell, 2016). *purR* in staphylococci was also previously reported to indirectly regulate biofilm formation through an indirect mechanism (Mack et al., 2007). In a recent study, mutants of *purEKCSQLFMNHD* operon, *purA*, *purB,* and *purR* genes obtained by genome-wide screening of transposon library in *S. aureus* USA300 were shown to possess altered microcolony (biofilm) formation and growth on an agar plate model (Wermser and Lopez, 2018). Interestingly, there was no direct remarkable influence of SKC3 on the transcription of *ica* locus (3 h) encoding the polysaccharide intercellular adhesin (PIA) responsible for biofilm formation in *S. epidermidis*. Instead, virulence factors like the phenol soluble modulins α and β (proinflammatory cytolysins) involved in biofilm structuring and detachment processes (Otto, 2009; Fey and Olson, 2010) and hemolysin (putative) were down-regulated. From these findings, it could be perceived that SKC3 could possibly repress the staphylococcal biofilm formation via downregulation of purine biosynthetic genes. However, further research on the regulatory linkage between purine metabolism and biofilm formation is needed.

Overall, it is evident from the data that SKC3 has multiple metabolic targets including several unexploited pathways. The antagonistic effect of SKC3 on staphylococcal metabolism is in line with the findings from previously reported antibiotic compounds. Various natural products and anti-staphylococcal compounds like betulinaldehyde, benzimidazole

derivatives, bisquaternary bisnapthalimide, isoquinolines, lupeol, rhodomyrtone, and stigmasterol were previously shown to interfere with staphylococcal metabolism (involving interference with processes like carbon metabolism, DNA metabolism and replication, fatty acid biosynthesis, purine metabolism, synthesis of ribosomal proteins, transport of compounds, etc.) (Cecil et al., 2011, 2015; Menzel et al., 2011; Sianglum et al., 2012; Chung et al., 2013; Adnan et al., 2017; Kong et al., 2018).

In conclusion, the anti-biofilm compound SKC3 was isolated from the chemically diverse strain *Streptomyces* sp. SBT348 with the aid of bioassay guided-fractionation. SKC3 exhibited antagonistic effects against growth and biofilm formation (at concentrations less than MICs) of several staphylococcal strains tested without exhibiting apparent *in vitro* and *in vivo* toxicity. Transcriptome analysis revealed the interference of SKC3 with several metabolic processes (carbon, protein, lipid, nucleotide, and energy metabolism) of staphylococci. However, further experimental data is needed to elucidate the exact anti-staphylococcal mode of action of SKC3.

AUTHOR CONTRIBUTIONS

TO, UA, UHO, UHU, KF, and WZ conceived and designed the experiments. SB, JS, and RB performed the experiments. SB, JS, RB, and TO analyzed the data. SB, JS, RB, UHO, KF, UHU, WZ, UA, and TO prepared the manuscript. SB, TO, UA, UHO, UHU, KF, and WZ 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.2018.01473/full#supplementary-material

REFERENCES

Abdelmohsen, U. R., Balasubramanian, S., Oelschlaeger, T. A., Grkovic, T., Pham, N. B., Quinn, R. J., et al. (2017). Potential of marine natural products against drug-resistant fungal, viral, and parasitic infections. *Lancet Infect. Dis.* 17, e30–e41. doi: 10.1016/S1473-3099(16)30323-1

Abdelmohsen, U. R., Bayer, K., and Hentschel, U. (2014a). Diversity, abundance and natural products of marine sponge-associated actinomycetes. *Nat. Prod. Rep.* 31, 381–399. doi: 10.1039/c3np70111e

Abdelmohsen, U. R., Grkovic, T., Balasubramanian, S., Kamel, M. S., Quinn, R. J., and Hentschel, U. (2015). Elicitation of secondary metabolism in actinomycetes. *Biotechnol. Adv.* 33, 798–811. doi: 10.1016/j.biotechadv.2015.06.003

Abdelmohsen, U. R., Yang, C., Horn, H., Hajjar, D., Ravasi, T., and Hentschel, U. (2014b). Actinomycetes from Red Sea sponges: sources for chemical and phylogenetic diversity. *Mar. Drugs* 12, 2771–2789. doi: 10.3390/md12052771

Adnan, S., Ibrahim, N., and Yaacob, W. A. (2017). Transcriptome analysis of methicillin-resistant *Staphylococcus aureus* in response to stigmasterol and lupeol. *J. Glob. Antimicrob. Resist.* 8, 48–54. doi: 10.1016/j.jgar.2016.10.006

Andrews, S. (2010). *FastQC: A Quality Control Tool for High Throughput Sequence Data*. Available at: http://www.bioinformatics.babraham.ac.uk?/projects/fastqc/ [accessed October 6, 2011].

Aparecida Procopio Gomes, L., Alves Figueiredo, L. M., Luiza Do Rosario Palma, A., Correa Geraldo, B. M., Isler Castro, K. C., Ruano de Oliveira Fugisaki, L., et al. (2016). *Punica granatum* L. (Pomegranate) extract: *in vivo* study of antimicrobial activity against *Porphyromonas gingivalis* in *Galleria mellonella* model. *Sci. World J.* 2016:8626987.

Balasubramanian, S., Othman, E. M., Kampik, D., Stopper, H., Hentschel, U., Ziebuhr, W., et al. (2017). Marine sponge-derived *Streptomyces* sp. SBT343 extract inhibits staphylococcal biofilm formation. *Front. Microbiol.* 8:236. doi: 10.3389/fmicb.2017.00236

Barros, M., Branquinho, R., Grosso, F., Peixe, L., and Novais, C. (2014). Linezolid-resistant *Staphylococcus epidermidis*, Portugal, 2012. *Emerg. Infect. Dis.* 20, 903–905. doi: 10.3201/eid2005.130783

Beau, J., Mahid, N., Burda, W. N., Harrington, L., Shaw, L. N., Mutka, T., et al. (2012). Epigenetic tailoring for the production of anti-infective cytosporones from the marine fungus *Leucostoma persoonii*. *Mar. Drugs* 10, 762–774. doi: 10.3390/md10040762

Becker, K., Heilmann, C., and Peters, G. (2014). Coagulase-negative staphylococci. *Clin. Microbiol. Rev.* 27, 870–926. doi: 10.1128/CMR.00109-13

Bjarnsholt, T., Ciofu, O., Molin, S., Givskov, M., and Hoiby, N. (2013). Applying insights from biofilm biology to drug development - can a new approach be developed? *Nat. Rev. Drug Discov.* 12, 791–808. doi: 10.1038/nrd4000

Casillo, A., Papa, R., Ricciardelli, A., Sannino, F., Ziaco, M., Tilotta, M., et al. (2017). Anti-biofilm activity of a long-chain fatty aldehyde from antarctic *Pseudoalteromonas haloplanktis* TAC125 against *Staphylococcus epidermidis* biofilm. *Front. Cell. Infect. Microbiol.* 7:46. doi: 10.3389/fcimb.2017.00046

Cecil, A., Ohlsen, K., Menzel, T., Francois, P., Schrenzel, J., Fischer, A., et al. (2015). Modelling antibiotic and cytotoxic isoquinoline effects in *Staphylococcus aureus*, *Staphylococcus epidermidis* and mammalian cells. *Int. J. Med. Microbiol.* 305, 96–109. doi: 10.1016/j.ijmm.2014.11.006

Cecil, A., Rikanovic, C., Ohlsen, K., Liang, C., Bernhardt, J., Oelschlaeger, T. A., et al. (2011). Modeling antibiotic and cytotoxic effects of the dimeric isoquinoline IQ-143 on metabolism and its regulation in *Staphylococcus aureus*, *Staphylococcus epidermidis* and human cells. *Genome Biol.* 12:R24. doi: 10.1186/gb-2011-12-3-r24

Cheng, C., Macintyre, L., Abdelmohsen, U. R., Horn, H., Polymenakou, P. N., Edrada-Ebel, R., et al. (2015). Biodiversity, anti-trypanosomal activity screening, and metabolomic profiling of actinomycetes isolated from mediterranean sponges. *PLoS One* 10:e0138528. doi: 10.1371/journal.pone.0138528

Cheng, C., Othman, E. M., Stopper, H., Edrada-Ebel, R., Hentschel, U., and Abdelmohsen, U. R. (2017). Isolation of petrocidin A, a new cytotoxic cyclic dipeptide from the marine sponge-derived bacterium *Streptomyces* sp. SBT348. *Mar. Drugs* 15:E383. doi: 10.3390/md15120383

Chung, P. Y., Chung, L. Y., and Navaratnam, P. (2013). Identification by gene expression profiling analysis, of novel targets in *Staphylococcus aureus* treated with betulinaldehyde. *Res. Microbiol.* 164, 319–326. doi: 10.1016/j.resmic.2013.01.005

Chung, Y. J., and Saier, M. H. Jr. (2002). Overexpression of the *Escherichia coli sugE* gene confers resistance to a narrow range of quaternary ammonium compounds. *J. Bacteriol.* 184, 2543–2545. doi: 10.1128/JB.184.9.2543-2545.2002

Dashti, Y., Grkovic, T., Abdelmohsen, U. R., Hentschel, U., and Quinn, R. J. (2014). Production of induced secondary metabolites by a co-culture of sponge-associated actinomycetes, *Actinokineospora* sp. EG49 and *Nocardiopsis* sp. RV163. *Mar. Drugs* 12, 3046–3059. doi: 10.3390/md12053046

Dyke, K. G., Jevons, M. P., and Parker, M. T. (1966). Penicillinase production and intrinsic resistance to penicillins in *Staphylococcus aureus*. *Lancet* 1, 835–838. doi: 10.1016/S0140-6736(66)90182-6

Edgar, R., Domrachev, M., and Lash, A. E. (2002). Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 30, 207–210. doi: 10.1093/nar/30.1.207

Fey, P. D., and Olson, M. E. (2010). Current concepts in biofilm formation of *Staphylococcus epidermidis*. *Future Microbiol.* 5, 917–933. doi: 10.2217/fmb.10.56

Fitzgerald, J. R., Monday, S. R., Foster, T. J., Bohach, G. A., Hartigan, P. J., Meaney, W. J., et al. (2001). Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. *J. Bacteriol.* 183, 63–70. doi: 10.1128/JB.183.1.63-70.2001

Flemming, H. C., and Wingender, J. (2010). The biofilm matrix. *Nat. Rev. Microbiol.* 8, 623–633. doi: 10.1038/nrmicro2415

Forstner, K. U., Vogel, J., and Sharma, C. M. (2014). READemption-a tool for the computational analysis of deep-sequencing-based transcriptome data. *Bioinformatics* 30, 3421–3423. doi: 10.1093/bioinformatics/btu533

Franca, A., Freitas, A. I., Henriques, A. F., and Cerca, N. (2012). Optimizing a qPCR gene expression quantification assay for *S. epidermidis* biofilms: a comparison between commercial kits and a customized protocol. *PLoS One* 7:e37480. doi: 10.1371/journal.pone.0037480

Franca, A., Perez-Cabezas, B., Correia, A., Pier, G. B., Cerca, N., and Vilanova, M. (2016). *Staphylococcus epidermidis* biofilm-released cells induce a prompt and more marked *in vivo* inflammatory-type response than planktonic or biofilm cells. *Front. Microbiol.* 7:1530. doi: 10.3389/fmicb.2016.01530

Freese, N. H., Norris, D. C., and Loraine, A. E. (2016). Integrated genome browser: visual analytics platform for genomics. *Bioinformatics* 32, 2089–2095. doi: 10.1093/bioinformatics/btw069

Ge, X., Kitten, T., Chen, Z., Lee, S. P., Munro, C. L., and Xu, P. (2008). Identification of *Streptococcus sanguinis* genes required for biofilm formation and examination of their role in endocarditis virulence. *Infect. Immun.* 76, 2551–2559. doi: 10.1128/IAI.00338-08

Gerwick, W. H., and Moore, B. S. (2012). Lessons from the past and charting the future of marine natural products drug discovery and chemical biology. *Chem. Biol.* 19, 85–98. doi: 10.1016/j.chembiol.2011.12.014

Gibreel, T. M., and Upton, M. (2013). Synthetic epidermicin NI01 can protect *Galleria mellonella* larvae from infection with *Staphylococcus aureus*. *J. Antimicrob. Chemother.* 68, 2269–2273. doi: 10.1093/jac/dkt195

Gomes, N. M., Bessa, L. J., Buttachon, S., Costa, P. M., Buaruang, J., Dethoup, T., et al. (2014). Antibacterial and antibiofilm activities of tryptoquivalines and meroditerpenes isolated from the marine-derived fungi *Neosartorya paulistensis*, *N. laciniosa*, *N. tsunodae*, and the soil fungi *N. fischeri* and *N. siamensis*. *Mar. Drugs* 12, 822–839. doi: 10.3390/md12020822

Hall-Stoodley, L., Costerton, J. W., and Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* 2, 95–108. doi: 10.1038/nrmicro821

Heilmann, C., Gerke, C., Perdreau-Remington, F., and Götz, F. (1996). Characterization of Tn917 insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. *Infect. Immun.* 64, 277–282.

Hoffmann, S., Otto, C., Kurtz, S., Sharma, C. M., Khaitovich, P., Vogel, J., et al. (2009). Fast mapping of short sequences with mismatches, insertions and deletions using index structures. *PLoS Comput. Biol.* 5:e1000502. doi: 10.1371/journal.pcbi.1000502

Huber, W., and Koella, J. C. (1993). A comparison of three methods of estimating EC50 in studies of drug resistance of malaria parasites. *Acta Trop.* 55, 257–261. doi: 10.1016/0001-706X(93)90083-N

Indraningrat, A. A., Smidt, H., and Sipkema, D. (2016). Bioprospecting sponge-associated microbes for antimicrobial compounds. *Mar. Drugs* 14:E87. doi: 10.3390/md14050087

Kim, J. K., Jang, H. A., Won, Y. J., Kikuchi, Y., Han, S. H., Kim, C. H., et al. (2014a). Purine biosynthesis-deficient *Burkholderia* mutants are incapable of symbiotic accomodation in the stinkbug. *ISME J.* 8, 552–563. doi: 10.1038/ismej.2013.168

Kim, J. K., Kwon, J. Y., Kim, S. Y., Han, S. H., Won, Y. J., Lee, J. H., et al. (2014b). Purine biosynthesis, biofilm formation, and persistence of an insect-microbe gut symbiosis. *Appl. Environ. Microbiol.* 80, 4374–4382. doi: 10.1128/AEM.00739-14

Kleinschmidt, S., Huygens, F., Faoagali, J., Rathnayake, I. U., and Hafner, L. M. (2015). *Staphylococcus epidermidis* as a cause of bacteremia. *Future Microbiol.* 10, 1859–1879. doi: 10.2217/fmb.15.98

Kodzius, R., and Gojobori, T. (2015). Marine metagenomics as a source for bioprospecting. *Mar. Genomics* 24(Pt 1), 21–30. doi: 10.1016/j.margen.2015.07.001

Kong, C., Chee, C. F., Richter, K., Thomas, N., Abd Rahman, N., and Nathan, S. (2018). Suppression of *Staphylococcus aureus* biofilm formation and virulence by a benzimidazole derivative, UM-C162. *Sci. Rep.* 8:2758. doi: 10.1038/s41598-018-21141-2

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., et al. (2001). Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* 357, 1225–1240. doi: 10.1016/S0140-6736(00)04403-2

Lage, H. (2003). ABC-transporters: implications on drug resistance from microorganisms to human cancers. *Int. J. Antimicrob. Agents* 22, 188–199. doi: 10.1016/S0924-8579(03)00203-6

Leary, J. T., Werger, M. M., Broach, W. H., Shaw, L. N., Santoni, B. G., Bernasek, T. L., et al. (2017). Complete eradication of biofilm from orthopedic materials. *J. Arthroplasty* 32, 2513–2518. doi: 10.1016/j.arth.2017.03.050

Lipinski, B., Hawiger, J., and Jeljaszewicz, J. (1967). Staphylococcal clumping with soluble fibrin monomer complexes. *J. Exp. Med.* 126, 979–988. doi: 10.1084/jem.126.5.979

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Mack, D., Davies, A. P., Harris, L. G., Rohde, H., Horstkotte, M. A., and Knobloch, J. K. (2007). Microbial interactions in *Staphylococcus epidermidis* biofilms. *Anal. Bioanal. Chem.* 387, 399–408. doi: 10.1007/s00216-006-0745-2

Mack, D., Siemssen, N., and Laufs, R. (1992). Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: evidence for functional relation to intercellular adhesion. *Infect Immun* 60, 2048–2057.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 17, 10–12. doi: 10.14806/ej.17.1.200

Martins, A., Vieira, H., Gaspar, H., and Santos, S. (2014). Marketed marine natural products in the pharmaceutical and cosmeceutical industries: tips for success. *Mar. Drugs* 12, 1066–1101. doi: 10.3390/md12021066

Mayer, A. M., Glaser, K. B., Cuevas, C., Jacobs, R. S., Kem, W., Little, R. D., et al. (2010). The odyssey of marine pharmaceuticals: a current pipeline perspective. *Trends Pharmacol. Sci.* 31, 255–265. doi: 10.1016/j.tips.2010.02.005

McDougal, L. K., Steward, C. D., Killgore, G. E., Chaitram, J. M., Mcallister, S. K., and Tenover, F. C. (2003). Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. *J. Clin. Microbiol.* 41, 5113–5120. doi: 10.1128/JCM.41.11.5113-5120.2003

Menzel, T. M., Tischer, M., Francois, P., Nickel, J., Schrenzel, J., Bruhn, H., et al. (2011). Mode of action of the novel bisquatenary bisnapthilimide MT02 against *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 55, 311–320. doi: 10.1128/AAC.00586-10

Namvar, A. E., Bastarahang, S., Abbasi, N., Ghehi, G. S., Farhadbakhtiarian, S., Arezi, P., et al. (2014). Clinical characteristics of *Staphylococcus epidermidis*: a systematic review. *GMS Hyg. Infect. Control* 9:Doc23. doi: 10.3205/dgkh000243

Nithya, C., Begum, M. F., and Pandian, S. K. (2010). Marine bacterial isolates inhibit biofilm formation and disrupt mature biofilms of *Pseudomonas aeruginosa* PAO1. *Appl. Microbiol. Biotechnol.* 88, 341–358. doi: 10.1007/s00253-010-2777-y

Otto, M. (2009). *Staphylococcus epidermidis*-the 'accidental' pathogen. *Nat. Rev. Microbiol.* 7, 555–567. doi: 10.1038/nrmicro2182

Otto, M. (2012). Molecular basis of *Staphylococcus epidermidis* infections. *Semin. Immunopathol.* 34, 201–214. doi: 10.1007/s00281-011-0296-2

Palomo, S., Gonzalez, I., De La Cruz, M., Martin, J., Tormo, J. R., Anderson, M., et al. (2013). Sponge-derived *Kocuria* and *Micrococcus* spp. as sources of the new thiazolyl peptide antibiotic kocurin. *Mar. Drugs* 11, 1071–1086. doi: 10.3390/md11041071

Percival, S. L., Hill, K. E., Malic, S., Thomas, D. W., and Williams, D. W. (2011). Antimicrobial tolerance and the significance of persister cells in recalcitrant chronic wound biofilms. *Wound Repair Regen.* 19, 1–9. doi: 10.1111/j.1524-475X.2010.00651.x

Percival, S. L., Suleman, L., Vuotto, C., and Donelli, G. (2015). Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control. *J. Med. Microbiol.* 64, 323–334. doi: 10.1099/jmm.0.000032

Putman, M., Van Veen, H. W., and Konings, W. N. (2000). Molecular properties of bacterial multidrug transporters. *Microbiol. Mol. Biol. Rev.* 64, 672–693. doi: 10.1128/MMBR.64.4.672-693.2000

Qin, N., Tan, X., Jiao, Y., Liu, L., Zhao, W., Yang, S., et al. (2014). RNA-Seq-based transcriptome analysis of methicillin-resistant *Staphylococcus aureus* biofilm inhibition by ursolic acid and resveratrol. *Sci. Rep.* 4:5467. doi: 10.1038/srep05467

Rahman, H., Austin, B., Mitchell, W. J., Morris, P. C., Jamieson, D. J., Adams, D. R., et al. (2010). Novel anti-infective compounds from marine bacteria. *Mar. Drugs* 8, 498–518. doi: 10.3390/md8030498

Rajput, A., Thakur, A., Sharma, S., and Kumar, M. (2018). aBiofilm: a resource of anti-biofilm agents and their potential implications in targeting antibiotic drug resistance. *Nucleic Acids Res.* 46, D894–D900. doi: 10.1093/nar/gkx1157

Rosenstein, R., Nerz, C., Biswas, L., Resch, A., Raddatz, G., Schuster, S. C., et al. (2009). Genome analysis of the meat starter culture bacterium *Staphylococcus carnosus* TM300. *Appl. Environ. Microbiol.* 75, 811–822. doi: 10.1128/AEM.01982-08

Ruisheng, A., and Grewal, P. S. (2011). *purL* gene expression affects biofilm formation and symbiotic persistence of *Photorhabdus temperata* in the nematode *Heterorhabditis bacteriophora*. *Microbiology* 157, 2595–2603. doi: 10.1099/mic.0.048959-0

Sabate Bresco, M., Harris, L. G., Thompson, K., Stanic, B., Morgenstern, M., O'mahony, L., et al. (2017). Pathogenic mechanisms and host interactions in *Staphylococcus epidermidis* device-related infection. *Front. Microbiol.* 8:1401. doi: 10.3389/fmicb.2017.01401

Sakimura, T., Kajiyama, S., Adachi, S., Chiba, K., Yonekura, A., Tomita, M., et al. (2015). Biofilm-forming *Staphylococcus epidermidis* expressing vancomycin resistance early after adhesion to a metal surface. *Biomed Res. Int.* 2015:943056. doi: 10.1155/2015/943056

Shida, T., Koseki, H., Yoda, I., Horiuchi, H., Sakoda, H., and Osaki, M. (2013). Adherence ability of *Staphylococcus epidermidis* on prosthetic biomaterials: an *in vitro* study. *Int. J. Nanomedicine* 8, 3955–3961. doi: 10.2147/IJN.S51994

Sianglum, W., Srimanote, P., Taylor, P. W., Rosado, H., and Voravuthikunchai, S. P. (2012). Transcriptome analysis of responses to rhodomyrtone in methicillin-resistant *Staphylococcus aureus*. *PLoS One* 7:e45744. doi: 10.1371/journal.pone.0045744

Skaf, J., Hamarsheh, O., Berninger, M., Balasubramanian, S., Oelschlaeger, T. A., and Holzgrabe, U. (2017). Improving anti-trypanosomal activity of alkamides isolated from *Achillea fragrantissima*. *Fitoterapia* 125, 191–198. doi: 10.1016/j.fitote.2017.11.001

Stewart, P. S., and Costerton, J. W. (2001). Antibiotic resistance of bacteria in biofilms. *Lancet* 358, 135–138. doi: 10.1016/S0140-6736(01)05321-1

Stowe, S. D., Richards, J. J., Tucker, A. T., Thompson, R., Melander, C., and Cavanagh, J. (2011). Anti-biofilm compounds derived from marine sponges. *Mar. Drugs* 9, 2010–2035. doi: 10.3390/md9102010

Sun, W., Zhang, F., He, L., Karthik, L., and Li, Z. (2015). Actinomycetes from the South China Sea sponges: isolation, diversity, and potential for aromatic polyketides discovery. *Front. Microbiol.* 6:1048. doi: 10.3389/fmicb.2015.01048

Thibane, V. S., Kock, J. L., Ells, R., Van Wyk, P. W., and Pohl, C. H. (2010). Effect of marine polyunsaturated fatty acids on biofilm formation of *Candida albicans* and *Candida dubliniensis*. *Mar. Drugs* 8, 2597–2604. doi: 10.3390/md8102597

Thompson, C. C., Kruger, R. H., and Thompson, F. L. (2017). Unlocking marine biotechnology in the developing world. *Trends Biotechnol.* 35, 1119–1121. doi: 10.1016/j.tibtech.2017.08.005

Tsai, C. J., Loh, J. M., and Proft, T. (2016). *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence* 7, 214–229. doi: 10.1080/21505594.2015.1135289

Vinh, D. C., and Embil, J. M. (2005). Device-related infections: a review. *J. Long Term Eff. Med. Implants* 15, 467–488. doi: 10.1615/JLongTermEffMedImplants.v15.i5.20

Wang, J., Nong, X. H., Amin, M., and Qi, S. H. (2018). Hygrocin C from marine-derived *Streptomyces* sp. SCSGAA 0027 inhibits biofilm formation in *Bacillus amyloliquefaciens* SCSGAB0082 isolated from South China Sea gorgonian. *Appl. Microbiol. Biotechnol.* 102, 1417–1427. doi: 10.1007/s00253-017-8672-z

Wermser, C., and Lopez, D. (2018). Identification of *Staphylococcus aureus* genes involved in the formation of structured macrocolonies. *Microbiology* 164, 801–815. doi: 10.1099/mic.0.000660

WHO (2014). *Antimicrobial Resistance: Global Report on Surveillance 2014*. Geneva: WHO.

Widerstrom, M. (2016). Significance of *Staphylococcus epidermidis* in health care-associated infections, from contaminant to clinically relevant pathogen: this is a wake-up call! *J. Clin. Microbiol.* 54, 1679–1681. doi: 10.1128/JCM.00743-16

Windolf, C. D., Logters, T., Scholz, M., Windolf, J., and Flohe, S. (2014). Lysostaphin-coated titan-implants preventing localized osteitis by *Staphylococcus aureus* in a mouse model. *PLoS One* 9:e115940. doi: 10.1371/journal.pone.0115940

Xi, L., Ruan, J., and Huang, Y. (2012). Diversity and biosynthetic potential of culturable actinomycetes associated with marine sponges in the China seas. *Int. J. Mol. Sci.* 13, 5917–5932. doi: 10.3390/ijms1305 5917

Yoshioka, S., and Newell, P. D. (2016). Disruption of *de novo* purine biosynthesis in *Pseudomonas fluorescens* Pf0-1 leads to reduced biofilm formation and a reduction in cell size of surface-attached but not planktonic cells. *PeerJ* 4:e1543. doi: 10.7717/peerj.1543

Yu, G., Wang, L. G., Han, Y., and He, Q. Y. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* 16, 284–287. doi: 10.1089/omi.2011.0118

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frontiers in Microbiology

ORIGINAL RESEARCH
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Production of Prodiginines Is Part of a Programmed Cell Death Process in *Streptomyces coelicolor*

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Actinobacteria are prolific producers of antitumor antibiotics with antiproliferative activity, but why these bacteria synthetize metabolites with this bioactivity has so far remained a mystery. In this work we raised the hypothesis that under certain circumstances, production of antiproliferative agents could be part of a genetically programmed death of the producing organism. While programmed cell death (PCD) has been well documented when *Streptomyces* species switch from vegetative (nutrition) to aerial (reproduction) growth, lethal determinants are yet to be discovered. Using DNA-damaging prodiginines of *Streptomyces coelicolor* as model system, we revealed that, under certain conditions, their biosynthesis is always triggered in the dying zone of the mycelial network prior to morphological differentiation, right after an initial round of cell death. The programmed massive death round of the vegetative mycelium is absent in a prodiginine non-producer (Δ*redD* strain), and mutant complementation restored both prodiginine production and cell death. The *redD* null mutant of *S. coelicolor* also showed increased DNA, RNA, and proteins synthesis when most of the mycelium of the wild-type strain was dead when prodiginines accumulated. Moreover, addition of the prodiginine synthesis inhibitors also resulted in enhanced accumulation of viable filaments. Overall, our data enable us to propose a model where the time-space production of prodiginines is programmed to be triggered by the perception of dead cells, and their biosynthesis further amplifies the PCD process. As prodiginine production coincides with the moment *S. coelicolor* undergoes morphogenesis, the production of these lethal compounds might be used to eradicate the obsolete part of the population in order to provide nutrients for development of the survivors. Hence, next to weapons in competition between organisms or signals in inter- and intra-species communications, we propose a third role for antibiotics (in the literal meaning of the word 'against life') i.e., elements involved in self-toxicity in order to control cell proliferation, and/or for PCD associated with developmental processes.

Keywords: antitumor antibiotics, secondary metabolites, DNA-damaging agents, cell death and differentiation, bacterial development, confocal laser microscopy

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INTRODUCTION

Traditionally, bacteria were regarded as unicellular microorganisms that rapidly grow and divide via binary fission. The concept of multicellularity among prokaryotes was recognized only three decades ago, and is particularly evident in actinobacteria, cyanobacteria, and myxobacteria (Shapiro, 1998; Claessen et al., 2014). These are bacteria with a complex life cycle and morphological and chemical differentiations that are switched on in response to environmental signals. A hallmark of multicellular organisms is programmed cell death or PCD (Bayles, 2014; Claessen et al., 2014; Lyons and Kolter, 2015). In *Bacillus subtilis*, sporulation is preceded by a form of PCD known as cannibalism in a subpopulation of the biofilm, and serves to produce the building blocks required for a new round of macromolecule synthesis to sustain developmental growth (González-Pastor et al., 2003; González-Pastor, 2011). In *Myxococcus*, three subpopulations that show division of labor arise, with cells differentiating into spores or into peripheral rods, while the remaining cells undergo PCD (Nariya and Inouye, 2008). In the cyanobacterial *Anabaena*, cell death is controlled by the circadian rhythm, which implies careful programming (Lee and Rhee, 1999).

Several rounds of PCD occur as part of the complex developmental program of the mycelial *Streptomyces* (Miguélez et al., 1999, 2000; Fernández and Sánchez, 2002; Manteca et al., 2005; Filippova and Vinogradova, 2017). Streptomycetes are filamentous Gram-positive high G+C bacteria that reproduce via sporulation. Their life cycle starts with a spore that germinates to grow out and form a mycelium consisting of multinucleoid vegetative hyphae. When reproduction is required, the older mycelium is used as a substrate to form aerial hyphae, which differentiate into chains of unigenomic spores. This morphological differentiation is accompanied by chemical differentiation, whereby many natural products, including antibiotics, anticancer compounds and antifungals, are produced. The lytic degradation of the vegetative mycelium is a clear manifestation of PCD, presumably to provide the nutrients necessary to produce the reproductive aerial hyphae (Méndez et al., 1985; Miguélez et al., 1999; Manteca et al., 2005; Chater, 2006).

The molecular triggers and genetic basis for PCD in mycelial bacteria are still largely unknown. 'Antibiotics' should be regarded as serious candidates for eliciting PCD processes. Antibiotic resistance is indeed mandatory for antibiotic makers such as streptomycetes, which infers that their 'weapons' against competitors are also often active against the producing species (Tomasz, 2006; Hopwood, 2007; Alkhatib et al., 2012; Mak et al., 2014; Ogawara, 2015; Sugiyama, 2015). To survive production of their natural products, *Streptomyces* possess self-defense mechanisms identical to those developed and acquired by human and animal pathogens (D'Costa et al., 2006; Wright, 2012; Surette and Wright, 2017). Self-toxicity - and therefore self-resistance - extends well beyond antibacterial agents; for example, anticancer molecules damaging DNA are also deadly to the producer (Galm et al., 2005). Mechanisms of resistance to antitumor antibiotics that destroy DNA have been identified in almost all producers and include (i) drug sequestration, (ii) drug inactivation (modification or destruction), (iii) drug efflux, and (iv) target repair or protection (Tenconi and Rigali, 2018).

Amongst the *Streptomyces* molecules currently explored in human disease therapy, the tripyrrole red-colored prodiginines or prodigiosin-like pigments (PdG) have gained interest due to their promising antitumor, immunosuppressive and anti-inflammatory properties (Montaner and Pérez-Tomás, 2003; Pérez-Tomás et al., 2003; Williamson et al., 2007; Pérez-Tomás and Viñas, 2010). Their topoisomerase-inhibiting activity by intercalating DNA and the many other ways they are harmful to cell components (Williamson et al., 2007; Darshan and Manonmani, 2016; Yang et al., 2017; Zhang et al., 2017) explains why prodiginines are toxic to so many different organisms and also display antimalarial, anthelmintic, antifungal, and antibacterial activities (Stankovic et al., 2014). Such a broad spectrum of activity suggests that the PdG-producing species might be sensitive to their own molecules. Indeed, in *Streptomyces coelicolor* the onset of expression of the *red* cluster (encoding the PdG biosynthetic pathway) coincides with the entrance of this species into a period of growth cessation – the so-called transition phase (Takano et al., 1992; Bibb, 1996; White and Bibb, 1997; Sun et al., 1999; Huang et al., 2001; Zhou et al., 2005; Williamson et al., 2006). In addition, prodiginine production coincides with a round of massive cell death in the course of which the *Streptomyces* multicellular filamentous network undergoes drastic morphological changes associated with the sporulation process. Time-space monitoring of *redD* expression, the activator of PdGs *red* biosynthetic genes, is also confined to aging, lysed filaments (Sun et al., 1999). Moreover, prodigiosin-like pigments are induced by stressing culture conditions like excess of metal ions and pH shock (Mo et al., 2013; Morgenstern et al., 2015), co-cultivation with competing microorganisms (Luti and Mavituna, 2011b; Schäberle et al., 2014), and feeding the medium with dead bacterial cells (Luti and Mavituna, 2011a). Finally, all other DNA-damaging agents have been shown to be toxic for the producing actinobacterial species (Tenconi and Rigali, 2018). The absence of resistance genes within the *red* cluster thus further supports the hypothesis that production of these compounds might be harshly destructive to *S. coelicolor*.

In the light of the known high cytotoxicity of prodiginines, it is remarkable that the DNA-damaging PdGs are not secreted by *S. coelicolor*, but rather accumulate internally (in the cytoplasm and within the membranes and cell wall), right at the time of growth cessation. The seemingly suicidal and at the same time well-programmed production of prodiginines suggests that these molecules may play a role in the control and/or progress of PCD. An advantage of using PdGs as our model to correlate toxin biosynthesis to PCD is that the timing and localization of their production is easily monitored *in situ* due to their red autofluorescence (Tenconi et al., 2013). In this work we demonstrate that prodiginine production correlates to dying filaments in time and space and that absence of PdGs reduces cell death in *S. coelicolor*, resulting in hyper-accumulation of viable filaments. We propose that prodiginines are main protagonists of a PCD process in the producing organisms.

MATERIALS AND METHODS

Strains and Culture Conditions

Streptomyces coelicolor M145 and its *redD* mutant M510 (Floriano and Bibb, 1996) were used as the wild-type strain and as PdGs non-producer, respectively. R2YE (Kieser et al., 2017) agar plates were used for phenotypic characterization. Where applicable, R2YE plates were covered with cellophane membranes (GE Osmonics Labstore, Ref K01CP09030). Plates were inoculated with 500 μl of a 2×10^7 cfu/ml spore suspension. Where applicable, *N*-acetylglucosamine was added to a final concentration of 25 mM in R2YE plates.

Complementation of the *redD* Mutant M510

The *S. coelicolor redD* mutant M510 was complemented by introducing plJ2587 harboring *redD* (SCO5877) with its native upstream region. A DNA fragment containing the *redD* upstream region (567 bp) was generated by PCR using primers (5′- GAATTCCCCCTGCTGCTCCAGGG -3′) and (5′- GGATCCCCCAATATGTTGATTTCCACGC -3′) with engineered EcoRI and BamHI sites, respectively, and cloned into pJET1.2 (Thermo Fisher Scientific). After sequence confirmation, the fragment was retrieved through EcoRI and BamHI restriction digest, gel purified, and cloned into an EcoRI/BamHI-linearized plJ2587 (van Wezel et al., 2000) upstream of *redD* resulting in plasmid pELT003. The complementation construct, as well as the empty plJ2587 plasmid, were introduced into the *redD* mutant through intergeneric conjugation as described previously (Tenconi et al., 2015). All thiostrepton resistant colonies transformed with pELT003 (gene *tsr* in plJ2587) presented the intracellular red pigmentation and red fluorescence associated with PdG production confirming the complementation of the *redD* mutant phenotype.

In situ Visualization by Confocal Fluorescence Microscopy

Samples were prepared as described previously (Tenconi et al., 2013). Samples were examined under Leica TCS-SP2 and Leica TCS-SP5 confocal laser-scanning microscopes. SYTO9 and SYTOX stained samples were examined at a wavelength of 488 for excitation and 530 nm (green) for emission. Red autofluorescence of PdGs and propidium iodide-stained samples were examined at a wavelength of 543 nm (Leica TCS-SP2) or 568 nm (Leica TCS-SP5) for excitation and 630 nm (red) for emission as described previously (Tenconi et al., 2012). Quantitative analyses were performed employing the Leica LAS-AF image analysis program. Image processing and 3D reconstruction of *Streptomyces* filaments were performed as described previously (Tenconi et al., 2013).

DNA, RNA, and Protein Extraction and Quantification

Total DNA, and intracellular and extracellular RNA were extracted using the phenol/chloroform/isoamyl alcohol protocol, mainly as described previously (Sambrook et al., 1989). The *S. coelicolor* mycelium (from 24, 32, 40, 48, 56, 65, and 72 h cultures) was scraped with a spatula from the R2YE agar plates covered with cellophane disks, put into 2 ml tubes and frozen at −70°C. 50 mg of mycelium were first subjected to lysozyme digestion (2 mg/ml final concentration in 2 ml of extracted buffer pH8), and then incubated with proteinase K (0.5 mg/ml; 1 h at 55°C) prior to nucleic acids extraction. DNA was removed from total nucleic acids extracted by using the kit Turbo DNA-free (Ambion). Proteins were extracted from 50 mg of mycelium as described previously (Tenconi et al., 2013) after sonication using 30 s pulse for 10 min (Bioruptor, Diagenode, Liège, Belgium) in 500 μl of extraction buffer. Protein concentration was determined by measuring absorbance at 280 nm.

RESULTS

In situ Visualization of Prodiginine Production During the Life Cycle of *S. coelicolor*

Prodiginine (PdG) production was monitored throughout the life cycle of *S. coelicolor*, making use of their red autofluorescence (RAF) as described previously (Tenconi et al., 2013). Spores (10^7 cfu) of *S. coelicolor* M145 were spread onto the surface of R2YE agar plates, and 0.5-mm thick slices of confluent solid cultures were collected at different time points and imaged via confocal fluorescence microscopy. *In situ* visualization of PdG production (under non-saturated excitation conditions) revealed that weak RAF appeared at the surface of the vegetative mycelium at around 36 h, and that this signal reached its maximum level at 50 h (**Figure 1**). From that time point onwards, the RAF intensity decreased abruptly to persist at approximately one third of its maximal intensity (**Figure 1** and **Supplementary Figure S1**). The accumulation of PdGs was therefore maximal (∼50 h) at the morphological transition phase, before the vegetative mycelium differentiates into aerial hyphae (between 50 and 64 h, **Figure 1**).

Time-Space Correlation Between Prodiginine Production and Cell Death

Simultaneous *in situ* quantification of RAF and dying cells revealed that the membrane-damaged filaments, which are permeable to SYTOX, reached their highest level at around 30 h of culture, about 20 h before the maximal intensity of RAF (∼50 h) (**Figures 2A,B**). This maximum of accumulation of dying filaments occurred ∼5 h after the peak of SYTO9 fluorescence (∼25 h), which, at time points prior to PdG production (see below), stains both live and membrane damaged (dying) cells (**Figures 2A,B**). Quantification of SYTO9 and SYTOX signals along the first 50 μm of the *S. coelicolor* culture revealed that both dyes present a peak of fluorescence at the same distance of ∼20–30 μm from the surface of the culture (**Figure 2B**). This spatial co-localization at an approximately 5 h interval suggests that dying filaments observed at 30 h emanated from those presenting the highest SYTO9 signal at 25 h. Quantification of PdGs along the same vertical axis showed that the earliest RAF signal detected at 36 h (under non-saturated excitation conditions **Figures 2A,B**)

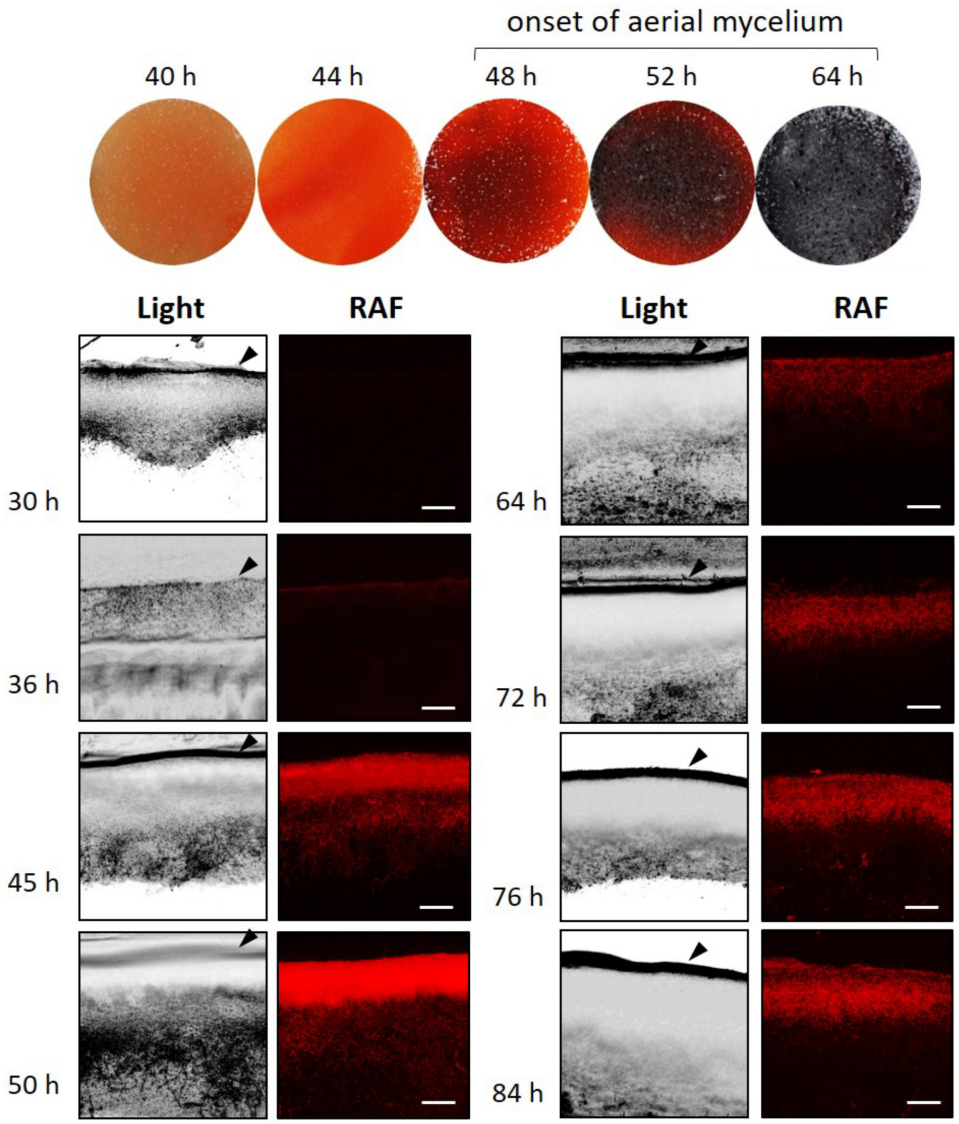

FIGURE 1 | Visualization and quantification of RAF throughout the life cycle of *Streptomyces coelicolor*. *In situ* visualization of RAF by confocal microscopy in cross section of a confluent *S. coelicolor* culture on R2YE agar plates. Bars, 40 μm. Arrows indicate the surface of the agar plate cultures. Phenotypes of *S. coelicolor* grown in R2YE agar plates are shown to visualize the delay between the onset of PdG production and the onset of the morphological differentiation (white mycelium appearing on the surface of the plate).

also arises at the same distance of ∼20–30 μm from the surface of the culture, right after the maximum of accumulation of dying cells (SYTOX-staining, **Figure 2**). Increasing the voltage on the photomultiplier tubes (PMT) during imaging of 30 h old samples revealed that the forced early RAF signal matched the thin 10 μm band of the culture where first dying filaments were seen (**Figure 2A**, right panel) supporting the idea that PdG biosynthesis spatially coincides with dying cells but would be posterior to the occurrence of this early cell death event.

At a later time point (40 h) qualitative analysis of PdG fluorescence and accumulation of dying cells (SYTOX stained) along the vertical axis of a confluent solid culture showed a clear correlation between RAF and the zone of cell death visible in the upper part of the culture (**Figure 3a**). Conversely, no or extremely weak RAF was seen in SYTO9 stained sections, and *vice versa* (**Figure 3a**). The fact that maximum RAF (50 h) coincided with (i) the lowest amount of SYTO9 (live and dead) staining, and (ii) maximal SYTOX (dead) staining (**Figure 3a**) further supports that PdGs might be associated with the massive round of cell death observed at the surface of the culture and preceding the morphological differentiation of *S. coelicolor*. To further demonstrate that PdGs accumulate in the dead zone at the surface of the *S. coelicolor* culture, we repeated the monitoring of dying cells using propidium iodide (PI) as alternative fluorescent dye for staining membrane-damaged filaments. PI displays maxima of excitation/emission of red fluorescence very close to the

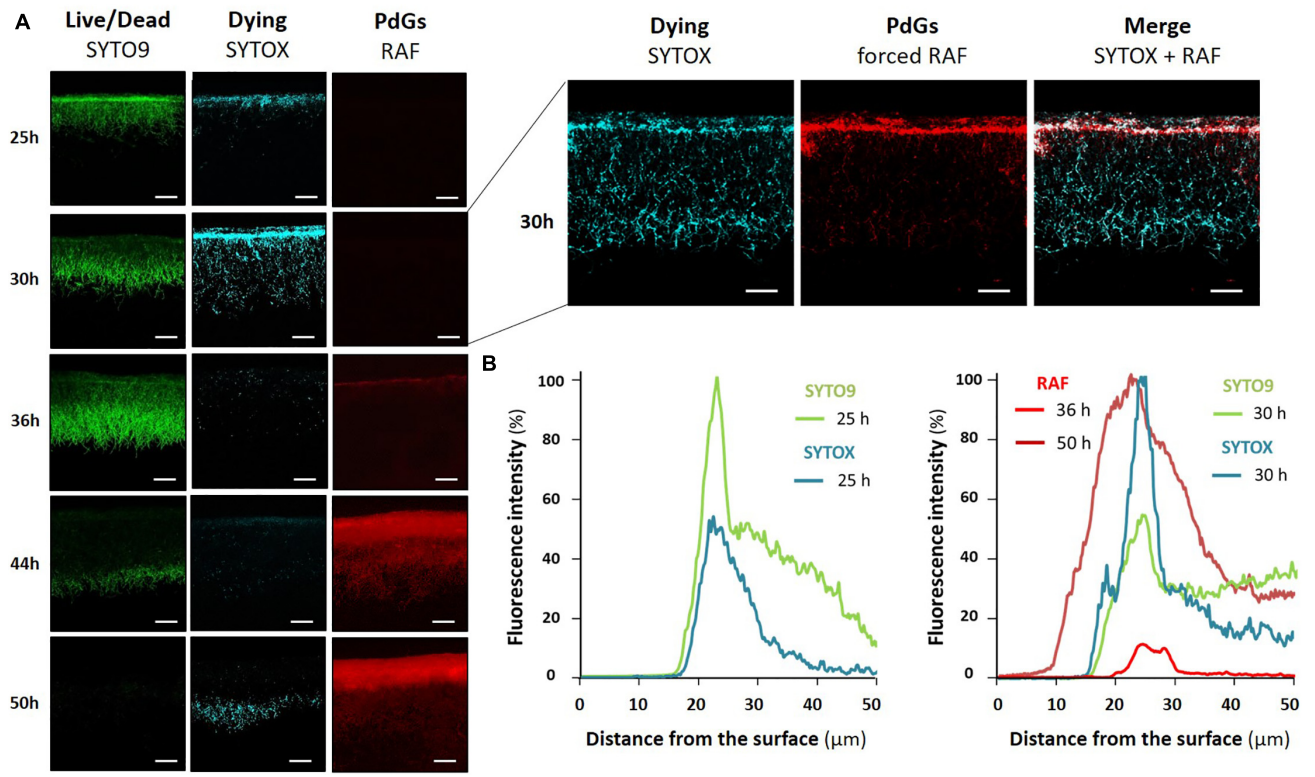

FIGURE 2 | Spatio-temporal occurrence of production of PdGs and cell death in *S. coelicolor*. **(A)** Images represent cross-sections of confluent lawns of *S. coelicolor* M145 grown on R2YE agar plates. Maximal fluorescence intensity associated with PdG biosynthesis (RAF), dying filaments (SYTOX-staining, blue-colored), or live/dead filaments (SYTO9-staining, green colored), was reached after 50, 30, and 25 h of growth, respectively. The voltage on the photomultiplier tubes (PMT) for RAF was fixed to 600 V. On the right panels the PMT was increased to 680 V in a 30 h old cross section in order to visualize RAF showing early PdG production that starts from the thin band of cell death. Bars, 40 μm. **(B)** Quantification of RAF, SYTO9, and SYTOX fluorescence signals across the first 50 μm of the *S. coelicolor* culture. Note that the peak of maximum RAF, SYTO9, and SYTOX occurred in the same thin band at a distance of about 20–30 μm from the surface of the culture.

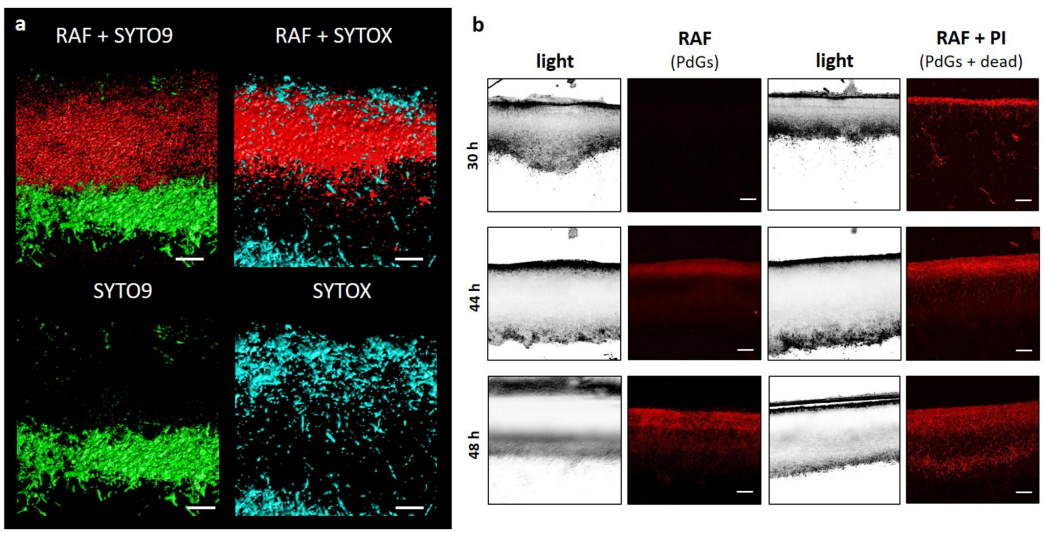

FIGURE 3 | Localization of the production of prodiginines in the dying zones of the *S. coelicolor* culture. **(a)** Fluorescence confocal micrographs (3D reconstruction) of two transverse sections of an *S. coelicolor* M145 culture grown for 40 h on R2YE agar plates. Left panel: RAF (red-colored) and staining of viable filaments (green-colored, SYTO9). Right panel: RAF (red-colored) and staining of dying filaments (blue-colored, SYTOX). Bar, 20 μM. **(b)** Visualization of the red fluorescence associated with PdG production (RAF) and propidium iodide staining (PI). Bar, 20 μM.

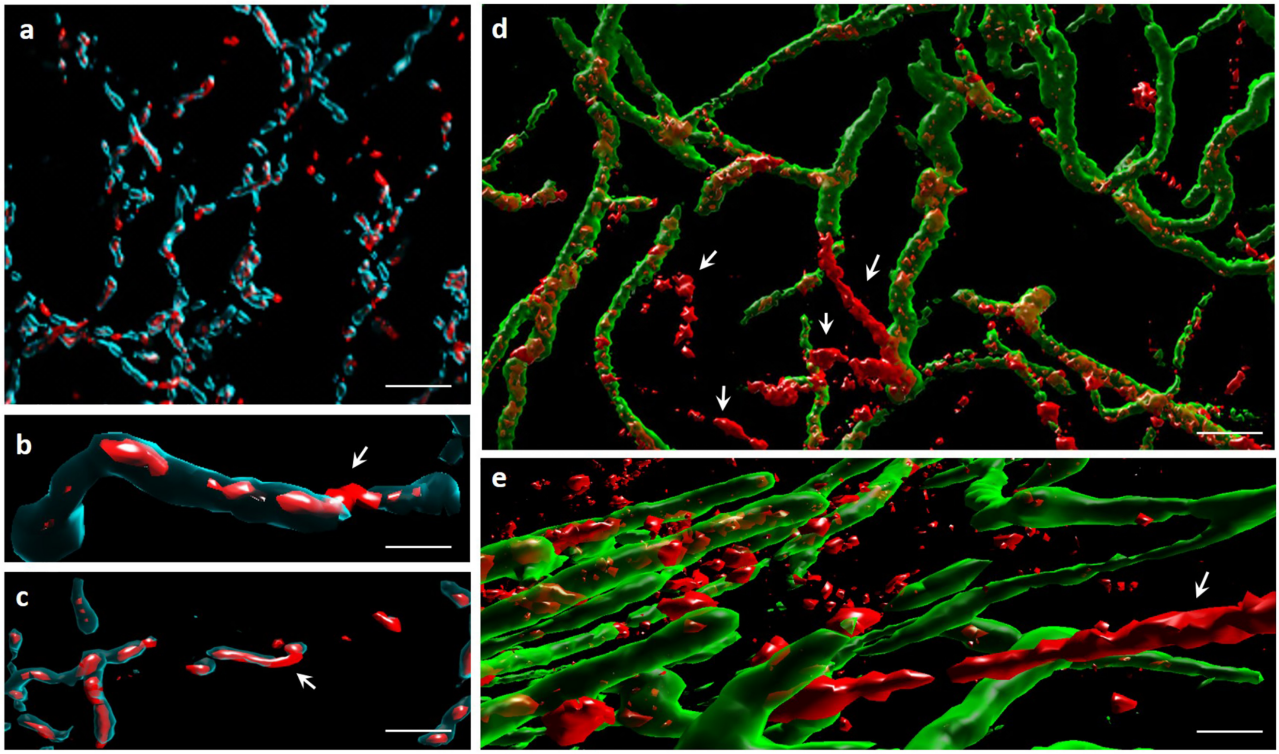

FIGURE 4 | Monitoring of PdG production at the filament size scale in *S. coelicolor*. **(a–c)** Filaments of *S. coelicolor* M145 inoculated on R2YE plates displaying both RAF and SYTOX (blue) staining. White arrows point to portion of filaments not stained with SYTOX and with abundant PdG production visualized by RAF. Bars, 10 μm **(a)**, 2 μm **(b)**, and 5 μm **(c)**. **(d,e)** Filaments of *S. coelicolor* M145 inoculated on R2YE plates displaying both RAF and SYTO9 (green) staining. White arrows point to 'ghost filaments' with abundant production of PdGs and therefore not stained with SYTO9. Bars, 2 μm **(d)**, and 1 μm **(e)**.

maxima of RAF of PdGs (Tenconi et al., 2013). Monitoring PI fluorescence will thus also reveal red fluorescence when PdGs are not produced. At 30 h of growth, prior to PdG production, PI display fluorescence at the upper part of the culture (**Figure 3b**) with a spatial pattern similar to the one observed with SYTOX staining (**Figure 2A**). At later time points, when PdGs are produced, the fluorescence of PI in the upper part of the culture (**Figure 3b**) is observed in the same zone as where RAF associated with PdGs was observed (**Figures 1**, **2**), the fluorescence signal being increased when both RAF and PI were recorded. That RAF recorded together with PI fluorescence staining dying filaments display similar spatio-temporal patterns as those recorded for RAF alone further supports that PdGs accumulate in the dead zone at the surface of the culture.

Monitoring of PdGs Biosynthesis at Filament Size Scale

We attempted to monitor PdG production at the filament size scale in order to assess if they are produced by dying or living filaments. Repetitive assays revealed that, at time points where *S. coelicolor* abundantly produces PdGs (from 40 h onwards), SYTO9 and SYTOX hardly stained any of the filaments that display RAF. Visualization of filaments that display both RAF, and SYTOX or SYTO9 fluorescence was only possible at lower mycelial density (below the zone of high fluorescence signals in the first 50–60 μm), or at the brief moment in the life cycle (30 h) when the level of PdGs is still low inside the filaments. Specimens where we could observe intracellular PdG production inside filaments stained with SYTO9 or SYTOX are presented at **Figure 4**. However, once PdG accumulated above a certain level inside the filaments, SYTOX and SYTO9 failed to stain, leading to partial or complete 'ghost' filaments that could only be visualized by RAF (**Figure 4**).

This inhibition of SYTO9 and SYTOX staining by PdGs is most likely the result of the DNA-intercalation ability of PdGs and/or because of the PdGs-induced destruction of nucleic acids (see below **Figure 6**). The observed lack of staining with SYTOX or SYTO9 once the RAF signal is high, and therefore when PdG production abundantly accumulated, led us to hypothesize that these DNA-damaging metabolites that remain intracellular, may be involved in the destruction of the DNA in the vegetative mycelium of *S. coelicolor*. However, RAF was more frequently observed inside filaments stained with SYTO9 compared to filaments stained with SYTOX, suggesting that their production occurs in living filaments, causing cell death most likely by damaging DNA, therefore causing SYTOX staining more casual once PdGs were produced. Alternatively, the rarely observed co-staining of PdGs-SYTO9 and PdGs-SYTOX at the filament scale could be attributed to the DNA-bound

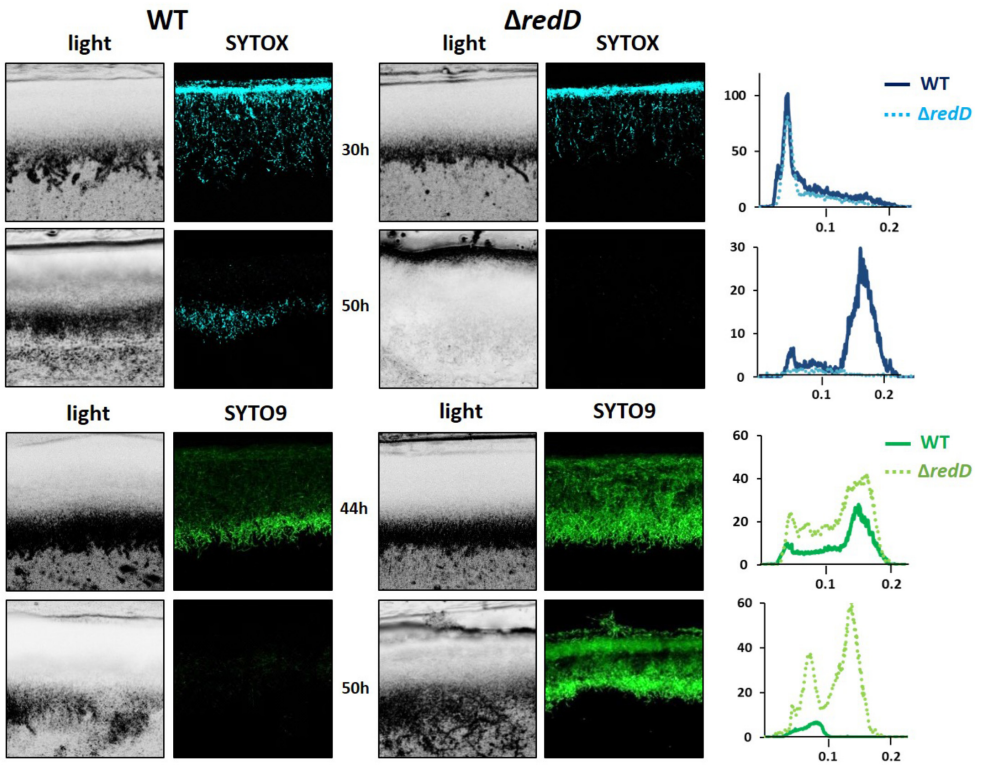

FIGURE 5 | Prodiginine biosynthesis causes massive loss of viable filaments. SYTOX (blue) and SYTO9 (green) staining of cross sections of culture of *S. coelicolor* M145 (wild-type, WT) and its *redD* null mutant inoculated in R2YE agar plates. Bars, 40 μm. Note that (i) the PCD round prior to PdG production also occurs at 30 h in the *redD* null mutant of *S. coelicolor*, and (ii) the higher accumulation of viable filaments (only stained by SYTO9) in the *redD* mutant. Quantification of the SYTOX and SYTO9 fluorescence signals are displayed in plots next to the microscopy pictures.

prodiginines that could sterically hinder SYTOX or SYTO9 dyes to access DNA.

PdGs Have Anti-proliferative Activity

The observed lack of staining with SYTOX or SYTO9 once the RAF signal is high, and therefore when PdG production abundantly accumulated, led us to hypothesize that these DNA-damaging metabolites that remain intracellular, may be involved in the destruction of the DNA in the vegetative mycelium of *S. coelicolor*. To investigate this, we first compared the accumulation of viable and dying filaments between *S. coelicolor* M145 and its *redD* null mutant M510, which fails to produce PdGs (**Figure 5**). The *redD* null mutant also displayed a band of dying hyphae at the upper surface of the culture at 30 h (**Figure 5**), which may be seen as an argument that the production of PdGs is a consequence, and not the cause, of the onset of cell death. However, at 50 h of growth, which corresponds to the time point of maximal RAF intensity in the wild-type strain (**Figures 1**, **2**), the PdG-non-producing strain M510 still displayed many SYTO9-stained cells, while the parental strain M145 showed only very weak staining along the vertical axis of the culture (**Figure 5**). Importantly, we did not observe any fluorescence when SYTOX was added after 50 h of growth in the *redD* null mutant (**Figure 5**), suggesting that all filaments stained by SYTO9 were viable. The absence of SYTO9 and SYTOX staining in the zone of the culture that displayed maximal RAF suggests that PdGs had caused widespread DNA destruction within the vegetative mycelium preventing the DNA-interacting commercial dyes to bind their molecular target. Complementation of the *redD* mutant restored PdG production and resulted in the subsequent massive loss of filaments stained with SYTO9 (**Supplementary Figure S2**) demonstrating that the absence of the massive death round in the *redD* mutant was caused by the loss of PdG production.

Additionally, we monitored and compared other markers of viability and metabolic activity between the wild-type strain M145 and the *redD* mutant, namely DNA, RNA, and protein synthesis (**Figure 6**). For this purpose, R2YE agar plates were covered with cellophane disks prior to inoculation with spores of either *S. coelicolor* M145 or its *redD* mutant M510, to allow collection of the biomass from the spent agar. The presence of the membranes on the top of the plates caused accelerated production of PdGs, with a peak in RAF intensity 10 h earlier than observed in plates not covered with cellophane disks (40 h instead of 50 h) (**Figure 6A** and **Supplementary Figure S1**). The genomic DNA collected prior (32 h), and during (40 and 58 h) PdG synthesis revealed less accumulation of DNA in the wild-type strain *S. coelicolor* once PdG are being produced while the amount of DNA remained constant in the *redD* mutant M510 (**Figure 6B**). Similarly, analysis of total RNA isolated from the wild-type strain *S. coelicolor* and its red mutant also revealed a

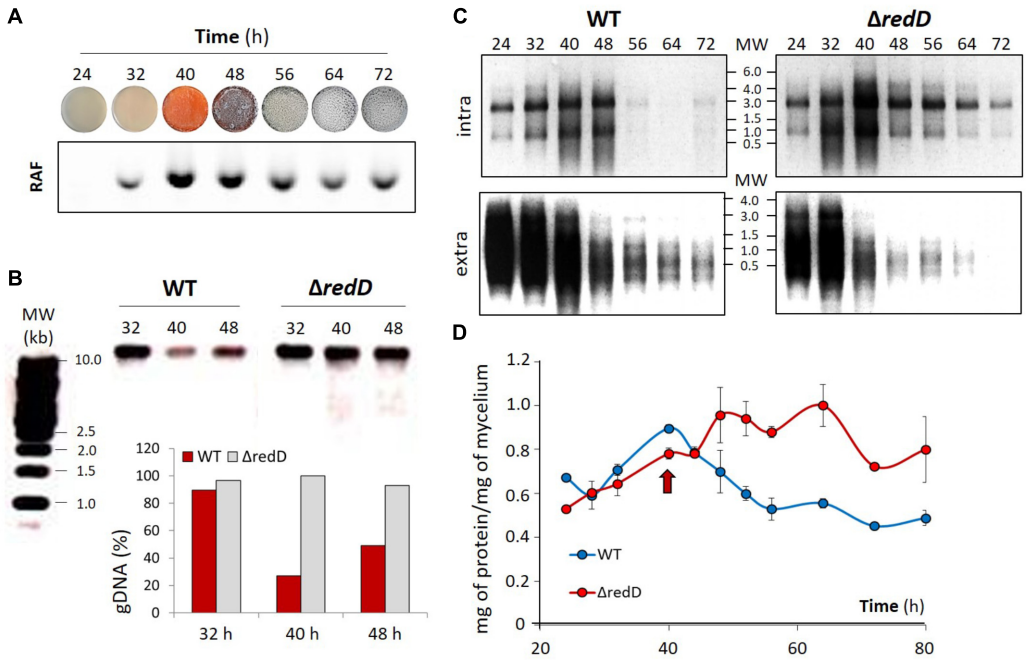

FIGURE 6 | Effect of the deletion of *redD* on DNA, RNA, and protein synthesis. **(A)** PdG production throughout the life cycle of *S. coelicolor*. RAF from mycelium extracts of *S. coelicolor* grown on the R2YE medium covered with cellophane disks. Extracts were deposited in an agarose gel and RAF was monitored after migration as described previously (56). **(B)** Ethidium bromide stained agarose gels and quantification showing the amount of genomic DNA extracted from 50 mg of mycelium of *S. coelicolor* M145 (WT) and its *redD* null mutant (Δ*redD*). 100% refers to the maximum of genomic DNA collected, i.e., in the *redD* mutant at 40 h. **(C)** Ethidium bromide stained agarose gels showing intracellular (top panels) and extracellular (bottom panels) RNA extracted from *S. coelicolor* M145 (WT) and its *redD* null mutant (Δ*redD*). **(D)** Quantification of the total protein content in crude extracts of mycelia of *S. coelicolor* WT (M145) and its *redD* null mutant. The red arrow indicates the timing of maximal PdGs biosynthesis.

drop in the accumulation of 16S and 23S rRNA in the wild-type strain right after the peak of PdG biosynthesis (**Figure 6C**). In contrast, after that time point, rRNA levels remained significantly higher in the *redD* mutant as compared to the parental strain (**Figure 6C**). Interestingly, the amount of RNA collected from the washed mycelium was instead significantly higher in the wild-type strain than in the *redD* mutant, suggesting more intense lysis of the parent and consequential accumulation of RNA outside the filaments (**Figure 6C**). Assessment of the total protein content of the intracellular crude extracts also revealed distinct profiles between the two strains, with the *redD* mutant displaying higher amounts throughout the time course as compared to the parental strain, which showed a drastic drop of protein accumulation just after the peak of RAF at 40 h (**Figure 6C**). The higher intracellular accumulation of macromolecules (DNA, RNAs, and proteins) strongly suggests that the metabolism of the PdG-non-producer remains active at stages of the life cycle where the large majority of the vegetative mycelium of the wild-type strain is encountering destructive processes.

Inhibiting Conditions for PdG Production Reduces Cell Death

To further test the idea that *S. coelicolor* itself is vulnerable to intracellular PdGs and that PdG are induced by cell death, we used a culture condition known to strongly reduce PdG production in *S. coelicolor* and assessed if it also correlates with reduced cell death and/or higher accumulation of viable filaments. 2.10^7 spores of *S. coelicolor* M145 were used to inoculate the R2YE solid medium with or without addition of the aminosugar *N*-acetylglucosamine (GlcNAc) known to block morphogenesis at the vegetative state and PdG production in *S. coelicolor* grown on rich media (Rigali et al., 2006, 2008) (**Figure 7**). *In situ* visualization of samples of confluent solid cultures collected at 42 h revealed that the presence of the morphogenesis-blocking agent GlcNAc resulted in a massive accumulation of SYTO9 stained filaments (**Figure 7**). As GlcNAc containing samples were devoid of the red fluorescence associated with PdG production and PI staining (**Figure 7**), it suggests that all SYTO9 stained filaments were viable. In contrast, without GlcNAc, the mycelium of *S. coelicolor* displayed important red fluorescence (from both PI and PdGs) associated with dying cells and presented a much lower growth as deduced from the thickness of the mycelial culture (**Figure 7**). The same experiment was previously performed by adding to the R2YE medium other types of PdG synthesis inhibitors such as phosphorylated sugars (Tenconi et al., 2012). The addition of phosphorylated nutrients (glycerol-3-P, glucose-6-P, Fructrose-1,6-BP) inhibited PdG production and reduced/delayed cell death (Tenconi et al., 2012). Importantly, the addition of counterpart non-phosphorylated nutrients neither reduced PdG synthesis nor cell death (Tenconi et al., 2012) suggesting that

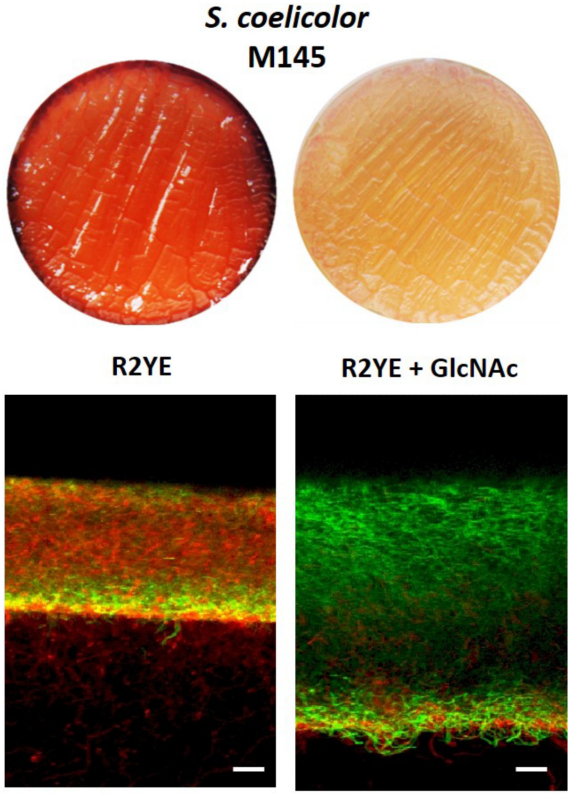

FIGURE 7 | Addition of the PdG biosynthesis inhibitor GlcNAc results in accumulation of viable filaments. Effect of GlcNAc (25 mM) on SYTO9 (green) filament staining, and PdG and PI (red) fluorescence. Bars, 20 μm. *S. coelicolor* M145 was inoculated in R2YE agar plates.

the observed phenomenon with phosphorylated sugars was not due to preventing nutrient starvation. Next to our study of the phenotype of the *redD* mutant, the use of PdG synthesis inhibitors also revealed that preventing or reducing PdG synthesis results in reduced cell death and higher accumulation of viable filaments.

DISCUSSION

Do natural products with anti-proliferative activities, such as PdGs, participate in the PCD process of the producing microorganism? As PdGs are not secreted but accumulate internally or remain in the membrane in *S. coelicolor*, this hypothesis is directly in line with the concept of these molecules being auto-destructive rather than acting on neighboring microbes. We found that, under the tested conditions, the production of PdGs is programmed to always coincide in space with an early and limited cell death event. PdG synthesis follows in time this early cell death event and their production amplifies the phenomenon by killing the overwhelming majority of the *S. coelicolor* culture. This broad and rapid destruction of the mycelium is assisted by the PdGs as in the PdG non-producing strain (the *redD* null mutant) cell death was attenuated, and DNA, RNA, and protein synthesis continued at time points where normally these processes are abolished in the parental strain. Loss of cell death and accumulation of viable filaments were also observed when the culture medium was supplemented with the PdG production inhibitors.

Cell Death Is the Trigger of PdG Biosynthesis

Next to phenotypic characterization of mutants, a complementary means to uncover the role(s) played by a secondary metabolite in nature is to identify signals that regulate its biosynthesis (i.e., '*what controls you will tell us who you really are*'). When we visualized RAF at an early time point, we found that the biosynthesis of PdGs is not triggered homogeneously but is instead spatially restricted to a very thin (<10 μM) band in the upper part of the *S. coelicolor* culture (**Figure 2**). This thin band of PdG production arises exactly where dying cells are constantly observed at the same moment of the life cycle of *S. coelicolor*. This observation corroborates previous findings that *redD* expression occurred only when a proportion of the hyphae had lysed, suggesting that transcription of *redD* is confined to aging vegetative mycelium (Sun et al., 1999). It has also been demonstrated that the production of PdGs of *S. coelicolor* is induced by the addition of dead cells in the culture medium (Luti and Mavituna, 2011a). The initial band of cell death where the production of PdGs started also corresponds to the thin band where we observed maximum SYTO9 and SYTOX staining at 25 and 30 h, respectively (**Figure 2**). PdG biosynthesis thus arises where the density of the population is maximal and therefore could be governed by a quorum sensing regulatory pathway. That synthesis of killing factors (PdGs) follows in time with the zone of the culture with the highest density of the population is most likely not just a coincidence. The high density observed at 25 h in the thin band in the upper part of the culture suggests highly active metabolism during aerobic growth with important production of reactive oxygen species (ROS) which could be the cause of the massive cell death observed in the same zone at 30 h (**Figure 2**) and proposed as triggered for the formation of the aerial mycelium of *Streptomyces* (Beites et al., 2015).

What Would Be the Evolutionary Advantage of Producing Cytotoxic Compounds Prior to Morphogenesis?

For *Streptomyces*, it is generally accepted that the death of the vegetative mycelium provides nutrients for the advancement of later stages of the life cycle, i.e., the erection of aerial hyphae and their subsequent maturation into spore chains (Méndez et al., 1985; Miguélez et al., 1999, 2000; Chater, 2006). However, we note that there is a current paucity of data that actually support this claim. At the same time, the toxicity of PdGs might also prevent scavenging of these newly liberated nutrients by other bacteria and/or fungi. The model above (i.e., chemical protection of its own food reservoir) is appealing as it rationalizes why a PCD event might precede morphogenic development and why a small molecule that is also toxic to other organisms might be an ideal mediator of this process. In this case, the killing agent

is a structurally complex compound whose synthesis requires a large cluster of 23 genes. Making PdGs might seem an expensive strategy to participate in a cell death process. But making a complex natural product is only 'expensive' if the evolutionary pay-off for making it is modest. However, if the pay-off is large, then it is a favorable strategy. So, for the PdGs if the pay-off is ensuring its own food reservoir during the sporulation process, then it might not be expensive at all.

Specific and Controlled Suicide

A microorganism that triggers its own cell death in a controlled manner has to make sure that (i) not all cells are killed by the process and (ii) it has to sense that the killing molecule is 'home made.' In other words, the compartments close to the future site of development have to undergo cell death in a controlled manner. It is important to note that the production of PdGs is indeed well controlled, and strictly correlates to the so-called transition phase in submerged cultures, and to the phase immediately preceding the onset of morphological development in solid-grown cultures (Takano et al., 1992; Bibb, 1996; Sun et al., 1999). This strongly suggests that the cell death caused by the PdGs is indeed programmed, in other words represents PCD in the true sense, as a way to eradicate the old mycelial biomass for the next growth phase, namely the aerial hyphae and spores. One could easily imagine that the physiological reaction to sensing the presence of a toxic compound must be very different if the molecule originates from competitors that share the same environmental niche or, instead, if the molecule is self-made. This also explains why microorganisms that undergo PCD to sustain metamorphosis would use their own molecule for signaling the timing of differentiation. If they would all use the same trigger their development would be synchronized, while their fitness to the environmental is different. Similarly, plants all have their own cocktail of hormones and physico-chemical parameters for inducing flowering. In addition, having a killing system not 'universal' (conserved molecule or mechanisms in all actinobacteria) allows also having its own mechanism of resistance [see examples of specific resistance to enediyene, daunorubicin and doxorubicin, mitomycins, bleomycins and other DNA-damaging antibiotics produced by Actinobacteria (Tenconi and Rigali, 2018)]. Therefore, a secret code in the form of a complex natural product with resistance is required.

However, a key experimental result presented here suggests that the reality is more complicated; the *redD* null mutant (which fails to produce PdGs) still undergoes an initial, limited round of PCD. Importantly, there are also many culture conditions where *S. coelicolor* does not produce PdGs but still undergoes PCD, which means that cell death can be completed in the absence of PdGs. When PdG biosynthesis is required remains unclear, but seems inextricably linked to growth conditions where microorganisms are facing challenging conditions. Indeed, previous works both in *Streptomyces* and *Serratia* species, reported that PdG biosynthesis is associated with diverse, stress-inducing culture conditions such as UV-light exposure, oxidative stress, nutrient depletion or competition with other microorganisms (Williamson et al., 2006; Luti and Mavituna, 2011a,b; Stankovic et al., 2014). In many cases authors have postulated that the ecophysiological role of PdGs is to protect the producing strain against these various stresses (Stankovic et al., 2014). How do the cell-eradicating properties of PdGs described in this work, and those also well documented for eukaryotic cell death or apoptosis (Williamson et al., 2007), fit with the proposed protective roles in various stressful conditions? Again, when part of the colony is challenged by lethal stress conditions, inducing controlled cell death in that part of the colony would be a good strategy to spare at least part of the population. In such a scenario, at least a fraction of the population might survive for dissemination to more appropriate living conditions.

CONCLUSION

The data presented here shows that PdGs are closely associated with PCD process of the producing organism. Specifically, an initial phase of PCD appears to serve as a 'detonator' that triggers the widespread self-killing of the *S. coelicolor* biomass through the action of the PdGs. Our work highlights for the first time genes encoding toxic determinants of the PCD phenomenon in mycelial bacteria. How exactly prodiginines are destructive to *S. coelicolor* still has to be clarified as these molecules are cytotoxic in multiple ways (Williamson et al., 2007; Darshan and Manonmani, 2016; Yang et al., 2017; Zhang et al., 2017). Providing answers to how some filaments survive the production of these lethal compounds and switch to a reproductive life-style are also key questions going forward. How much the use of cytotoxic "secondary metabolites" in PCD processes is widespread in microorganisms is difficult to estimate and this question is currently under investigation. The proposed role in PCD of antitumor antibiotics could be played by other types of antibiotics (targeting the cell wall, the translational machinery,. . .) which increases the possibility that the observed phenomenon is not unique to prodiginines and *S. coelicolor*. Hence, next to weapons in warfare between organisms or molecules involved in inter- and intra-species communications, our work proposes a third role for antibiortic (in the literal meaning of the word 'against life') that is elements required for self-toxicity in PCD events either necessary to maintain an appropriate growth balance, to cope with stress conditions, or as part of developmental programs.

AUTHOR CONTRIBUTIONS

ET and SR designed the experiments. ET, assisted by CH, performed the experiments. All authors interpreted the data and wrote the manuscript.

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REFERENCES

Alkhatib, Z., Abts, A., Mavaro, A., Schmitt, L., and Smits, S. H. J. (2012). Lantibiotics: how do producers become self-protected? *J. Biotechnol.* 159, 145–154. doi: 10.1016/j.jbiotec.2012.01.032

Bayles, K. W. (2014). Bacterial programmed cell death: making sense of a paradox. *Nat. Rev. Microbiol.* 12, 63–69. doi: 10.1038/nrmicro3136

Beites, T., Oliveira, P., Rioseras, B., Pires, S. D. S., Oliveira, R., Tamagnini, P., et al. (2015). *Streptomyces natalensis* programmed cell death and morphological differentiation are dependent on oxidative stress. *Sci. Rep.* 5:12887. doi: 10.1038/srep12887

Bibb, M. (1996). 1995 Colworth Prize Lecture. The regulation of antibiotic production in *Streptomyces coelicolor* A3(2). *Microbiology* 142(Pt 6), 1335–1344. doi: 10.1099/13500872-142-6-1335

Chater, K. F. (2006). *Streptomyces* inside-out: a new perspective on the bacteria that provide us with antibiotics. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 361, 761–768. doi: 10.1098/rstb.2005.1758

Claessen, D., Rozen, D. E., Kuipers, O. P., Søgaard-Andersen, L., and van Wezel, G. P. (2014). Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies. *Nat. Rev. Microbiol.* 12, 115–124. doi: 10.1038/nrmicro3178

Darshan, N., and Manonmani, H. K. (2016). Prodigiosin inhibits motility and activates bacterial cell death revealing molecular biomarkers of programmed cell death. *AMB Exp.* 6:50. doi: 10.1186/s13568-016-0222-z

D'Costa, V. M., McGrann, K. M., Hughes, D. W., and Wright, G. D. (2006). Sampling the antibiotic resistome. *Science* 311, 374–377. doi: 10.1126/science.1120800

Fernández, M., and Sánchez, J. (2002). Nuclease activities and cell death processes associated with the development of surface cultures of *Streptomyces antibioticus* ETH 7451. *Microbiology* 148, 405–412. doi: 10.1099/00221287-148-2-405

Filippova, S. N., and Vinogradova, K. A. (2017). Programmed cell death as one of the stages of streptomycete differentiation. *Microbiology* 86, 439–454. doi: 10.1134/S0026261717040075

Floriano, B., and Bibb, M. (1996). *afsR* is a pleiotropic but conditionally required regulatory gene for antibiotic production in *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* 21, 385–396. doi: 10.1046/j.1365-2958.1996.6491364.x

Galm, U., Hager, M. H., Van Lanen, S. G., Ju, J., Thorson, J. S., and Shen, B. (2005). Antitumor antibiotics: bleomycin, enediynes, and mitomycin. *Chem. Rev.* 105, 739–758. doi: 10.1021/cr030117g

González-Pastor, J. E. (2011). Cannibalism: a social behavior in sporulating *Bacillus subtilis*. *FEMS Microbiol. Rev.* 35, 415–424. doi: 10.1111/j.1574-6976.2010.00253.x

González-Pastor, J. E., Hobbs, E. C., and Losick, R. (2003). *Cannibalism* by sporulating bacteria. *Science* 301, 510–513. doi: 10.1126/science.1086462

Hopwood, D. A. (2007). How do antibiotic-producing bacteria ensure their self-resistance before antibiotic biosynthesis incapacitates them? *Mol. Microbiol.* 63, 937–940. doi: 10.1111/j.1365-2958.2006.05584.x

Huang, J., Lih, C. J., Pan, K. H., and Cohen, S. N. (2001). Global analysis of growth phase responsive gene expression and regulation of antibiotic biosynthetic pathways in *Streptomyces coelicolor* using DNA microarrays. *Genes Dev.* 15, 3183–3192. doi: 10.1101/gad.943401

Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2017). *Practical Streptomyces Genetics*. Norwich: John Innes Foundation.

Lee, D.-Y., and Rhee, G.-Y. (1999). Circadian rhythm in growth and death of *Anabaena Flos-Aquae* (cyanobacteria). *J. Phycol.* 35, 694–699. doi: 10.1046/j.1529-8817.1999.3540694.x

Luti, K. J., and Mavituna, F. (2011a). Elicitation of *Streptomyces coelicolor* with dead cells of *Bacillus subtilis* and *Staphylococcus aureus* in a bioreactor increases production of undecylprodigiosin. *Appl. Microbiol. Biotechnol.* 90, 461–466. doi: 10.1007/s00253-010-3032-r2

Luti, K. J., and Mavituna, F. (2011b). *Streptomyces coelicolor* increases the production of undecylprodigiosin when interacted with *Bacillus subtilis*. *Biotechnol. Lett.* 33, 113–118. doi: 10.1007/s10529-010-0401-y

Lyons, N. A., and Kolter, R. (2015). On the evolution of bacterial multicellularity. *Curr. Opin. Microbiol.* 24, 21–28. doi: 10.1016/j.mib.2014.12.007

Mak, S., Xu, Y., and Nodwell, J. R. (2014). The expression of antibiotic resistance genes in antibiotic-producing bacteria. *Mol. Microbiol.* 93, 391–402. doi: 10.1111/mmi.12689

Manteca, A., Fernández, M., and Sánchez, J. (2005). A death round affecting a young compartmentalized mycelium precedes aerial mycelium dismantling in confluent surface cultures of *Streptomyces antibioticus*. *Microbiology* 151, 3689–3697. doi: 10.1099/mic.0.28045-0

Méndez, C., Braña, A. F., Manzanal, M. B., and Hardisson, C. (1985). Role of substrate mycelium in colony development in *Streptomyces*. *Can. J. Microbiol.* 31, 446–450. doi: 10.1139/m85-083

Miguélez, E. M., Hardisson, C., and Manzanal, M. B. (1999). Hyphal death during colony development in *Streptomyces antibioticus*: morphological evidence for the existence of a process of cell deletion in a multicellular prokaryote. *J. Cell Biol.* 145, 515–525. doi: 10.1083/jcb.145.3.515

Miguélez, E. M., Hardisson, C., and Manzanal, M. B. (2000). Streptomycetes: a new model to study cell death. *Int. Microbiol.* 3, 153–158.

Mo, S., Kim, J., and Oh, C.-H. (2013). Different effects of acidic pH shock on the prodiginine production in *Streptomyces coelicolor* M511 and SJM1 mutants. *J. Microbiol. Biotechnol.* 23, 1454–1459. doi: 10.4014/jmb.1307.07067

Montaner, B., and Pérez-Tomás, R. (2003). The prodigiosins: a new family of anticancer drugs. *Curr. Cancer Drug Targets* 3, 57–65. doi: 10.2174/1568009033333772

Morgenstern, A., Paetz, C., Behrend, A., and Spiteller, D. (2015). Divalent transition-metal-ion stress induces prodigiosin biosynthesis in *Streptomyces coelicolor* M145: formation of coeligiosins. *Chemistry* 21, 6027–6032. doi: 10.1002/chem.201405733

Nariya, H., and Inouye, M. (2008). MazF, an mRNA interferase, mediates programmed cell death during multicellular *Myxococcus* development. *Cell* 132, 55–66. doi: 10.1016/j.cell.2007.11.044

Ogawara, H. (2015). Penicillin-binding proteins in *Actinobacteria*. *J. Antibiot.* 68, 223–245. doi: 10.1038/ja.2014.148

Pérez-Tomás, R., Montaner, B., Llagostera, E., and Soto-Cerrato, V. (2003). The prodigiosins, proapoptotic drugs with anticancer properties. *Biochem. Pharmacol.* 66, 1447–1452. doi: 10.1016/S0006-2952(03)00496-9

Pérez-Tomás, R., and Viñas, M. (2010). New insights on the antitumoral properties of prodiginines. *Curr. Med. Chem.* 17, 2222–2231. doi: 10.2174/092986710791331103

Rigali, S., Nothaft, H., Noens, E. E., Schlicht, M., Colson, S., Müller, M., et al. (2006). The sugar phosphotransferase system of *Streptomyces coelicolor* is regulated by the GntR-family regulator DasR and links N-acetylglucosamine metabolism to

the control of development. *Mol. Microbiol.* 61, 1237–1251. doi: 10.1111/j.1365-2958.2006.05319.x

Rigali, S., Titgemeyer, F., Barends, S., Mulder, S., Thomae, A. W., Hopwood, D. A., et al. (2008). Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*. *EMBO Rep.* 9, 670–675. doi: 10.1038/embor.2008.83

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Available at: https://www.cabdirect.org/cabdirect/abstract/19901616061 [accessed December 26, 2017].

Schäberle, T. F., Orland, A., and König, G. M. (2014). Enhanced production of undecylprodigiosin in *Streptomyces coelicolor* by co-cultivation with the corallopyronin A-producing myxobacterium, *Corallococcus coralloides*. *Biotechnol. Lett.* 36, 641–648. doi: 10.1007/s10529-013-1406-r0

Shapiro, J. A. (1998). Thinking about bacterial populations as multicellular organisms. *Annu. Rev. Microbiol.* 52, 81–104. doi: 10.1146/annurev.micro.52.1.81

Stankovic, N., Senerovic, L., Ilic-Tomic, T., Vasiljevic, B., and Nikodinovic-Runic, J. (2014). Properties and applications of undecylprodigiosin and other bacterial prodigiosins. *Appl. Microbiol. Biotechnol.* 98, 3841–3858. doi: 10.1007/s00253-014-5590-1

Sugiyama, M. (2015). Structural biological study of self-resistance determinants in antibiotic-producing actinomycetes. *J. Antibiot.* 68, 543–550. doi: 10.1038/ja.2015.32

Sun, J., Kelemen, G. H., Fernández-Abalos, J. M., and Bibb, M. J. (1999). Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). *Microbiology* 145(Pt 9), 2221–2227. doi: 10.1099/00221287-145-9-2221

Surette, M. D., and Wright, G. D. (2017). Lessons from the environmental antibiotic resistome. *Annu. Rev. Microbiol.* 71, 309–329. doi: 10.1146/annurev-micro-090816-093420

Takano, E., Gramajo, H. C., Strauch, E., Andres, N., White, J., and Bibb, M. J. (1992). Transcriptional regulation of the *redD* transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* 6, 2797–2804. doi: 10.1111/j.1365-2958.1992.tb01459.x

Tenconi, E., Guichard, P., Motte, P., Matagne, A., and Rigali, S. (2013). Use of red autofluorescence for monitoring prodiginine biosynthesis. *J. Microbiol. Methods* 93, 138–143. doi: 10.1016/j.mimet.2013.02.012

Tenconi, E., Jourdan, S., Motte, P., Virolle, M.-J., and Rigali, S. (2012). Extracellular sugar phosphates are assimilated by *Streptomyces* in a PhoP-dependent manner. *Antonie Van Leeuwenhoek* 102, 425–433. doi: 10.1007/s10482-012-9763-r6

Tenconi, E., and Rigali, S. (2018). Self-resistance mechanisms to DNA-damaging antitumor antibiotics in actinobacteria. *Curr. Opin. Microbiol.* 45, 100–108. doi: 10.1016/j.mib.2018.03.003

Tenconi, E., Urem, M., Świątek-Połatyńska, M. A., Titgemeyer, F., Muller, Y. A., van Wezel, G. P., et al. (2015). Multiple allosteric effectors control the affinity of DasR for its target sites. *Biochem. Biophys. Res. Commun.* 464, 324–329. doi: 10.1016/j.bbrc.2015.06.152

Tomasz, A. (2006). Microbiology. Weapons of microbial drug resistance abound in soil flora. *Science* 311, 342–343. doi: 10.1126/science.1123982

van Wezel, G. P., White, J., Hoogvliet, G., and Bibb, M. J. (2000). Application of *redD*, the transcriptional activator gene of the undecylprodigiosin biosynthetic pathway, as a reporter for transcriptional activity in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. *J. Mol. Microbiol. Biotechnol.* 2, 551–556.

White, J., and Bibb, M. (1997). *bldA* dependence of undecylprodigiosin production in *Streptomyces coelicolor* A3(2) involves a pathway-specific regulatory cascade. *J. Bacteriol.* 179, 627–633. doi: 10.1128/jb.179.3.627-633.1997

Williamson, N. R., Fineran, P. C., Gristwood, T., Chawrai, S. R., Leeper, F. J., and Salmond, G. P. C. (2007). Anticancer and immunosuppressive properties of bacterial prodiginines. *Future Microbiol.* 2, 605–618. doi: 10.2217/17460913.2.6.605

Williamson, N. R., Fineran, P. C., Leeper, F. J., and Salmond, G. P. C. (2006). The biosynthesis and regulation of bacterial prodiginines. *Nat. Rev. Microbiol.* 4, 887–899. doi: 10.1038/nrmicro1531

Wright, G. D. (2012). The origins of antibiotic resistance. *Handb. Exp. Pharmacol* 211, 13–30. doi: 10.1007/978-3-642-28951-4_2

Yang, K., Chen, Q., Zhang, D., Zhang, H., Lei, X., Chen, Z., et al. (2017). The algicidal mechanism of prodigiosin from *Hahella* sp. KA22 against *Microcystis aeruginosa*. *Sci. Rep.* 7:7750. doi: 10.1038/s41598-017-08132-5

Zhang, H., Wang, H., Zheng, W., Yao, Z., Peng, Y., Zhang, S., et al. (2017). Toxic effects of prodigiosin secreted by *Hahella* sp. KA22 on Harmful Alga *Phaeocystis globosa*. *Front. Microbiol.* 8:999. doi: 10.3389/fmicb.2017.00999

Zhou, L., Li, Y., Li, Y., and Wu, D. (2005). Spatio-temporal expression of the pathway-specific regulatory gene *redD* in *S. coelicolor*. *J. Zhejiang Univ. Sci. B* 6, 464–469. doi: 10.1631/jzus.2005.B0464

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REVIEW
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Streptomyces as a Prominent Resource of Future Anti-MRSA Drugs

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Methicillin-resistant *Staphylococcus aureus* (MRSA) pose a significant health threat as they tend to cause severe infections in vulnerable populations and are difficult to treat due to a limited range of effective antibiotics and also their ability to form biofilm. These organisms were once limited to hospital acquired infections but are now widely present in the community and even in animals. Furthermore, these organisms are constantly evolving to develop resistance to more antibiotics. This results in a need for new clinically useful antibiotics and one potential source are the *Streptomyces* which have already been the source of several anti-MRSA drugs including vancomycin. There remain large numbers of *Streptomyces* potentially undiscovered in underexplored regions such as mangrove, deserts, marine, and freshwater environments as well as endophytes. Organisms from these regions also face significant challenges to survival which often result in the production of novel bioactive compounds, several of which have already shown promise in drug development. We review the various mechanisms of antibiotic resistance in MRSA and all the known compounds isolated from *Streptomyces* with anti-MRSA activity with a focus on those from underexplored regions. The isolation of the full array of compounds *Streptomyces* are potentially capable of producing in the laboratory has proven a challenge, we also review techniques that have been used to overcome this obstacle including genetic cluster analysis. Additionally, we review the *in vivo* work done thus far with promising compounds of *Streptomyces* origin as well as the animal models that could be used for this work.

Keywords: *Streptomyces*, methicillin-resistant *Staphylococcus aureus*, antibiotics, anti-MRSA, Actinobacteria

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) show resistance to almost all therapeutic β-lactams and other classes of antibiotics. MRSA was first reported in 1961 (Jevons, 1961), when a β-lactamase producing strain of *S. aureus* which had previously been methicillin sensitive, developed methicillin resistance. The fact that this occurred only a year after the introduction of the semi-synthetic penicillin was truly a harbinger of the specter of drug resistance that would haunt healthcare providers in the years to come. MRSA has since been isolated in many hospitals around

the world and currently represents a serious healthcare problem. It is particularly prevalent (>50%) in South America, Romania, and Japan and is becoming increasingly widespread in other countries (Lee et al., 2018). Concerns have also been raised over the emergence of MRSA among livestock due to the extensive use of antibiotics to prevent and treat infections (Conceição et al., 2017). Recently, cases of MRSA have been reported outside of the hospital settings, mainly affecting young, healthy individuals (Braun et al., 2016; Braun and Kahanov, 2018).

While MRSA generally do not cause severe disease, there are limited therapeutic options available to MRSA infections making all infections, even mild ones, noteworthy. By definition, MRSA are resistant to penicillin-like antibiotics, and they have now been noted to be developing resistance to other existing classes of antibiotics (Kaur and Chate, 2015). There is a constant hunt for new antibiotics but in the last few decades, only a limited number have been added to the clinician's arsenal; among them are linezolid in 2000 (Lee and Caffrey, 2017), daptomycin (a lipopeptide) in 2003 (Frankenfeld et al., 2018) and ceftaroline in 2010 (Long et al., 2014). Presently, vancomycin remains the most important first-line therapy for severe MRSA infection. However, the emergence of MRSA with reduced susceptibility to vancomycin (Ghahremani et al., 2018) as well as daptomycin (Roch et al., 2017) and linezolid resistance (De Dios Caballero et al., 2015) have been reported. Given that bacteria naturally evolve toward developing resistance to all antibiotics they are exposed to, there is a critical need for research focusing on the search of novel antibacterial agents as well as innovative approaches to combat MRSA. In light of the pressing need for new anti-MRSA drugs, the World Health Organization has also included MRSA as an important antibiotic-resistant bacteria requiring the urgent need for new drugs (WHO, 2017).

Natural sources such as microbes, plants, and animals have contributed immensely to the development of current drugs (Gu et al., 2013; Tang et al., 2016; Ma et al., 2018; Tan et al., 2018). Among these natural sources, microbes, particularly those belonging to the gram-positive Actinobacteria phylum, stand out as a rich source of drugs (Bérdy, 2012). The genus *Streptomyces* is categorized under the phylum Actinobacteria (Waksman and Henrici, 1943); they currently represent the most widely studied genus under the Actinobacteria phylum with 843 species and 38 subspecies to date (LPSN, 2018). The vast diversity within this genus based on its sheer numbers is particularly evident when compared with other genera: *Micromonospora* genus has 84 species and 7 subspecies, *Propionibacterium* has only 16 species and 4 subspecies, while *Salinispora* has 3 species (LPSN, 2018) at the time of writing (June 2018). Based on historical evidence, *Streptomyces* seem to be a viable target in the hunt for new drugs as they represent the source of 75% of clinically useful antibiotics presently available (Janardhan et al., 2014). One of the newer antibiotics currently in use, daptomycin, represents the latest contribution of *Streptomyces* in the fight against pathogenic microbes—it was discovered in the 1980s and approved by the US Food and Drug Regulatory Administration (US FDA) for clinical use in 2003 (Frankenfeld et al., 2018). To date, *Streptomyces*-derived daptomycin remains the only naturally produced antibiotic of a novel class introduced since 2003 and it is currently considered a first-line drug for treatment of MRSA bacteremia (Choo and Chambers, 2016).

Streptomyces has a large genome, which logically contains many biosynthetic gene clusters (Bentley et al., 2002; Ikeda et al., 2003), a further indication of their potential ability to produce large numbers of compounds with diverse biological activities. However, under traditional culture condition, only a few compounds have so far been isolated—far less than what is expected based on the genome. Various methods discussed later are being used to overcome this problem. An additional recent concern is that analysis of *Streptomyces* from terrestrial soil has limited yield of new compounds but instead leads to rediscovery of known compounds. To overcome this problem, researchers now focus on isolating *Streptomyces* from underexplored ecosystems (Hong et al., 2009). Researchers are also attempting to utilize current genetic tools to identify gene clusters of promising compounds. Once identified, these biosynthetic gene clusters are modified in order to improve the efficacy of the compounds, produce better analogs of compounds, or increase the yield of the compound of interest (Alexander et al., 2010; Yang et al., 2017). Besides that, *in situ* computer-models have been used successfully to determine mechanism of action of some promising anti-MRSA compounds isolated from *Streptomyces*. Newer animal models have also been used to determine *in vivo* efficacy and toxicity of anti-MRSA compounds produced by *Streptomyces*. This review aims to highlight the potential of *Streptomyces* as a resource to combat MRSA—we look at all the anti-MRSA compounds derived from *Streptomyces* since the 1990s. We also discuss the ecological niches where the source organisms may be found, mechanism of actions of anti-MRSA compounds produced by *Streptomyces* and newer interventions for MRSA infection.

EPIDEMIOLOGY OF MRSA

Hospital-acquired MRSA (HA-MRSA) is now among the most problematic bacterial infections to treat (Kaur and Chate, 2015) and, alarmingly, it is responsible for about 20–80% of hospital infections (Krishnamurthy et al., 2014). Even though the incidence of HA-MRSA is reported to have reduced by 54.2% in the USA (Dantes et al., 2013), statistics from other parts of the world indicate this is not the general trend. For example, studies in South Africa, India and Pakistan revealed 52, 54.8, and 50% of hospital-acquired infections, respectively, were attributed to MRSA (Laxminarayan et al., 2013). According to the European Centre for Disease Prevention and Control (ECDC), the number of cases of MRSA infection varies greatly between the northern and southern regions of Europe. For example, Norway and Iceland were reported to have the lowest case of MRSA infection of 1.2 and 1.3%, respectively. Romania and Portugal represent the opposite end of the spectrum with the highest rate of cases reported with figures of 50.5 and 43.6%, respectively, in regard to invasive infections (ECDC, 2016). Most often, in hospital settings, MRSA occurs as a secondary infection and is most prevalent among the elderly, post-surgical and immunocompromised patients (Krishnamurthy et al., 2014).

These secondary infections lead to increased healthcare costs, resulting from prolonged hospital stay and additional antibiotics (Nelson et al., 2015). In Japan alone, a recent comprehensive comparative cost analysis for MRSA has been estimated to be greater than all other non-MRSA infection (Uematsu et al., 2017). As an indication of the scale of the threat posed by these organisms, in the USA, MRSA kills more people than HIV and TB combined (Boucher and Corey, 2008). In hospitals, controlling the spread of MRSA is further complicated by their ability to form biofilms on the surfaces of medical devices. These biofilms tend to be resistant to disinfectants and may act as reservoirs for growing MRSA colonies that can be transferred to another host (Suzuki et al., 2015). The carriage rate in hospitals is estimated to be around 20–60% (Pathare et al., 2016) with the resultant implication that a large proportion of health care workers (Shibabaw et al., 2013; Khanal et al., 2015; El Aila et al., 2017) and patients (Ho and Hong Kong intensive care unit antimicrobial resistance study (HK-ICARE) Group, 2003; Aslam et al., 2013; Moyo et al., 2017) are carriers of MRSA. The most common way MRSA enters a host is through a breach in the skin (Datta et al., 2014) and tends to develop into an infection when there is immunodeficiency in the host. As a result of its tendency to affect more vulnerable patient populations, MRSA has not only become a difficult disease to treat but also a costly one. While immunocompromised individuals are at higher risk of MRSA infection, worryingly there have been recent reports of MRSA infection among healthy individuals, especially children (Davoodabadi et al., 2016).

About 20 years after the first reported case of MRSA, the organism was confirmed to have spread beyond the hospital environment to the community. The earliest report of community acquired MRSA (CA-MRSA) was in Detroit, Michigan, USA in 1980 (Saravolatz et al., 1982). Several other community-based infections were reported not long after—in the community of native Indians (Taylor et al., 1990), then in 1989-1991 it was found among the Aborigines of Western Australia (Udo et al., 1993) and after that it was detected in Europe (Stegger et al., 2014). While HA-MRSA cases seem to be on the decline in the USA, CA-MRSA has emerged more strongly in communities around the world. Currently, 2 out of 100 people are carriers of CA-MRSA (CDC, 2016); which is particularly worrying as CA-MRSA can more easily spread than HA-MRSA. It has been suggested that there may be a more mobile genetic element in CA-MRSA as compared to HA-MRSA (Udo and Boswihi, 2017; Boswihi and Udo, 2018). According to CDC, a CA-MRSA is categorized as such if infection is evident on admission or a MRSA culture was obtained within 48 h of admission, with no history of admissions or medical treatment requiring invasive procedures (Gorwitz et al., 2006).

Carrier status is not limited to humans as there is evidence to suggest that animals have also become carriers of MRSA, thus creating the possibility of spread of MRSA from animals to humans. The steady increase in the global population has resulted in ever increasing demands for food supply. As part of the efforts to increase the yield of livestock production, antibiotics have been increasingly used to prevent infection. However, the uncontrolled use of antibiotics has encouraged the development of antibiotic resistance including the emergence of MRSA within these animals (Van Boeckel et al., 2015). These livestock (Conceição et al., 2017) and their products tend to become reservoirs of MRSA (Asiimwe et al., 2017). Cases of animal-associated MRSA transfer to humans have been reported (Loncaric et al., 2013; Van Duijkeren et al., 2015). A few cases where MRSA was recovered from free-living animals (Wardyn et al., 2012; Porrero et al., 2013) and pets (Bierowiec et al., 2016) have been reported. This additional reservoir of MRSA in animals in the community creates an additional threat to public health.

THE MECHANISMS OF ANTIBIOTIC RESISTANCE IN MRSA

The notion that "there is a pill for every ill" has led to a widespread public perception that medication of some sort is necessary to cure all forms of illnesses. A particularly pertinent example is the use of antibiotics for virtually any infection—including mild bacterial infections that do not warrant antibiotic treatment and even viral infections. This practice is a major cause of the rapid rise of antibiotic resistant bacteria. As a countermeasure, many countries have imposed tight regulations on the use and sale of antibiotics; however even in these countries, prescribers were found to overprescribe antibiotics. Given this scenario, it is unsurprising that in countries with less stringent regulations, there is a tendency to abuse antibiotics. This overuse of antibiotics in humans and animals has accelerated development of antibiotic resistance (Ventola, 2015).

Antibiotic resistance in and of itself is actually a natural phenomenon forming part of bacteria's inbuilt machinery to help them to adapt to new and changing environments. Soil bacteria possess an inbuilt "*resistome gene*" that helps them express resistance mechanisms in response to external events (Nesme and Simonet, 2015). Resistome genes present in soil bacteria can be horizontally transferred to pathogenic bacteria over a period of time. In the presence of antibiotics, these organisms also tend to develop resistance through an antibiotic resistance gene (Nesme and Simonet, 2015). This demonstrates that antibiotic resistance is inherent in bacteria and underlines the need for constant research to help develop a new supply of effective antibiotics in order to treat infections, including MRSA that are resistant to almost all β-lactam antibiotics. However, in order to effectively develop new therapies, it is crucial to first understand the various mechanisms of drug resistance and the elements of the bacterial cells that are new potential targets for drug development.

MRSA Mechanism of Resistance to β-Lactams

There have been studies exploring the origins of the methicillin resistance gene in MRSA. Studying sets of MRSA isolates revealed one single clone (Kreiswirth et al., 1993), with the most probable ancestral source dating back to *Staphylococcus sciuri*. Yet another study found support for *S. fleurettii* as the most probable origin (Tsubakishita et al., 2010). The study by Rolo et al. (2017) provided support that the Staphylococcal Chromosomal cassette (SCC*mec*), a mobile genetic element, evolved from three

Staphylococcus species—*S. vitulinus, S. fleuretti,* and *S. sciuri*. Since its original development, the antibiotic resistance gene remains highly conserved in chromosomes of MRSA, and is currently used as a marker for screening and identifying MRSA isolates (Koupahi et al., 2016; Luo et al., 2017). Up to the present, 11 types of SCC*mec* (40-60Kb) have been identified (IWG-SCC, 1999). In the relevant types, the SCC*mec* is the region which contains the *mec*A gene which is responsible for the expression of PBP2a—an altered penicillin binding protein which has a low affinity for β-lactam antibiotics. On the basis of genetics, it is the expression of PBP2a that differentiates MRSA from other *S. aureus* strains, and confers resistance to most of β-lactams and other classes of antibiotics. The expression of PBP2a encoded on *mecA* gene is regulated by genes identified as *mecR1* and *mecI* on the *mecA* element, inducing and repressing transcription of PBP2a protein (Lee et al., 2018). Among the 11 SCC*mec* types (Mkrtchyan et al., 2015), 5 are known to be epidemic (Rachman et al., 2017).

S. aureus strains usually have 4 PBPs (PBP1, PBP2, PBP3, and PBP4) which play an important role in the biosynthesis of peptidoglycans—the structural frame of cell walls in gram-positive bacteria. As the name suggests, peptidoglycans are chains of glycans, namely *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), cross-linked by peptides (Peacock and Paterson, 2015). Two biochemical reactions involved in cell wall synthesis are transglycosylation and transpeptidation, which are carried out by the enzymes transglycosylase and transpeptidase (PBP2), respectively. The former enzyme catalyses the elongation of glycans while PBP2 follows up from this action, crosslinking glycans at β1-4 NAM via 5 glycine amino acids (Fishovitz et al., 2014). This is key to our discussion as PBP2, or transpeptidase, is the target site of β-lactam antibiotics. In methicillin-sensitive *Staphylococcus aureus* (MSSA), the β-lactam class of antibiotics target the PBP2 causing cell death of susceptible *S. aureus.* In MRSA, however, the resistance-conferring PBP2a is overexpressed in the presence of β-lactams resulting in the cells still being able to generate sufficient cross-linking to survive.

The peptidoglycan layers are much thicker (20–80 nm) in gram-positive bacteria as compared to gram negative (1.5–10 nm; Mai-Prochnow et al., 2016), giving rise to the thick cell walls characteristic of gram positive bacteria. Peptidoglycans in gram-positive bacteria give shape and protect the cell from osmotic pressure and subsequent rupture and leaking of contents (Lovering et al., 2012). The β-lactam group of antibiotics prevent formation of this thick protective peptidoglycan layer by preventing cross linking of the glycan chains. Unfortunately, MRSA have developed resistance to almost all the β-lactam groups of antibiotics because SCC*mec* is a mobile element, it can be either horizontally transferred or vertically transferred (Grumann et al., 2014; Howden et al., 2014). The SCC*mec* types I, IV and V are the smallest of the 5 types and only express resistance toward the β-lactam groups of antibiotics; they therefore remain susceptible to other classes of antibiotics (Zuma et al., 2017). CA-MRSA have been found to contain smaller *mec*A IV and V genes compared to HA-MRSA, thus conferring the ability to move their mobile genetic elements much faster and with greater ease between various *S. aureus* chromosomes forming the basis of CA-MRSA's tendency to spread rapidly and more readily. This is demonstrated by the fact that cases of CA-MRSA are known to be widespread in Europe, USA, and other continents (Tavares et al., 2010; Dukic et al., 2013; San Sit et al., 2017). As for HA-MRSA, they possess SCC*mec* variants (SCC*mec* types II and III) which are larger in size, and thus providing additional genetic capacity which increases the likelihood of the presence of transposons and resistance genes (Hiramatsu et al., 2013). A specific region on the SCC*mec* known as the J region was shown to have antibiotic resistance genes of other classes of antibiotics (IWG-SCC, 1999).

Resistance to Other Antibiotics

MRSA have been shown to have begun developing resistance to several important classes of antibiotics which are being used for treatment of severe MRSA infection including vancomycin, daptomycin, linezolid, tigecycline, and ceftaroline. Furthermore, MRSA strains were found to be resistant toward aminoglycosides, tetracyclines, lincosamides, and streptorubin B. This overwhelming spectrum of resistance imposes a huge burden on healthcare institutions. There are a few common mechanisms of resistance that pathogenic bacteria can develop when they exposed to antibiotics. The three main resistance mechanisms currently known are the inactivation of antibiotics by enzymes, efflux pumps that reduce intracellular drug concentration as well as alteration of target site. MRSA have been shown to employ all of these strategies.

To date, vancomycin remains the most important antibiotic for the treatment of severe MRSA infections. Vancomycin belongs to the glycopeptide antibiotic class that target the peptidoglycan layer of the bacterial cell wall. Hence, like β-lactam antibiotics, their effect is bactericidal. There appears to be a range of the level of resistance to vancomycin by *S. aureus* with the resistant organisms being either vancomycin-intermediate *S. aureus* (VISA) or vancomycin-resistant *S. aureus* (VRSA) with MIC $\leq$ 2 and 4–8 μg/μL, respectively (CLSI, 2015). The acquisition of vancomycin resistance in MRSA was shown to be different between the vancomycin resistant and the vancomycin intermediate strains. The vancomycin-resistant MRSA was found to contain the *vanA* operon (*vanA, vanH, vanY, vanX, vanZ*) and can either be acquired from vancomycin-resistant enterococci (VRE) via transposon 1546 or through horizontal transfer of original VRE plasmid, consequently leading to an alteration of the precursor of cell wall peptidoglycan, specifically the depsipeptide D-Ala-D-Lac. On the other hand, the emergence of vancomycin-intermediate MRSA involves chromosomal point mutations of polygenes resulting in the thickening of the peptidoglycan cell walls (McGuinness et al., 2017).

The second most important anti-MRSA drug is daptomycin which belongs to the lipopeptide antibiotic class (Schriever et al., 2005). Daptomycin targets the cell membrane causing depolarization and destabilization of the cell membrane leading to bactericidal effect (Alborn et al., 1991). The mechanism of resistance is multifactorial and involves a stepwise mutation of multiple genes (Bæk et al., 2015). According to Cafiso et al. (2014), *dltABCD* genes is a common

pathway for reduced susceptibility of daptomycin, whereas *mprF* gene mutation was expressed in only certain strains that were tested (Cafiso et al., 2014). Another yet important gene mutation that was related to reduced daptomycin susceptibility is the RNA polymerase subunits (*rpoB*) (Cui et al., 2010). It is important to note that the incidence of daptomycin resistance is rare with absence of outbreak to date. Studies have demonstrated that this may be due to the involvement of high fitness cost in the development of daptomycin resistance required for dissemination (Roch et al., 2017).

Tigecycline is the first semi-synthetic antibiotic under the minocycline antibiotic class. Studies have reported MRSA to have overexpression of efflux pump through the mutations of both *mep*R and *mep*A genes resulting in overexpression of *mep*A and derepression of *mep*R. Furthermore, studies have shown the mutation of the ribosomal protein at S10 (Argudín et al., 2018). Study by Dabul et al. (2018) found out that mutation in *rpsJ* was not observed in their MRSA strain studied, but only the efflux mechanism was determined (Dabul et al., 2018). Interestingly, tigecycline resistance is not associated with fitness cost as compared with daptomycin (Dabul et al., 2018).

Ceftaroline belongs to the cephalosporin antibiotics and has been recently approved by FDA. Unlike other β-lactam antibiotics that target cell wall of *S. aureus*, ceftaroline has a high affinity toward the PBP2a of MRSA (Saravolatz et al., 2011). The ceftaroline susceptibility is defined as MIC of $\leq$ 1mg/L, intermediate resistance at MIC of 2 and $\geq$ 4 mg/L (CLSI, 2018). According to Alm et al. (2014), ceftaroline resistance is developed firstly by a mutation at the non-penicillin binding site of PBP2a and followed by a mutation at the active site (Long et al., 2014).

Antibiotics that inhibit protein synthesis are aminoglycosides, tetracyclines, macrolides, clindamycin, oxazolidones, and rifampin. Aminoglycoside resistance occurs via enzymatic inactivation—the most predominant aminoglycoside modifying enzyme is the aminoglycoside acetyl transferase responsible for aminoglycoside resistance in MRSA (Mahdiyoun et al., 2016). Tetracycline resistance in MRSA also occurs through a variety of mechanisms, including drug efflux and ribosomal protection mediated by *tetK* (Ullah et al., 2012) and *tetM* (Ong et al., 2017), respectively. Macrolide resistance in MRSA appears to be related to methylation of ribosomes by enzymes encoded on erythromycin resistance methylase (*erm*) genes. Studies have shown that MRSA strains express either dominant *ermA* (Lim et al., 2012) or *ermC* genes (Da Paz Pereira et al., 2016; Osman et al., 2015;), which is common among macrolide, lincosamide and streptogramin B (MLS_B) resistant organisms. Linezolid belongs to the oxazolidone class of antibiotics and is a protein synthesis inhibitor (Kloss et al., 1999) with bacteriostatic action. The resistance gene responsible for linezolid resistance was identified as *cfr* gene, encoding a methyltransferase that modifies the 23S rRNA site of the 50S ribosomal subunit, preventing linezolid to bind to it (Toh et al., 2007; Quiles-Melero et al., 2012). Work on these organisms reported the presence of *cfr* gene in chromosomes of clinical human MRSA isolates as being responsible for mediating resistance in MRSA (Morales et al., 2010). Rifampin is a first-line drug for treatment of TB which is also used clinically to treat severe MRSA as an adjunct to vancomycin. Studies identified the *rpoB* gene as conferring resistance to rifampin by point mutation in the conserved region for ß-subunit of RNA polymerase (Van Rensburg et al., 2012).

Fluoroquinolones belong to the quinolone antibiotics that target DNA gyrases involved in bacterial DNA synthesis (Hooper and Jacoby, 2016). Resistance in MRSA appears to be mediated through a combination of mechanisms such as alteration of target site and prevention of drug access to the bacterial cell by efflux pump. NorA is one of the multidrug efflux pumps identified in MRSA. This pump belongs to major facilitator superfamily (MFS) which extrudes quinolone compounds. An additional mechanism of quinolone resistance in MRSA is the alteration of topoisomerase IV which is the primary target site for quinolone. This appears to be predominantly the result of a mutation occurring in the quinolone resistance determinant region (QRDR) of *parC* gene (Hashem et al., 2013; Hooper and Jacoby, 2016).

QUORUM SENSING IN MRSA

Quorum sensing is the means through which bacteria sense the external environment in order to adapt to changes or stress and these may include pH, antibiotics, minerals Abisado et al., 2018; Igarashi et al., 2013) and even cell population density (Rutherford and Bassler, 2012). Regulating these changes helps bacteria to survive in critical conditions. The two-component signaling (TCS) system called histidine kinase sensor and its cognate response regulator (Utsumi, 2017) facilitate the regulation of changes that is required by bacteria in order to cope with the outside environment. To date, there are many TCSs found in bacterial populace (Fabret and Hoch, 1998; Lange et al., 1999; Kawada-Matsuo et al., 2013; Guo et al., 2017), of which however, the WalK/WalR system stand out as an important TCS for regulating cell wall metabolism (Zheng et al., 2015). Interestingly, it is so far found in gram-positive bacteria with low G + C content such as *S. aureus*. Among the 16 TCSs found in *S. aureus*, the WalK/WalR was observed to be single most important regulator for virulence and cell wall metabolism among others functions (Ji et al., 2016). In cell wall metabolism, the WalK/WalR system activates autolysins known as peptidoglycan hydrolases that facilitates restructuring of peptidoglycan layer of the cell wall and promotes continuous cell growth and division (Utsumi, 2017). Since the cell wall provides the structural support necessary for cell survival, the WalK/WalR system therefore plays a significant role in gram-positive bacteria such as MRSA. Further, WalK is a master regulator for cell wall metabolism as nine cell wall metabolism genes are dependent on WalK/WalR (Utsumi, 2017). Previous studies have shown that inhibiting the WalK/WalR system is detrimental and bactericidal to cells (Gotoh et al., 2010; Igarashi et al., 2013). Therefore, WalK/WalR system is a promising target for drug development of anti-MRSA therapy. Among other TCSs are those that regulate biofilm formation and virulence factors that may offer potential for future antibiotics to treat MRSA-related infections.

Biofilm and Virulence of MRSA

Biofilm refers to a community of microbes that is firmly attached to surfaces and surrounded by a matrix of biopolymers (Flemming et al., 2016). Even though biofilm was first proposed in the 1970's by Costerton (Costerton et al., 1978), the importance of biofilm only became more apparent recently when genes responsible for expressing biofilm were characterized (Cucarella et al., 2001; Atshan et al., 2012; McCourt et al., 2014). Biofilm is a pressing medical problem in hospital settings, especially with regards to HA-MRSA infections, as it facilitates the persistence of MRSA in hospitals. Biofilm confers bacteria protection from extracellular threats such as antibiotics, disinfectants and the human immune response. The biofilm environment also allows bacterial communities to communicate with each other through quorum sensing molecules, for nutrients and space. Furthermore, the transfer of antibiotic resistance genes within the biofilm is also possible. These factors result in these organisms within biofilms demonstrating 1,000-times resistance to normal antibiotic doses (Wu et al., 2015). The communities of microbes then grow and mature within the matrix. Once the organisms mature, they detach from the biofilm and seek a new residence to colonize where they once again begin the process of biofilm formation (Otto, 2013). In hospitals, their ability to form biofilms on the surface of medical devices is a major concern (Suzuki et al., 2015) as insertion of medical devices infected with MRSA into the human body are a reservoir of infection that is extremely difficult to treat. It has been estimated that about 65–80% of human infections with MRSA are associated to biofilm formation (Jamal et al., 2017).

Biofilm, along with adhesion, are in fact two important mechanisms identified so far for successful colonization of host tissue or artificial surfaces (Mirzaee et al., 2014), and researchers have identified genes and factors responsible for these. Proteins found on the surface of MRSA, particularly fibronectin binding proteins A (*fnbp*A) and B (*fnbp*B), autolysin enzyme and extracellular DNA (eDNA) are largely responsible for adhesion and biofilm formation. Unlike biofilm in MSSA, biofilm in MRSA is dependent on fibronectin surface protein, for adhesion to surfaces and are encoded on the *fnbp*A and *fnbp*B genes. Other proteins implicated in adhesion are the clumping factors A and B encoded on *clf* AB genes, accumulation associated protein (*aap*) and protein A (SpA). The biofilm formation in MRSA is mostly proteinaceous matrix derived from extracellular DNA (eDNA) and *fnbp* (McCarthy et al., 2015). Special proteins observed in MRSA are responsible for lysing cells and providing eDNA for the structural component of biofilm. Furthermore, studies have shown that expression of *mec*A in MRSA isolates leads to marked repression of global accessory genes (*agr*) and subsequent reduction in expression of virulence genes (McCarthy et al., 2015) while an activation of the Staphylococcal accessory regulator gene (*sarA*) is observed. Because virulence expression of *agr* loci is relatively reduced in HA-MRSA compared to CA-MRSA, it is believed that they are less virulent in nature but remain active biofilm producers. This may well prove to be a survival strategy HA-MRSA use, especially in healthcare institutions. Developing drugs that target proteins and signaling molecules in biofilm formation may prove beneficial in addressing MRSA infections.

Virulence is the ability of harmful microbes to invade and colonize the human body and they do so by producing virulence factors- small molecules or structures (Allen et al., 2014). Virulence factors of MRSA vary in degree depending on the type of MRSA and the condition of the host. An important virulence factor is Panton-Valentine Leukocidin (PVL), first described by Panton and Valentine (1932) and is increasingly found in CA-MRSA (Grumann et al., 2014). Infections with organisms carrying PVL tend to progress from mild skin and soft tissue infection (SSTI) such as boils and cellulitis, to more severe invasive infection such as severe abscesses, necrotizing pneumonia and increased complications in pneumonia (Haider and Wright, 2013). PVL in association with other leukocidin protein LukS and LukF, enables MRSA to invade cells causing severe invasive infection (Zhang et al., 2018). Other important virulence factors secreted by MRSA are Staphylococcal enterotoxins serotypes A-Q(SEs), toxic shock syndrome toxin (TSST), cytolytic toxins (hemolysis), exfoliative toxins and enzymes causing food-poisoning related diarrhea and emesis, low blood pressure and shock, bleeding and red blistering of skin, respectively (Otto, 2014).

The alarming reports of MRSA cases emerging in hospitals, communities and animals combined with the limited availability of antibiotics to treat MRSA has made it a very important infectious disease globally. At present, vancomycin remains the most important first-line therapy for severe MRSA infection (Boswihi and Udo, 2018). In order to successfully seek novel antibiotics and adjuvant therapies to treat resistant infections, it is necessary to home in on promising techniques and sources. The current search techniques for interesting compounds from *Streptomyces* should make use of new genetic tools and chemistry to accelerate the search for new treatments of MRSA. In terms of sources, microbes particularly *Streptomyces* have been demonstrated to be prolific producers of new compounds in the past and seem to show promise to continue in the present. Compounds with anti-MRSA activity isolated from *Streptomyces* are summarized in **Supplementary Table 1**. It is hoped that by utilizing advancements of genetic and chemical technology, these anti-MRSA compounds from *Streptomyces* can be further improved to be able to be used in clinical settings.

WHAT ARE *STREPTOMYCES*?

Streptomyces are filamentous gram-positive bacteria that are categorized under the phylum Actinobacteria (Waksman and Henrici, 1943). To date, there are about 843 species and 38 subspecies with validly published names in bacterio.net (LPSN, 2018) at the time of writing (June 2018). *Streptomyces* is known to be a very robust genus of bacteria in terms of their ability to thrive in inhospitable soil conditions. They have been isolated from soil samples from the richly biodiverse tropical regions to the far reaches of the Arctic Circle. The soil samples from these ecoregions seem to hold promising *Streptomyces* with interesting chemical and genetic make-up (Ser et al., 2015a,

2016b,c, 2017, 2018a) for drug discovery work. It was logical to begin a search for *Streptomyces* by investigating soil samples because soil is readily available and is known to be a rich source of microbes (Hibbing et al., 2010). Further, *Streptomyces* are saprophytic bacteria that thrives on dead and decaying materials. Most studies investigating the microbial diversity of soil samples have reported Actinobacteria, particularly *Streptomyces* as the predominant species. The fact that *Streptomyces* can be found in almost all places studied thus far, suggest that they are a highly competent bacterial species. On a molecular level, this demonstrates their superior genetic and metabolic potential that allows them to dominate the microbial population. To understand the mechanisms that led to the latter observation, researchers began studying related genes and proteins that could contribute to their versatility and ability to thrive. One of the interesting findings, which is now considered as a characteristic of *Streptomyces*, are their high guanine plus cytosine (G + C) genomic content. On average, they carry 70% G + C content which is considered very high as compared to 40% G + C content of *Bacillus subtilis* the model gram-positive bacteria (Kunst et al., 1997). In the most widely studied species *S. coelicor*, the G + C content measures up to 72.1% (Bentley et al., 2002). The high G + C content in *Streptomyces* species is believed to have accumulated over time by a selection process of adaptation to new environment; and is thought to have granted them overarching dominance in soil.

They also express powerful secretory systems constituting 40% of the ATP-binding cassette (ABC) transporters and MFS (Zhou et al., 2016). As a result, they have attracted interest from the biotechnology industry in the production of recombinant proteins. One example is the use of *Streptomyces lividans* as a potential producer for recombinant proteins due to the possession of better excretory system as compared to the traditionally used *Escherichia coli* (Anné et al., 2014). In terms of their ecological role, *Streptomyces* utilize these secretory systems, to regulate the intake of nutrients, expel toxins and secrete enzymes. These enzymes help break down tough plant and animal materials into soluble substances that can be easily taken up through their mycelia. Once in the mycelial cells, they are utilized for basic metabolic processes to produce ethanol, amino acids, nucleotides, organic acids and vitamins. Under stressful conditions, when soil nutrients become scarce, *Streptomyces* initiate a process called programmed cell death (PCD), whereby autolysin enzymes break down mycelial cells into amino acids and sugars, providing the building blocks for aerial hyphae that will eventually produce and carry spores. This process ensures that *Streptomyces* continue to grow, reproduce and survive under stressful conditions. A key point of interest from a drug development point of view is the biosynthesis of secondary metabolites that accompanies PCD. As the name suggests, secondary metabolites are derived from primary metabolites and cons diverse compounds with different biological activities (Ser et al., 2016b, 2018b; Tan et al., 2016). Some of these compounds are antibiotics and are today perceived to play an ecological role in terms of suppressing potential competitors by killing them or inhibiting their metabolic growth. This behavior becomes essential in protecting their food supply from other microbes and it demonstrates a metabolic pathway that is highly organized and well-co-ordinated, giving rise to their dominance (Ilic-Tomic et al., 2015). It is therefore not surprising to see that *Streptomyces* have a large genome size in order to facilitate production of the range of regulatory proteins and extracellular enzymes these processes require. Many of these secondary metabolites have been successfully used as antibiotics in treating infections in humans and animals (Cho et al., 2012), though resistance has now developed to many of them. The role of *Streptomyces* as an important antibiotic producer warrants their continued exploration as a promising source in the search for new anti-MRSA antibiotics.

Streptomyces first contributed to our antibiotic arsenal in 1940 with the discovery of the naturally produced antibiotic streptomycin from soil-derived *Streptomyces griseus* (Woodruff, 2014). Since then, 2 Nobel Prizes have been awarded to researchers for the study of *Streptomyces* (Woodruff, 2014; Dlugónska, 2015). *Streptomyces* have made invaluable contributions to conventional medicines (Janardhan et al., 2014) with 75% of antibiotics clinically used in humans having their origins in *Streptomyces* derived compounds while 60% have been used in animals (Cho et al., 2012; Ser et al., 2015b; Law et al., 2017a,b).

Although artificial synthesis of new drugs is theoretically promising, natural sources such as microbes remain the better producers of antibiotics as they provide lead molecules for development of current antibiotics (Ser et al., 2016a). According to the review of Bérdy, the success rate of drugs produced through chemical synthesis compared to microbes is 0.005 to 1.6 (Bérdy, 2012). Even today, microbe-derived chemical compounds continue to inspire the development of new antibiotics (Newman and Cragg, 2016). For example, some of the newly approved drugs from the year 2000 onwards are actually synthetic analogs of antibiotics produced by *Streptomyces* namely tigecycline, everolimus, miglustat, daptomycin, biapenem, ertapenem, pimecrolimos, and ceftarolinefosamil (De Lima Procópio et al., 2012; Kumar and Chopra, 2013). Similarly, *Streptomyces* are also the source of some drugs that are currently undergoing clinical trials using combination formulation of cephalosporin-lactam (e.g., ceftazidime-avibactam and ceftolozane-tazobactam), a new cephalosporin siderophore S649266, omadacycline (phase 2 trial) and eravacycline (Fernandes and Martens, 2017). Fosfomycin was previously isolated from *S. fradiae* in 1969 and is currently undergoing comparative phase 3 clinical trials to evaluate its intravenous preparation against piperacillin/ tazobactam in the treatment of chronic urinary tract infection and acute pyelonephritis in hospitalized adults (Fernandes and Martens, 2017).

Over the years, the number of compounds reported from *Streptomyces* have significantly reduced, resulting in fewer drugs approved for clinical uses. A clear example is the US FDA approved drug daptomycin-the only new class of antibiotic introduced since 2003. Overall, *Streptomyces* have produced an estimated 10, 400 bioactive compounds since the discovery of streptomycin. The number of compounds has waxed and waned over the years—bioactive compounds isolated from *Streptomyces* increased from 2,900 between 1940 and 1974 to

5,100 compounds in the years 1975–2000, before dropping to 2,400 compounds in the period between 2001 and 2010 (Bérdy, 2012). The decrease seen was mainly attributed to rediscovery of compounds from *Streptomyces* isolated from soil, particularly from the land (Bérdy, 2012). Given that genome mining has demonstrated the potential of *Streptomyces* to produce many more compounds than what is currently observed, the search for new bioactive compounds from *Streptomyces* continued with focus on other understudied ecosystem. Researchers began to focus on understudied environments particularly those with harsh condition postulating that the challenge to survival in these situations is the driving force for speciation resulting in a rich diversity of microbiological sources including *Streptomyces* (Hong et al., 2009). In the following paragraphs, we focus on the various ecological sources of *Streptomyces* with anti-MRSA activity.

ECOLOGICAL SOURCES OF *STREPTOMYCES* WITH ANTI-MRSA POTENTIALS

Based on existing literature, *Streptomyces* derived from numerous ecological sources are active producers of natural compounds that exhibit anti-MRSA activity. These ecological sources include soil collected from terrestrial regions such as tropical forests, marine regions encompassing marine sediments and symbionts as well as newer understudied ecological niches such as endophytes, freshwater, deserts and mangrove ecosystem. A schematic diagram shown in **Figure 1** is included to give an overview of the extent of work in the area of *Streptomyces* as potential sources of anti-MRSA agents.

Terrestrial Soil

Literature analysis shows that prior to the year 2000, *Streptomyces*-based research mainly focused on terrestrial soil (Raja et al., 2003). The enthusiasm for sampling terrestrial soil *Streptomyces* for the search of newer anti-MRSA seems to have gained momentum around the 1990s. The studies presented in **Supplementary Table 1** are reflective of published work undertaken from 1990 to the current year. In total, 86 *Streptomyces* species investigated have demonstrated promising anti-MRSA activity. From studies carried out so far, 37 of those promising *Streptomyces* strains isolated from terrestrial soil demonstrated moderate to potent anti-MRSA action. In Korea, the emergence of multi-drug resistant *S. aureus* (MDRSA) prompted the search for newer sources of anti-MRSA drugs. In an attempt to seek new treatment for the MRSA strains in their country, Lee et al. (1997) began investigating *Streptomyces* extracts from local soil against clinical isolates of MDRSA. Their work led to the successful isolation of *Streptomyces* sp. HW-003 from the soil of the primary mountain forest Gyebangsan (1,577 m), a region known to have soil which has a fine texture of organic matter (Yang et al., 2014). Work on HW-003 then yielded the active compound AMRSA1 which showed potent anti-MRSA activity at 0.01–0.1 μg/mL (Lee et al., 1997) and is by far the most potent anti-MRSA compound isolated from terrestrial soil samples. Its structure however, remains unknown to date. Other promising compounds isolated from terrestrial soil include polyketomycin.

Marine Environments

Natural product researchers have also begun to explore the marine ecosystem which clearly represents an alternative source for novel *Streptomyces* as it covers 70% of the earth's surface (Beygmoradi and Homaei, 2017); especially considering that 90% of the underwater life-forms are still awaiting discovery (Mora et al., 2011; Li, 2016; NOAA, 2018). A literature search from 1990 to the present year yielded reports indicating 42 *Streptomyces* strains from the marine environment with compounds showing moderate to potent anti-MRSA activity. This strongly suggests an increasing trend in discovery of anti-MRSA compounds over time from the marine ecosystem, particularly the marine sediments.

Newer and Underexplored Ecological Sources

Natural product researchers are now exploring understudied ecosystems for potentially new *Streptomyces* that display new chemistry and biological activity–these include that biodiverse environments such as the freshwater, endophytes, deserts and mangrove ecosystem.

Freshwater Environment

Freshwater ecosystems are natural water runways such as rivers, lakes and streams except saltwater (IPCC, 2007). According to the literature, only 4 *Streptomyces* species have so far been isolated from freshwater and their compounds tested for anti-MRSA activity (Malik et al., 2008; Zhu et al., 2013). One of these was *Streptomyces* sp. MC004 which was isolated from an acidic coral mine drainage which produced angucyclic quinones including angumicynone B which showed anti-MRSA activity at MIC of 12.5 μg/mL (Park et al., 2014). *Streptomyces fulvissimus* MTCC7336 produced a high molecular weight glycopeptide with anti-MRSA activity determined by disk diffusion method (19.00 ± 1.0 mm).

Endophytes

Endophytes are microbes that live inside plant tissues. They are assumed to form mutual relationship with host plant by producing defensive compounds in order to ward off potential plant pathogens. To date, they remain an understudied source for new compounds (Gouda et al., 2016). Based on the literature, only 3 endophytic *Streptomyces* have so far shown anti-MRSA activity. Interesting compounds derived from endophytes were previously reviewed by Martinez- Klimova (Martinez-Klimova et al., 2017) who found that the majority of endophytes isolated under Actinobacteria phylum were *Streptomyces* (Martinez-Klimova et al., 2017). *Streptomyces* sp. SUK25 was isolated from the root sample of *Zingiber spectabile* in Malaysia, producing compounds cyclo-(tryptophanyl-prolyl) and chloramphenicol. *Streptomyces* sp. SUK06, was isolated from *Thottea grandiflora* in Malaysia on the basis of its

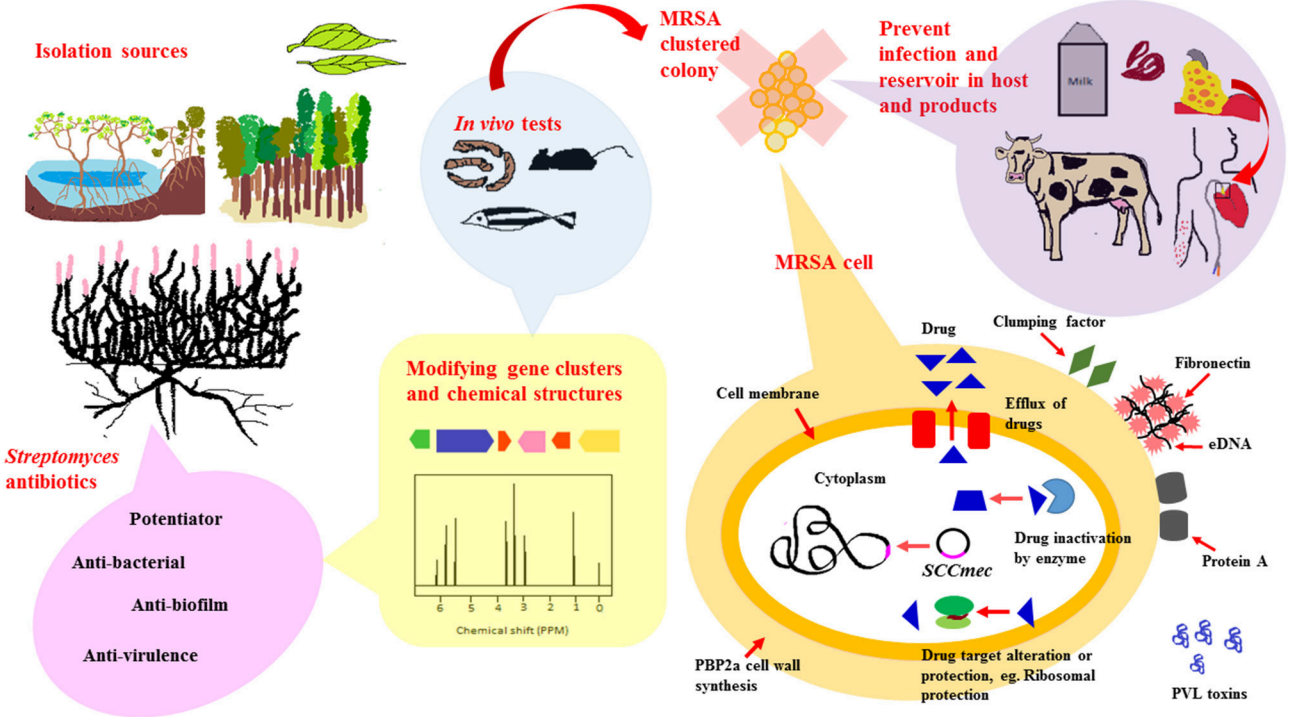

FIGURE 1 | *Streptomyces* as potential sources for newer anti-MRSA compounds. To date, anti-MRSA compounds produced by *Streptomyces* have been isolated from various ecological sources that include terrestrial, marine, mangrove ecosystems and endophytes. *Streptomyces* are widely known for their ability to produce diverse range of antibiotic-like compounds. These compounds exert their anti-MRSA activity via different mode of actions, either by direct killing, synergistic or potentiator, anti-biofilm and anti-virulence properties. Recent advancement of technology, genetic and chemical modification, have facilitated production of antibiotics isolated from *Streptomyces* to form new chemical entities with improved anti-MRSA potency. These newly modified anti-MRSA compounds are further subjected to animal models-mice, silkworm and zebrafish, to validate their clinical efficacy prior to clinical trials. MRSA carry a mobile genetic element, Staphylococcal chromosomal cassette (*SCCmec*), that can be horizontally transferred from one bacteria to another. In addition to that, they are also capable of developing resistance mechanisms to non-β-lactam antibiotics, either via (i) Efflux of intracellular drug concentration (ii) Enzyme that inactivates drugs (iii) Altered drug target. Developing antibiotics that target these resistance mechanism, biofilm formation and virulence, would markedly reduce spread and infection of MRSA in hosts and animal products.

medicinal use- wound healing, skin infection or curing fever. The careful selection of medicinal plants resulted in isolation of *Streptomyces* having significant antimicrobial activity with a zone of inhibition of 37 mm (Ghadin et al., 2008). There are also reports of marine *Streptomyces* endophytes with anti-MRSA activity, for example *Streptomyces sundarbabensis* WR1L1S8 from the *Ficus* species or the brown alga. Among the 22 marine alga it was found that *Streptomyces* from *Ficus* species demonstrated promising antimicrobial activity inclusive of anti-MRSA activity (Djinni et al., 2013). The work so far suggests the benefits of focusing the search for endophytes rather than randomly selecting plants. Thus far, medicinal plants and algal, particularly *Ficus* species have been shown to be potential sources of endophytes with promising anti-MRSA activity.

Desert

Recently, deserts have become interesting sources of sampling *Streptomyces* with anti-MRSA activity. Deserts are barren dry land with extremely high temperature, making it difficult for plants and other organisms to survive there (Smith, 2018). Microbes or any form of desert lifeforms therefore must have developed unique metabolic pathways to allow them to tolerate extreme levels of heat and temperature. *Streptomyces* sp. C34 which was isolated from the Chilean hyper-arid Atacama desert soil produced chaxamycins with potent anti-MRSA activity with MIC of 0.13 μg/mL-0.25 μg/mL (Rateb et al., 2011). We did note there is no current published work on *Streptomyces* with anti-MRSA activity from the Arctic. It is therefore suggested that apart from desert, researchers interested in anti-MRSA compounds from *Streptomyces* may also want to focus on the Arctic.

Mangrove Ecosystem

Mangroves represent an additional untapped ecological niche which may play an important role in harboring organisms which may produce potential contributors for lead molecules for new anti-MRSA drugs (Ser et al., 2015c; Tan et al., 2015). Mangroves are a very interesting ecological niche because they lie at an interface of terrestrial and marine ecosystems, hence, the prospect of finding novel *Streptomyces* that are adapted to survive this environment is exciting. It is likely that the chemical ecology in this environment will be very different from that of terrestrial as well as marine environments. Furthermore, mangrove also represents a viable research platform as it

encompasses 75% of tropical coastlines and 25% of the world's coastlines. Despite the well-documented flora and fauna, the microbial diversity of mangrove forest remains underexplored (Xu et al., 2009; Lee et al., 2014a,b; Zainal et al., 2016; Tan et al., 2017). Based on the literature, a few anti-MRSA compounds have been reported from the mangrove forests (**Supplementary Table 1**).

This demonstrates that these hitherto underexplored fields offer potential for future therapeutic drugs targeting MRSA. Some of these anti-MRSA compounds have been further studied for their mechanism of action and are highlighted in the following paragraphs.

ANTI-MRSA COMPOUNDS DERIVED FROM *STREPTOMYCES* AND THEIR MECHANISMS OF ACTIONS

Existing literature so far have highlighted 124 compounds produced by *Streptomyces* which show moderate to potent anti-MRSA activity (**Supplementary Table 1**). A numerical analysis of these compounds shows that polyketides (PKS) form the largest group (53), the next being non-ribosomal peptides (NRPS) while others include smaller proportions of alkaloids, hybrids of PKS/NRPS and PKS/terpenoids. In fact, polyketide and NRPS biosynthetic pathways are the source of most *Streptomyces* derived conventional antibiotics; and logically these classes also constitute a large fraction of the substances demonstrating anti-MRSA activity. Among the molecules identified were several compounds—such as polyketomycin, heliquinomycin, griseusin A, 4′deacetyl griseusin, citreamicin θ A, chaxamycin D, nosiheptide, nosokomycin, and marinopyrrole A—which have been reported to be potent anti-MRSA compounds and exhibit lower MIC than several clinically used antibiotics such as vancomycin. Vancomycin has been used as a standard for testing of effectiveness of new compounds against MRSA (CLSI, 2015). The chemical structures of these bioactive compounds are depicted in **Figure 2A (1-12)**, **Figure 2B (13-24)**.

PKS Compounds With Anti-MRSA Activity

Polyketides (PKS) are common natural products among *Streptomyces* species and their synthesis is catalysed by enzymes known as polyketide synthase (PKS). These enzymes function together in a consecutive manner along the metabolic pathway. PKS shares a common pattern of biosynthetic steps with fatty acid synthase (FAS) (Jenke-Kodama et al., 2005), basically catalysing step-by-step condensation of simple carboxylic acids. Yet in PKS, additional modifications to structures are possible using specialized enzymes, different starter and extender units, reduction and cyclization reactions resulting in a wide array of antibiotic compounds with antibacterial actions. It is not surprising therefore, to find polyketides from *Streptomyces* with potent anti-MRSA activity such as polyketomycin.

Polyketomycin

The second most potent anti-MRSA compound known is polyketomycin (**1**) which was isolated from *Streptomyces* sp. MK277-AF1 found in a soil sample collected in the Kanagawa prefecture in Japan and has shown an MIC of 0.025–0.2 μg/mL (Momose et al., 1998). Polyketomycin (**1**) produced by *Streptomyces* was extensively studied and used as a model to identify gene clusters of potentially important antibiotics. The use of genomic mining has allowed the identification of biosynthetic gene clusters of antibiotic compounds from *Streptomyces*. *Streptomyces coelicolor*, the most extensively studied *Streptomyces* was found to potentially have 20 gene clusters (Bentley et al., 2002), indicating its potential for production of about 20 secondary metabolites.

Biosynthetic pathways of compounds that have shown promising biological activities are studied to identify their respective biosynthetic gene clusters. As such, polyketomycin as a polyketide is composed of a tetracyclic ring, 2 monosaccharides and a dimethyl salicylic acid. Based on the chemical structure, Paululat et al. (1999) postulated the biosynthetic gene clusters by firstly predicting the enzymes involved in the biosynthetic pathway. For example, the tetracyclic moiety and dimethyl salicylic acid moiety were assumed to derive from a PKS II and PKS I, respectively. The two sugar moieties, which are 6-deoxysugars, might have been synthesized from glucose via a pathway involving a TDP-glucose-4, 6-dehydratase during biosynthesis and attached by two glycosyltransferases. Once the gene clusters have been identified, alterations can be made to the natural pathways to create modified chemical structures with improved clinical efficacy using nature's biosynthetic machinery (Greule et al., 2017).

Polyketomycin (**1**) seems to be attractive not only because of its potent anti-MRSA activity and naturally high yield (4.3 g/L) but also because of the composition of its biosynthetic product. The polyketide synthase of the aglycone and the enzymes of the salicylic acid moiety combine different activities from known pathways of other strains in a new manner (Paululat et al., 1999). Hence, polyketomycin has been proposed to be a good target for combinatorial biosynthesis to modify its existing structure to increase its potential applications. Combinatorial biosynthesis utilizes genetic engineering to bring about changes to biosynthetic pathways of isolated compounds and hence, produce new structures (Floss, 2006).

Although polyketomycin was discovered in 1998, its mechanism of action has not been determined. It is however known that polyketomycin belongs to the anthracycline group of antibiotics which are already well-known for their anti-tumor properties. Current anticancer drugs doxorubicin, daunarobucin, and rubidazone are clinically used for treating cancer whose mechanism is defined by the inhibition of DNA polymerase. Hence, it is likely that polyketomycin belonging to anthracycline antibiotic can exhibit the same mechanism of action toward MRSA; this was suggested by another study investigating anti-malarial activity of polyketomycin (Otoguro et al., 2003). Recent discovery of other anthracyclines from *Streptomyces* include cervimycins and DMI-2 isolated from *Streptomyces* sp. 560 and *Streptomyces tendae* HKI-179, respectively. Interestingly the anthracyclines mentioned have a *p*-quinone D ring in common (Daum et al., 2009) which may be the element giving rise to the anti-MRSA activity observed.

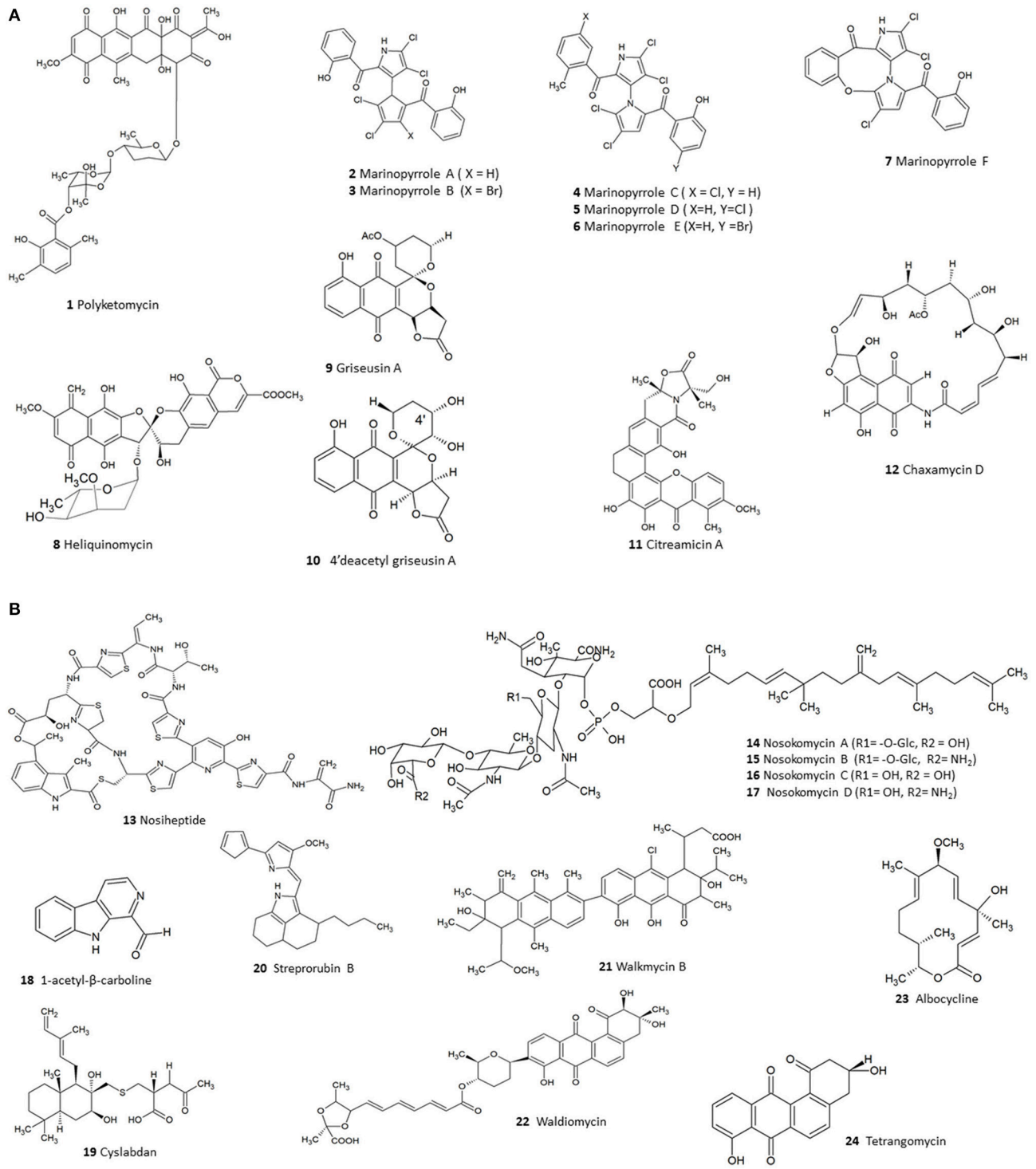

FIGURE 2 | (A) The chemical structures of compounds isolated from *Streptomyces* with moderate to potent anti-MRSA compounds **(1–12)**. **(B)**. The chemical structures of compounds isolated from *Streptomyces* with moderate to potent anti-MRSA compounds **(13–24)**.

Heliquinomycin

Heliquinomycin (**8**), a polyketide isolated from *Streptomyces* sp. MJ929 in Japan, exhibited low MIC of < 0.05–0.1 μg/mL (**Supplementary Table 1**). Interestingly, it was found to inhibit the human DNA helicase, a known target for tumor growth. Heliquinomycin belongs to the rubromycin family which are

known for their activity against telomerase and retroviral reverse transcriptase which may have led to further investigation of anti-cancer instead of MRSA properties. To date, no separate mechanism of action has been afforded to heliquinomycin on MRSA (Chino et al., 1996, 1997, 1998).

Griseusin A and 4′deacetyl Griseusin A

Aromatic polyketide griseusin A (9) and 4′deacetyl griseusin A (10) were isolated from *S. griseus* M33-5 found in the Turkish soil (Urgen et al., 2010). Griseusin A and 4′deacetyl griseusin belong to the griseusin antibiotics and demonstrate anti-MRSA activity of 1 and 0.5 μg/mL, respectively (**Supplementary Table 1**). Griseusin compounds represent a subclass of pyranonaphthoquinones and include griseusin A and B whose structures were initially characterized from *S. griseus* in 1976 (Tsuji et al., 1976). Griseusins are also known for their potent anticancer properties and are commonly found distributed among actinomycetes particularly *Streptomyces* and *Nocardiopsis* sp. (Ding et al., 2012) as well as fungi. For example, griseusins were previously isolated from *S. griseus* K-63, 3–5, *S. griseus* MJ361-48F3, *Streptomyces* sp. IFM 11307, actinomycete strain MJ932-SF3, *Nocardiopsis* sp. and *Penicillium* sp. Recent studies discovered newer griseusin E and 4′deacetyl griseusin A in *Streptomyces* IFM 11307 with 4′deacetyl griseusin shown to be the most effective to overcome tumor necrosis factor related apoptosis-inducing ligand (TRAIL) resistance. Their antibiotic activity and the fact that they are one of the complex pyranonapthoquinones, has prompted interest in their chemical synthesis. The first successful total synthesis of griseusin A was published in 1983 (Kometani et al., 1983) followed by synthesis of analogs of griseusin A (Brimble et al., 1999). Further interest into PKS of griseusin by *S. griseus* K-63 led to the identification of 5 *gris* genes that encode for griseusin. It is known that PKS type 2 (PKS II) compounds are mostly aromatic in nature. Yet the aromatic PKS of griseusin are found to be programmed differently from other aromatic PKS. This sparked renewed interest in these compounds and studies were carried out to identify programming mechanism of these 5 genes (Yu et al., 1994). However, the mechanism of action of these compounds with respect to their anti-MRSA activity is still elusive.

Citreamicin θ A

The citreamicin group of xanthone antibiotics (polycyclic aromatic antibiotics) were first isolated in 1989 from *Micromonospora citrea* in lake Manyara, Tanzania (Carter et al., 1990). Among the citreamicins isolated at that time were α, β, γ, ζ, and η. All four compounds shared a common xanthone nucleus and methoxy group at position at C17 except citreamicin η which has a hydrogen instead. All displayed anti-MRSA activity with citreamicin η being the most potent anti-MRSA compound with MIC of < 0.015 μg/mL even though it lacked a methoxy group at position 17 (Carter et al., 1990). Yet another analog of citreamicin known as neocitreamicin I and its derivate neocitreamicin II were reported in 2008 with anti-MRSA MIC at 0.12–0.50 and 0.5–1.0 μg/mL, respectively. These neocitreamicin also lacked a methoxy group at C17 indicating that modification on C17 does not significantly affect their anti-MRSA activity (Peoples et al., 2008). The recent discovery of marine-derived citreamicin analogs citreamicin θ A (**11**), citreamicin θ B, citreaglycon A, and dehydrocitreaglycon A from *Streptomyces caelestis* isolated from the Red Sea exhibited potent anti-MRSA activity especially citreamicin θ A (**11**) and citreamicin θ B at 0.25 μg/mL. Furthermore, anti-MRSA activity of the compounds of citreamicin θ A (**11**) and θ B were assumed to be due to 5 membered ring group which was absent in citreaglycon A and dehydrocitreaglycon A (Liu et al., 2012). Citreamicin δ and ε were also isolated in 2008 from *Streptomyces vinaceus* from a soil sample obtained from a river. They were observed to be structurally similar to citreamicin η except for the relocation of a methoxy group but yet showed anti-MRSA activity in the range of $\leq$0.5–2 and 0.12–0.25 μg/mL (Hopp et al., 2008). Despite its potent anti-MRSA activity, its mechanism of action remains unknown at present time. However, it is speculated that the 5 membered ring confers citreamicin its potent anti-MRSA activity. Chemical synthesis has proven difficult to date since its discovery in 1989 with research only achieving a 11-step synthesis of the pentacyclic core of citreamicin η (Blumberg and Martin, 2017).

Chaxamycin D

Chaxamycin D (**12**) was originally isolated from *Streptomyces* sp. strain C34 from soil samples from the Chilean hyper-arid Atacama Desert (**Supplementary Table 1**; Rateb et al., 2011). Chaxamycin D (**12**) belongs to the ansamycin group of compounds, which also includes the potent anti-TB drug rifamycin. Even though its MIC is lower than 2 μg/mL, activity was compared to rifampicin having an MIC of 0.002–4 μg/mL. Ansamycins are widely known for their anti-tumor properties via inhibition of heat shock protein 90 (Hsp90) via selective interaction with the ATP-binding pocket in the *N*-terminal domain; and *in situ* molecular studies of chaxamycin demonstrated that it also possesses this property. Due to its promising anti-MRSA activity, further studies have been carried out to determine the gene cluster of chaxamycin, as was done with polyketomycin. However, this experiment used mutational analysis (mutasynthesis) in the natural producer and heterologous expression in *S. coelicolor* A3 (2) strain M1152. Restoration of chaxamycin production in a non-producing cxmK mutant was achieved by supplementing the growth medium with 3-amino-5-hydroxybenzoic acid (Castro et al., 2015). Mutasynthesis is a method that attempts to modify the structure of a known compound, by feeding the microbe with structural analog precursors with the aim to improve its pharmacological property. In a similar study carried out by Feng et al. (2014) mutasynthesis was used where *Streptomyces ansochromogenes* were fed with structural analog precursors of the anti-fungal compound nikkomycins. Although the nikkomycins produced through mutasynthesis was the same as the one produced in the natural host, the resultant showed better stability (Feng et al., 2014). Hence, mutasynthesis can be used to potentially

improve the pharmacological properties of promising anti-MRSA compounds.

NRPS compounds With Anti-MRSA Activity

NRPS include clinically important drugs such as daptomycin, bleomycin and cyclosporine. The synthetic pathway for NRPS compounds involves a system of enzymes that utilizes 500 non-protein building blocks apart from the 20 amino acids to form diverse linear and cyclic peptides with a wide array of biological activities (Strieker et al., 2010) through postranslational modification of peptides such as acylation, halogenation, or hydroxylation. Non-ribosomal peptides often have cyclic and/or branched structures and can contain non-proteinogenic amino acids including D-amino acids. From the literature reviewed (**Supplementary Table 1**), compounds AMRSA1 (Lee et al., 1997) and nosiheptide (Haste et al., 2012) showed anti-MRSA activity at 0.01–0.1 and $\leq$0.25 μg/mL, respectively. These 2 compounds are among the most potent anti-MRSA compounds known, having lower MIC readings compared to positive controls used. Given that the study on AMRSA1 is still limited, only nosiheptide is further discussed as follows.

Nosiheptide

Nosiheptide (**13**) was isolated from *Streptomyces* sp. CNT 373 isolated from marine sediments off the coast of Fiji (Haste et al., 2012), as part of the marine drug discovery project by Fenical and Jensen. This protein synthesis inhibitor (Cundliffe and Thompson, 1981), had a rapid bactericidal action in the first 6 h and a post-antibiotic effect (PAE) exceeding 9 h. In addition, its MIC level remained unaffected in the presence of 20% serum suggesting it may have promise in clinical settings. One of the surprising elements of nosiheptide is its lack of cytotoxicity. It is assumed that most of the compounds isolated from marine environments possess cytotoxic properties, relevant in anti-cancer drugs. After the discovery of nosiheptide (**13**) in 1970 under the name multhiomycin (Tanaka et al., 1970; Endo and Yonehara, 1978) it was approved for use as a growth promoter in animal feeds. Its anti-MRSA activity was only determined later on by Haste et al. (2012). The mechanism of action of nosiheptide is closely related to thiostrepton which acts on 50S ribosomes, however, thiostrepton shows a higher affinity than nosiheptide, with 30% and 70% inhibition of binding, respectively (Cundliffe and Thompson, 1981). Despite their similarity of mechanism of action, they differ in the biosynthetic pathways for the formation of the terminal amide group. While nosiheptide (**13**) carries an endogenous amide moiety, thiostrepton has its amide moiety incorporated by an asparagine synthase-like protein (Liu et al., 2015). Total synthesis of nosiheptide (**13**) was achieved by initial formation of a linear precursor and followed by macrocyclization to form the compound (Wojtas et al., 2016). Anti-MRSA activity was maintained after synthesis of nosiheptide (**13**). Successful total synthesis of nosiheptide paves the way to modify structures in order to derive newer antibiotics for clinical use. Nosiheptide has already proven to be effective in animal models (20 mg/kg) in a murine model, whereby intraperitoneal MRSA was treated with nosiheptide (**13**) with positive results (Haste et al., 2012).

Nosokomycin B, A Phosphoglycolipid Compounds With Anti-MRSA Activity

Nosokomycins A (**14**), B (**15**), C (**16**), and D (**17**) were isolated from *Streptomyces* sp. K04-0144 found in the soil of Japan. The MIC with respect to MRSA for nosokomycins A-D was 0.125 μg/mL while vancomycin and imipenem were 0.5 and 16 μg/mL, respectively; indicating nosokomycin has more potent *in vitro* anti-MRSA activity than vancomycin (**Supplementary Table 1**; Uchida et al., 2010). In their subsequent investigation of nosokomycin's anti-MRSA activity against 54 MRSA strains, nosokomycin B (**15**) was clearly the most potent, followed by nosokomycins A (**14**), D (**17**), and C (**16**) (Uchida et al., 2014). The activity of nosokomycin has been studied in detail relating to its chemical structure; in terms of its structure-activity relationship, it was observed that the presence of a glucose residue at R1 and an amino residue at R2 was necessary for potent anti-MRSA activity. Nosokomycin belongs to a family of compounds known as moenomycins. Structurally, nosokomycin B (**15**) exists to be identical to a semisynthetic moenomycin A derivative, which lacks the chromophoric cyclopentenone moiety (Uchida et al., 2010). Moenomycin A possesses a glucose residue at R1 and a chromophoric cyclopentenone residue via an amide bond at R2, and shows weak bactericidal activity against MRSA. Even though the MIC of moenomycins against gram-positive bacteria were of submicrogram concentrations, there was one study on MIC of moenomycin A against MRSA, ATCC 43300 which showed an MIC of 4 μg/mL (Vancomycin = 1 μg/mL). Given that nosokomycin A (**14**) and B (**15**) are biosynthetic intermediates of moenomycin A, it was unexpected that while nosokomycins were isolated, no members of the moenomycins were isolated from producing *Streptomyces* strain K04-0144 (Uchida et al., 2010). The authors suggested that the genes for moenomycin A are lacking or not activated in the producing strain.

An interesting feature of moenomycins is their underlying mechanism of antibacterial activity. They seem to target the peptidoglycan glycosyltransferases directly, and they are the only natural products known to do so. This differs from vancomycin which targets the substrates for these enzymes and therefore acts indirectly. While vancomycin acts by competing with substrate *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) for the enzyme, moenomycins directly bind to the enzyme itself. It was shown that nosokomycin B has submicrogram MIC concentration even lower than vancomycin. Moenomycins have great therapeutic potential as studies have shown that it is necessary to target the active site of the enzyme to achieve stronger therapeutic effect; and the work done so far supports that moenomycins bind to the active site of the enzyme (Uchida et al., 2010).

The moenomycin class have a unique structure and mechanism of action resulting in potent anti-MRSA effect, yet they do not make good drug candidates in their natural form because of their poor pharmacokinetic profile. The C25 lipid chain which is responsible for the potent MRSA of moenomycin is also the cause of its long half-life and low bioavailability. It was assumed that the long lipid chain helped

the drug to anchor itself onto the cytoplasmic membrane. Modifying the lipid chain by decreasing the carbon chain was shown to improve pharmacokinetic properties but led to the loss of activity (Fuse et al., 2010; Galley et al., 2014). According to the literature reviewed, there are no reports of studies looking into the gene clusters for nosokomycin B as a means to improve its pharmacokinetic properties. This may be because the nosokomycins A and B were identified as intermediates of moenomycin biosynthesis. Therefore, the gene clusters for producing MmA can be used to improve the pharmacokinetics of both MmA and nosokomycin. Also, to date no pharmacokinetic study exists to determine its potential in human therapy–moenomycins are currently used only in veterinary settings (Uchida et al., 2010).

Marinopyrrole A, An Alkaloid With Anti-MRSA Activity

The research group by Hughes et al. (2010) isolated marinopyrroles with anti-MRSA activity from obligate marine *Streptomyces* sp. CNQ-418 which was in turn isolated from marine sediments at 51 m depth off the coast of La Jolla, California (Hughes et al., 2008, 2010). The 6 active compounds identified as marinopyrroles A-F (**2-7**) belong to the 1, 3′ bispyrroles alkaloid—they are densely halogenated and axially chiral metabolites that contain an uncommon bispyrrole. The need for sea water is essential in the biosynthesis of the dense halogenated backbone structure. These sets of compounds are the first naturally occurring 1, 3′ bipyrroles. Apart from their intriguing chemistry, they exhibit potent anti-MRSA activity with MIC <1 μg/mL. Marinopyrrole C exhibited the lowest MIC at 0.16 μg/mL followed by marinopyrrole A at MIC 0.31 μg/mL (**Supplementary Table 1**; Hughes et al., 2010). This group of compounds have attracted a lot of interest due to their structure, antibiotic and antitumor activities (Cheng et al., 2010). However, they are unsuitable for clinical use in their natural form as they are neutralized by 20% human serum (Haste et al., 2011b). Numerous chemical and genetic studies have since begun to work on ways to modify the structure to overcome this problem. For example, some of the studies focused on total synthesis of marinopyrroles A and B as well as their analogs (Nicolaou et al., 2011). The first total synthesis was carried out by Cheng et al. (2010) and the biosynthesis of marinopyrrole was successfully carried out by Yamanaka et al. (2012).

Marinopyrroles are the first naturally-occurring microbial compounds classified under the group of 1, 3′-bipyrroles and found to show potent antibacterial activity against MRSA (Hughes et al., 2008). These marine-derived compounds were produced from an obligate marine *Streptomyces* sp. strain CNQ-418 isolated from marine sediment. The considerable attention given toward this interesting group of compounds have also led to the successful chemical synthesis of a series of novel marinopyrrole derivatives (Hughes et al., 2010; Nicolaou et al., 2011) exhibiting promising biological properties including anti-MRSA activity (Haste et al., 2011b; Liu et al., 2014).

In 2008, marinopyrroles A (**2**) and B (**3**) were both isolated from the extract of *Streptomyces* strain CNQ- 418 in a seawater-based culture broth and their structure determined. Since then, marinopyrroles C- F (**4-7**) have been identified from *Streptomyces* under optimized culturing conditions**.** As the major metabolite, the antibacterial activity of marinopyrrole A (**2**) has been studied extensively against a variety of clinically important MRSA strains. It was observed that marinopyrrole A (**2**) showed potent concentration-dependent bactericidal activity against clinically relevant HA-MRSA and CA-MRSA strains (Haste et al., 2011b). Marinopyrrole A displayed substantial concentration- dependent killing against MRSA strain TCH1516 and was far more rapid in its antibiotic action than either vancomycin or linezolid. Using time-kill assay, marinopyrrole A at 10× MIC (3.75 μg/ml) showed a 2-log-unit kill of MRSA TCH1516 within 9 h, while the activity of vancomycin at 10 MIC (20 μg/ml) was much slower, reducing the initial inoculum by only about 10-fold. Treatment with marinopyrrole A at 20× MIC (7.5 μg/ml) reduced the initial inoculum by nearly 6- log-fold within 9 h. Moreover, marinopyrrole A (**2**) exhibits a prolonged PAE effect measures the effects of antibiotics at set times after exposure (**Supplementary Table 1**). This may be a more clinically significant measure than MIC which only demonstrates an all-or nothing relationship with a constant antibiotic concentration and therefore represents threshold concentration only. In this case, marinopyrrole A (**2**) at 1×, 10×, or 20× MIC exhibited a concentration-dependent PAE against MRSA strain TCH1516. A longer recovery rate of MRSA culture was noted with use of marinopyrrole compared to vancomycin and linezolid between 4 and 6 h at 20× MIC (**Supplementary Table 1**).

In terms of its clinical applications, marinopyrrole A (**2**) was shown to have affinity toward binding to plastic surfaces, allowing it to be applied as an antibiotic-lock agent which is relevant for the use as a topical agent or in local therapy of device-related MRSA infection. However, the study demonstrated the potential uses of marinopyrrole A (**2**) as a systemic antibacterial therapy may be hampered by its poor pharmacological profile in which its antibacterial efficacy was markedly reduced by serum. However, recent work has successfully modified structure of marinopyrrole and its derivatives resulting in retained or improved anti-MRSA activity and a reduced susceptibility to serum inactivation (Cheng C. et al., 2013; Liu et al., 2014). The overall evidence so far suggests that this group of unique natural products, marinopyrroles from marine origin *Streptomyces* bacteria, serve as important chemical structures for future modification and optimization in the continuous effort to develop new antibiotic therapy against MRSA.

COMBINATORIAL THERAPY/SYNERGISM: CONVENTIONAL ANTIBIOTICS + NEW *STREPTOMYCES* DERIVED NATURAL PRODUCTS

An alternative to developing new anti-MRSA compounds is to focus on improving the effectiveness of currently available antibiotics such as β-lactams. Since developing a totally new drug is costly and time-consuming, a more cost-effective approach would be to enhance the anti-MRSA activity of existing drugs

as measured by a significantly reduced MIC indicating the bacteria become increasingly susceptible to the levels of drug concentration in blood and tissues.

Antimicrobial drug combination approaches aimed at improving the effectiveness of anti-MRSA drugs (reducing MIC) has become a potential area of research in drug discovery. The urgent need to have new antibiotics to eradicate MRSA infection has further validated this area of research, especially when MRSA is resistant to most of the β-lactam antibiotics. Potentiating or enhancing the effectiveness of existing antibiotics through synergism studies has been used to identify new and better combinations with enhanced ability to eradicate infections. Synergism is a favorable effect observed in antimicrobial combination studies whereby activity is significantly greater when two agents are combined than that provided by the sum of each agent alone. Synergy is more likely to manifest when the ratio of the concentration of each antibiotic to the MIC of that antibiotic was same for all components of the mixture (Jain et al., 2011).

The rationale for combination of antibiotic therapy for bacterial infections in general is that it enhances activity, reduces toxicity, may prevent the emergence of resistance or even enable treatment of a polymicrobial infection. The checkerboard is a useful method for studying synergism since a number of combination concentrations can be prepared and studied via broth 2-fold dilution with synergism detected as no visible growth (Stein et al., 2015). According to the Clinical Laboratory Standards Institute guidelines for broth microdilution, the MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of the organism as detected with the naked eye (CLSI, 2015).

1-Acetyl-β-carboline and Penicillins

Streptomyces sp. 04DHS2 was isolated from marine sediment and was shown to produce the alkaloid 1-acetyl-β-carboline. Used alone, 1-acetyl-β-carboline (**18**) exhibited an MIC of 64 μg/mL (**Supplementary Table 1**). However, when used in conjunction with several other penicillins using the checkerboard method, 1-acetyl-β-carboline (**18**) showed synergistic action based on the fractional inhibitory concentration (FIC) index (Shin et al., 2010). The study showed synergism between the combinations of 1-acetyl-β-carboline (**18**) with either ampicillin or penicillin against 14 MRSA strains (Shin et al., 2010). Given that MRSA have become resistant to the penicillin group of antibiotics, the study also revealed the potential of the synergistic effect of 1-acetyl-β-carboline (**18**) in restoring the antibacterial property of β- lactams against MRSA.

Cyslabdan and Carbapenems

Besides the checkerboard method, comparison between the MIC of the antibiotic when used alone and in combination was also performed to investigate the synergistic effects of the potentiating agent. Such was the case for investigating the effectiveness of cyslabdan as a potentiator of β-lactams. Cyslabdan **(19)** is described as a non-antibiotic compound belonging to the labdan-type diterpene. It was isolated from *Streptomyces* sp. K04-0144 and shown to enhance the antibacterial activity of carbapenems against MRSA K24 (**Supplementary Table 1**). Among the carbapenems, the anti-MRSA activity of imipenem was synergistically enhanced the most when combined with cyslabdan. Cyslabdan (**19**) exhibited weak anti-MRSA activity (MIC of 64 μg/ml) and no effect on MRSA at 10 μg/ml. When in combination with cyslabdan (**19**), the anti-MRSA activity of imipenem activity was enhanced with a reduction in MIC from 16 to 0.015 μg/ml (Fukumoto et al., 2008). This has large potential impact on health economics as carbapenems like imipenem, being the most potent of the β-lactams, have a high production cost, making the drug even more expensive to purchase. The cost of treatment could be significantly reduced if the effective dose of imipenem needed can be reduced with the combination use of a potentiator. These findings also prompted further studies on the mechanism of action via which cyslabdan (**19**) potentiates the anti-MRSA activity of imipenem (Koyama et al., 2012). The study showed that cyslabdan (**19**) appears to be the first known inhibitor to FemA enzyme, which is involved in pentaglycine interpeptide bridge formation during the synthesis of peptidoglycan. Therefore, the biosynthetic pathway of pentaglycine interpeptide bridge formation was suggested to be a potential target for rendering MRSA susceptible toward β-lactams.

ANTI-BIOFILM AND ANTI-VIRULENCE ACTIVITIES

Biofilm formation is a key virulence determinant of MRSA; creating drugs able to induce disruption in the process of biofilm formation or its structure has become a potential area of research. This is because biofilm protects the MRSA from biocidals such as antibiotics and the immune system. Compounds from *Streptomyces* such as streptorubin B (**20**) have shown promising results in disrupting biofilm formation. Streptorubin B was originally isolated from *Streptomyces* sp. strain MC11024, and even though its MIC was 32 μg/mL, which is considerably higher than the vancomycin standard MIC of ≤ 2 μg/ml it demonstrated potential as a biofilm inhibitor by reducing the biofilm of MRSA to only 27% at 1 μg/mL (Suzuki et al., 2015). Since streptorubin B (**20**) was tested against one strain of MRSA, that is MRSA 315, increasing the number of strains tested will further demonstrate its inhibitory effect against MRSA biofilm. Streptorubin B (**20**) belongs to the previously discovered prodiginine class of pigmented antibiotics, known for their anti-tumor (Kojiri et al., 1993; Nakajima et al., 1993) and immunosuppressant actions (Songia et al., 1997) and now it has also shown potential as an anti-MRSA agent. Naturally occurring prodiginines have been isolated from actinomycetes, *Serratia*, *Pseudomonas,* and *Vibrio* among other bacterial species (Gerber and Lechevalier, 1976). From the limited data available, it seems justified to explore streptorubin B further to establish its role as an anti-MRSA agent.

A recent study showed that the ethyl acetate extract from *Streptomyces* sp. SBT343 significantly reduced the biofilm formation of several Staphylococcal species including USA300 (MRSA) at the maximum inhibitory concentration of 125 μg/mL. However, it did not display an inhibitory effect

on the bacterial growth of *Staphylococcus* species as well as gram-negative *Pseudomonas aeruginosa*; overall indicating that it has selective anti-biofilm activity against Staphylococcal bacteria (Balasubramanian et al., 2017). Aside from high selectivity, no clinically relevant levels of cytotoxicity have been demonstrated, making this a promising source for an anti-biofilm against Staphylococcus species. Biofilms tend to complicate the therapeutic regime leading to increased duration of treatment, whereby the drug is required to penetrate through the biofilm to reach its target site. Most often, this can lead to treatment failure and consequently death. This has become a concern when treating severe MRSA infection with mainline drugs such as vancomycin. Using additional antibiotics with vancomycin, has been shown to dramatically improve the efficacy of vancomycin in both *in vitro* (Pistella et al., 2005) and *in vivo* mouse model (Shi et al., 2014). Given that vancomycin-intermediate and vancomycin-resistant *S. aureus* (VISA and VRSA) strains have been reported in some parts of the world, the need to develop new antibiotics is critical.

Quorum Sensing Inhibitors

Apart from drugs targeting the formation and structure of biofilm, cell wall metabolism is another new focus of research for novel antibiotics. The WalK/WalR pathway is the main signaling pathway in low G + C content gram-positive bacteria such as MRSA and is responsible for cell wall metabolism and biofilm formation. It is assumed that inhibiting WalK/WalR consequently causes cell death. Studies investigating the potential of *Streptomyces* as producers of WalK/R inhibitors identified walkmycin B (**21**) (Okada et al., 2010) and waldiomycin (**22**) (Igarashi et al., 2013) as potent (0.39 μg/mL) and moderate (16 μg/mL) anti-MRSA agents, respectively.

The quorum sensing pathway offers a new potential target of new anti-MRSA compounds. Since quorum sensing plays a vital role in biofilm formation, it can be used as a potential drug target. In fact, studies have been carried out screening *Streptomyces* extracts that can inhibit the quorum sensing pathway in some gram positive and gram-negative bacteria (Hassan et al., 2016; Miao et al., 2017).

Attenuation of Virulence Factors

Newer strategies to overcome MRSA infection involves attenuation of virulence factors rather than direct inhibition of the cell. Compounds isolated from *Streptomyces* such as albocycline (**23**) (Reusser, 1969) were recently shown to selectively block cell wall synthesis in *S. aureus* (Koyama et al., 2013). Recently identified metabolic pathways unique to *S. aureus* present alternative potential drug targets in combating antibiotic resistance. For example, the newly established biosynthetic pathway of heme—an essential iron carrying molecule—unique to gram-positive bacteria (Dailey et al., 2015). In addition, dehydrosqualine desaturase (CrtM) which is responsible for catalysing the first step in the synthesis of the yellow pigment staphyloxanthin (a virulence factor for *S. aureus)* may represent a potential target for new therapies. Studies provide evidence that *S. aureus* strains that produce staphyloxanthin are more resistant toward the host's immune system. Inhibition of this enzyme prevents the synthesis of staphyloxanthin. Using *in situ* docking models, the natural compound tetrangomycin (**24**) produced by *Streptomyces* sp. CAH29 was demonstrated to bind to the dehydrosqualene synthase enzyme (Özakin et al., 2016).

IN VIVO EVIDENCE OF ANTI-MRSA ACTIVITIES OF *STREPTOMYCES*

The *in vivo* stage of drug development involves the use of animals to investigate the safety and efficacy of new drugs in living mammalian organisms prior to clinical trials. This ensures that drugs are reasonably safe and effective before they are tested in human beings. However, prior to animal testing, promising drug candidates should, of course, demonstrate at least reasonable *in vitro* performance in terms of key parameters including MIC, rapid bactericidal activity and PAE (Guo et al., 2016).

These *in vivo* studies are necessary, because a compound that shows great potential *in vitro*, may not actually be effective in a biological system (Haste et al., 2011a,b). Unlike *in vitro* testing, in an actual biological system, a drug must first reach the target site where they can bind to and carry out their actions in order to have an effect; hence the pharmacokinetic processes of absorption, distribution, metabolism and excretion greatly influence their effectiveness and distinguishes the findings from *in vitro* and *in vivo* studies. Given that these drugs are usually intended ultimately for human use, it is important to select a model that mimics humans as closely as possible for the *in vivo* studies (Rosenthal and Brown, 2007).

Nosiheptide, which showed great promise *in vitro*, was tested *in vivo* on a murine model of intraperitoneal MRSA infection, where the test mice were given intraperitoneal injections of nosiheptide (20 mg/kg)—only 1 out of 10 infected mice died at the end of the study as compared to the controls where 6 out of 10 died on day 1. This provides evidence of significant *in vivo* activity of nosiheptide warranting further study (Haste et al., 2012). *In vivo* models are also used to determine toxicity of compounds isolated. Several of these compounds mentioned in **Supplementary Table 1** are heliquinomycin, vinylamycin, polyketomycin, methylsulfomycin, lactinomycin, and gargantulide. Some of these compounds tested indicated toxicity in mice model, rendering them non-viable. Other examples include gargantulide isolated from marine *Streptomyces* which demonstrated a potent MIC of 2 μg/mL *in vitro*, methylsulfomycin which (at 25 mg/kg) was inconclusive due to precipitation formed while polyketomycin exhibited acute toxicity.

Moreover, the *in vivo* model selection needs to bear in mind several factors—mainly cost, time taken, and ethical considerations (Ferdowsian and Beck, 2011). Mammalian models tend to be more expensive and require a longer study period. Additionally, it is no longer possible to use mammalian models for most preliminary drug trials as the animal protection Act has tightened regulation of use of animal in experiments especially in European Union countries in 1998, where it is forbidden to use healthy animals in *in vivo* models (Uchida et al., 2014).

To overcome these problems, researchers have focused on non-mammalian animals such as Zebrafish, *Caenorhabditis elegans*, *Drosophila melanogaster* and silkworms as alternative hosts for *in vivo* screening systems. According to the literature reviewed in this paper (**Supplementary Table 1**), only silkworm and zebrafish have so far been employed for the screening of anti-MRSA compounds produced by *Streptomyces*. Uchida et al. (2014) demonstrated that a silkworm model could be successfully used in primary screening of new anti-MRSA agents—in this case, 4 potent anti-MRSA agents, nosokomycins A-D (MIC 0.125 μg/mL) (**Supplementary Table 1**) which were isolated from *Streptomyces*. Silkworm larvae infected with MRSA alone exhibited a lifespan of <3 days, compared to a survival rate of 3 days with vancomycin or other effective drugs including the nosokomycins (Uchida et al., 2014); the team also performed agar diffusion assay and also used a mouse model to provide supporting evidence of the reliability of the silkworm assay. The authors suggest that using a silkworm model as an *in vivo*-mimic assay may provide a more reliable prediction of actual *in vivo* activity compared to the traditional disk diffusion method. Hence, alternate animal models such as silkworms can become potential as alternate animal models in drug discovery and development of new anti-MRSA antibiotics.

Zebrafish embryos are an alternative model which is becoming increasingly popular in drug discovery work; they were initially introduced back in the 1970s but only recently became a model of choice in research on areas of genetically mutated diseases such as cancer. The advantages of this model are that they are cheap compared to rodents, they share 80% of genes with humans, and are much faster compared to trials on rodents. The zebrafish produce 200 eggs/week and so provides a reasonable source for research and they are easy to maintain. Additionally, the embryos are transparent making it easier to see the effects of test drugs on their organs and tissues. Zebrafish embryos were used to demonstrate the anti-MRSA activity of C23 (a purified compound from *Streptomyces rubrolavendulae* ICN3) as MRSA-infected zebrafish embryos survived with minimal toxicity effects in the presence of purified molecule C23 at 10 μg/ml (Kannan et al., 2014).

FUTURE DIRECTIONS/STRATEGIES

Streptomyces remain one of the most promising natural producers of antibiotics. Given the problems in rediscovery of known antibiotics from *Streptomyces*, it has been suggested that there may be a need to activate silent antibiotic gene clusters which may have gone dormant. This is possible with newer methods available such as: (1) The addition of signaling molecule *N*-acetylglucosamine in culture media of *Streptomyces clavuligerus, Streptomyces collinus, Streptomyces griseus, Streptomyces hygroscopicus,* and *Streptomyces venezuelae* have led to the activation of genes responsible for antibiotic productions. However, the same study also proved that this method does not work in all *Streptomyces* species (Rigali et al., 2008). In another study, Imai et al. (2015) showed that when *Streptomyces* are grown in the presence of lincomycin at sub-inhibitory concentration (1/12 or 1/3 its MIC) they produce compounds initially not produced in lincomycin-free media (Imai et al., 2015). (2) Similar results were seen with the addition of bacterial hormone gamma-butyrolactones (γ-butyrolactones), that serve as the main signaling molecules in *Streptomyces* species for the regulation of antibiotics by binding to cytoplasmic γ-butyrolactone receptors, whereby γ-butyrolactone induced gene expression of target genes and production of antibiotics in *Streptomyces* (Takano, 2006; Horinouchi and Beppu, 2007). Apart from γ-butyrolactones, the other 2 major groups of signaling hormones are the furans (e.g., 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids) (Corre et al., 2008) and gamma-butenolides (γ-butenolides). In 2011, Kitani et al. (2011) was able to show that the γ-butenolide avenolide was responsible for stimulating the production of avermectin in *Streptomyces avermitilis* (Kitani et al., 2011). (3) Co-culture techniques are a powerful method of enhancing the biological activity of *Streptomyces* by allowing them to grow with another microbe in the same media (Sung et al., 2017). Sung et al. (2017) successfully used this method to enhance the anti-MRSA activity of extracts from marine *Streptomyces* sp. PTY087I2 M when grown in the presence of MRSA. The MIC was significantly reduced from 50 to 12.5 μg/mL, when grown under traditional culture condition and in co-culture system, respectively. These new approaches open possibilities to discover interesting anti-MRSA compounds from *Streptomyces* bacteria.

Also, understanding the biosynthetic pathways of important antibiotics is gaining increasing importance in the drug discovery process. A decade ago, cloning and identification of biosynthetic gene clusters was time-consuming and genome sequencing was expensive. Today, however, fast genome sequencing and genome mining have accelerated the identification of gene clusters–and knowledge of gene clusters provides crucial insights into modifying already existing antibiotics in order to improve them (Greule et al., 2017). These compounds may be effective in their ecological role to ward off competitors, but may need further modification in order to be viable antimicrobials in the human biological systems in terms of pharmacokinetics and dynamics. Apart from the new methods already mentioned in the paper having led to improved activity or increased yield, there are also reports of other approaches used recently to promote discovery of new compounds from *Streptomyces*. There are also reports of new methods of co-transformation carried out in *Streptomyces* sp. (Kallifidas et al., 2012). Despite the use of whole genome sequencing to identify the gene clusters, heterologous expression of these gene clusters often end up remaining silent. To overcome this problem, Kallifidas et al. (2012) used environmental DNA clones (eDNA), particularly those containing PKS type transcription factors and introduced them into *S. albus* harboring its corresponding minimal PKS containing clone. This method ultimately led to the production and isolation of tetarimycin in *S. albus*. In another example, Zhao et al. (2018) used a gene editing tool called the Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 (CRISPR-Cas9) to introduce *kasO* promoter (97 base pair) successfully in *Streptomyces roseosporus* NRRL 15998. This promoter region

then allowed for the activation of silent *aurR1* gene cluster, which when expressed, led to the production of auroramycin (Zhao et al., 2018). This was only possible after the biosynthetic gene cluster of auroramycin (*aurR1*) was identified (Zhao et al., 2018). These methods can further improve already known anti-MRSA compounds from *Streptomyces* and also allow for discovery of unknown compounds that are currently not produced because of the fact that these genes remain silent under traditional culture condition.

Given that antibiotic resistance is an inherent property of bacteria, the need to develop newer anti-microbial agents over time is inevitable. However, it is vital to understand that abuse and misuse of antibiotics have greatly accelerated the development of antibiotic resistance. As such, stewardship programs play a key role in creating greater public awareness of the importance of compliance to antibiotics, consequences of overprescribing and sanitary practices. Efforts to reduce the development of antibiotic resistance will help preserve the effective lifespan of last resort antibiotics that are still effective against MRSA such as vancomycin.

CONCLUSION

In conclusion, MRSA poses a serious healthcare threat especially with the emergence of vancomycin resistant strains. *Streptomyces* have historically been and continue to play an important role as a source of new antibiotics—including compounds active against MRSA. Given the emerging trend of MRSA across the globe, the search for better therapeutic agents should be exploring all available resources—including *Streptomyces* from underexplored territory which are likely to yield exciting new compounds. This seems a promising approach since studies have shown that ecological variation provides a more suitable approach to accelerate the discovery rate of promising antibiotics. In this review, a number of new ecological niches have been discussed. Our review also revealed a spectrum of compounds isolated from *Streptomyces* with anti-MRSA activity and summarized the extent of studies so far carried out on these compounds.

Future work should focus on utilizing newly introduced genetic and chemical tools such as genome sequencing and mining and combinatorial biosynthesis to help create new and more effective antibiotics to address the emergence of antibiotic resistant infectious bacteria such as MRSA. Additionally, potential areas of research that remain yet to be undertaken have also been mentioned. The authors hope that future work can be focused on those underexplored niches, isolating new compounds and utilizing new technologies—genomic mining, combinatorial biosynthesis, mutasynthesis, to improve pharmacological properties of promising anti-MRSA compounds and thereby increase the number of successful anti-MRSA drugs for the treatment and halt spread of MRSA infection.

AUTHOR CONTRIBUTIONS

HK, LT-HT, TK, K-GC, PP, B-HG and L-HL performed the literature search, data analysis as well the manuscript writing. Technical supports and proofreading were contributed by PP, B-HG, and L-HL. L-HL and B-HG founded the research project.

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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.2018.02221/full#supplementary-material

REFERENCES

Abdulkhair, W. M., and Alghuthaymi, M. A. (2016). Double inhibitory effect of extracellular protein of marine *Streptomyces tendae* against different strains of MRSA. *Der Pharmacia Lettre* 8, 11–20.

Abisado, R. G., Benomar, S., Klaus, J. R., Dandekar, A. A., and Chandler, J. R. (2018). Bacterial quorum sensing and microbial community interactions. *mBio* 9, e02331-17. doi: 10.1128/mBio.02331-17

Ahmad, S. J., Lian, H. H., Basri, D. F., and Zin, N. M. (2015). Mode of action of endophytic *Streptomyces* sp., SUK 25 extracts against MRSA; microscopic, biochemical and time-kill analysis. *Int. J. Pharm. Sci. Rev. Res.* 30, 11–17.

Alborn, W. E., Allen, N. E., and Preston, D. A. (1991). Daptomycin disrupts membrane potential in growing *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 35, 2282–2287.

Alexander, D. C., Rock, J., He, X., Brian, P., Miao, V., and Baltz, R. H. (2010). Development of a genetic system for combinatorial biosynthesis of lipopeptides in *Streptomyces fradiae* and heterologous expression of the A54145 biosynthesis gene cluster. *Appl. Environ. Microbiol.* 76, 6877–6887. doi: 10.1128/AEM.01248-10

Allen, R. C., Popat, R., Diggle, S. P., and Brown, S. P. (2014). Targeting virulence: can we make evolution-proof drugs? *Nat. Rev. Microbiol.* 12, 300. doi: 10.1038/nrmicro3232

Alm, R. A., Mclaughlin, R. E., Kos, V. N., Sader, H. S., Iaconis, J. P., and Lahiri, S. D. (2014). Analysis of *Staphylococcus aureus* clinical isolates with reduced susceptibility to ceftaroline: an epidemiological and structural perspective. *J. Antimicrob. Chemother.* 69, 2065–2075. doi: 10.1093/jac/dku114

Alshaibani, M. M., Jalil, J., Sidik, N. M., Edrada-Ebel, R., and Zin, N. M. (2016). Isolation and characterization of cyclo-(tryptophanyl-prolyl) and chloramphenicol from *Streptomyces* sp. SUK 25 with antimethicillin-resistant *Staphylococcus aureus* activity. *Drug Des. Devel. Ther.* 10, 1817–1827. doi: 10.2147/dddt.s101212

Anné, J., Vrancken, K., Van Mellaert, L., Van Impe, J., and Bernaerts, K. (2014). Protein secretion biotechnology in Gram-positive bacteria with special emphasis on *Streptomyces lividans*. *Biochim. Biophys. Acta* 1843, 1750–1761. doi: 10.1016/j.bbamcr.2013.12.023

Argudín, M. A., Roisin, S., Dodémont, M., Nonhoff, C., Deplano, A., and Denis, O. (2018). Mutations at the ribosomal S10 gene in clinical strains of *Staphylococcus aureus* with reduced susceptibility to tigecycline. *Antimicrob. Agents Chemother* 62:e01852-17. doi: 10.1128/AAC.01852-17

Asiimwe, B. B., Baldan, R., Trovato, A., and Cirillo, D. M. (2017). Prevalence and molecular characteristics of *Staphylococcus aureus*, including methicillin resistant strains, isolated from bulk can milk and raw milk products in pastoral communities of South-West Uganda. *BMC Infect. Dis.* 17:422. doi: 10.1186/s12879-017-2524-4

Aslam, N., Izhar, M., and Mehdi, N. (2013). Frequency of methicillin-resistant *Staphylococcus aureus* nasal colonization among patients suffering from methicillin resistant *Staphylococcus aureus* bacteraemia. *Pak. J. Med. Sci.* 29, 1430–1432. doi: 10.12669/pjms.296.3911

Atshan, S. S., Nor Shamsudin, M., Sekawi, Z., Lung, L. T. T., Hamat, R. A., Karunanidhi, A., et al. (2012). Prevalence of adhesion and regulation of biofilm-related genes in different clones of *Staphylococcus aureus*. *Biomed Res. Int.* 2012:976972. doi: 10.1155/2012/976972

Bæk, K. T., Thøgersen, L., Mogenssen, R. G., Mellergaard, M., Thomsen, L. E., Petersen, A., et al. (2015). Step-wise decrease in daptomycin susceptibility in clinical *Staphylococcus aureus* isolates associated with an initial mutation in rpoB and a compensatory inactivation of the clpX gene. *Antimicrob. Agents Chemother.* 59, 6983–6991. doi: 10.1128/AAC.01303-15

Balasubramanian, S., Othman, E. M., Kampik, D., Stopper, H., Hentschel, U., Ziebuhr, W., et al. (2017). Marine sponge-derived *Streptomyces* sp. SBT343 extract inhibits Staphylococcal biofilm formation. *Front. Microbiol.* 8:236. doi: 10.3389/fmicb.2017.00236

Bentley, S. D., Chater, K. F., Cerdeno-Tarraga, A. M., Challis, G. L., Thomson, N. R., James, K. D., et al. (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3 (2). *Nature* 417, 141–147. doi: 10.1038/417141a

Bérdy, J. (2012). Thoughts and facts about antibiotics: where we are now and where we are heading. *J. Antibiot.* 65, 385–395. doi: 10.1038/ja.2012.27

Beygmoradi, A., and Homaei, A. (2017). Marine microbes as a valuable resource for brand new industrial biocatalysts. *Biocatal. Agric. Biotechnol.* 11, 131–152. doi: 10.1016/j.bcab.2017.06.013

Bierowiec, K., Płoneczka-Janeczko, K., and Rypuła, K. (2016). Is the colonisation of *Staphylococcus aureus* in pets associated with their close contact with owners? *PLoS ONE* 11:e0156052. doi: 10.1371/journal.pone.0156052

Blumberg, S., and Martin, S. F. (2017). Synthesis of the pentacyclic core of citreamicin η. *Org. Lett.* 19, 790–793. doi: 10.1021/acs.orglett.6b03760

Boswihi, S. S., and Udo, E. E. (2018). Methicillin-resistant *Staphylococcus aureus:* an update on the epidemiology, treatment options and infection control. *CMRP* 8, 18–24. doi: 10.1016/j.cmrp.2018.01.001

Boucher, H. W., and Corey, G. R. (2008). Epidemiology of methicillin-resistant *Staphylococcus aureus*. *Clin. Infect. Dis.* 46, S344–S349. doi: 10.1086/533590

Braun, T., and Kahanov, L. (2018). CA-MRSA infection rates and management among student-athletes. *Med. Sci. Sports Exerc.* 50, 1802–1809. doi: 10.1249/MSS.0000000000001649

Braun, T., Kahanov, L., Dannelly, K., and Lauber, C. (2016). CA-MRSA infection incidence and care in high school and intercollegiate athletics. *Med. Sci. Sports Exerc.* 48, 1530–1538. doi: 10.1249/MSS.0000000000000940

Brimble, M. A., Nairn, M. R., and Park, J. (1999). Synthesis of analogues of griseusin A. *Org. Lett.* 1, 1459–1462. doi: 10.1021/ol991020c

Cafiso, V., Bertuccio, T., Purrello, S., Campanile, F., Mammina, C., Sartor, A., et al. (2014). dltA overexpression: a strain-independent keystone of daptomycin resistance in methicillin-resistant *Staphylococcus aureus*. *Int. J. Antimicrob. Agents* 43, 26–31. doi: 10.1016/j.ijantimicag.2013.10.001

Carter, G. T., Nietsche, J. A., Williams, D. R., and Borders, D. B. (1990). Citreamicins, novel antibiotics from *Micromonospora citrea*: isolation, characterization, and structure determination. *J. Antibiot.* 43, 504–512. doi: 10.7164/antibiotics.43.504

Castro, J. F., Razmilic, V., Gomez-Escribano, J. P., Andrews, B., Asenjo, J. A., and Bibb, M. J. (2015). Identification and heterologous expression of the chaxamycin biosynthesis gene cluster from *Streptomyces leeuwenhoekii*. *Appl. Environ. Microbiol.* 81, 5820–5831. doi: 10.1128/AEM.01039-15

CDC (2016). *General Information About MRSA in the Community*. Available online at: https://www.cdc.gov/mrsa/community/index.html (Accessed March 6, 2018).

Chawawisit, K., Bhoopong, P., Phupong, W., and Lertcanawanichakul, M. (2015). Anti-MRSA activity, mode of action and cytotoxicity of 2, 4-Di-tert-butylphenol produced by *Streptomyces* sp. KB1. *Int. J. Pharm. Sci. Rev. Res.* 35, 114–119.

Chawawisit, K., Bhoopong, P., Phupong, W., and Lertcanawanichakul, M. (2016). Combination effect between 2, 4-Di-tert-butylphenol produced by *Streptomyces* sp. KB1 TISTR 2304 and vancomycin against methicillin-resistant *Staphylococcus aureus* (MRSA). *Int. J. Pharmacol.* 12, 838–844. doi: 10.3923/ijp.2016.838.844

Cheng, C., Liu, Y., Song, H., Pan, L., Li, J., Qin, Y., et al. (2013). Marinopyrrole derivatives as potential antibiotic agents against methicillin-resistant *Staphylococcus aureus* (II). *Mar. Drugs* 11, 2927–2948. doi: 10.3390/md11082927

Cheng, C., Pan, L., Chen, Y., Song, H., Qin, Y., and Li, R. (2010). Total synthesis of (±)-marinopyrrole a and its library as potential antibiotic and anticancer agents. *J. Comb. Chem.* 12, 541–547. doi: 10.1021/cc100052j

Cheng, Y. B., Jensen, P. R., and Fenical, W. (2013). Cytotoxic and antimicrobial napyradiomycins from two marine-derived *Streptomyces* strains. *Eur. J. Org. Chem.* 3751–3757. doi: 10.1002/ejoc.201300349

Chino, M., Nishikawa, K., Tsuchida, T., Sawa, R., Nakamura, H., Nakamura, K. T., et al. (1997). Heliquinomycin, a new inhibitor of DNA helicase, produced by *Streptomyces* sp. MJ929-SF2. II. structure determination of heliquinomycin. *J. Antibiot.* 50, 143–146. doi: 10.7164/antibiotics.50.143

Chino, M., Nishikawa, K., Umekita, M., Hayashi, C., Yamazaki, T., Tsuchida, T., et al. (1996). Heliquinomycin, a new inhibitor of DNA helicase, produced by *Streptomyces* sp. MJ929-SF2. I. Taxonomy, production, isolation, physico-chemical properties and biological activities. *J. Antibiot.* 49, 752–757. doi: 10.7164/antibiotics.49.752

Chino, M., Nishikawa, K., Yamada, A., Ohsono, M., Sawa, T., Hanaoka, F., et al. (1998). Effect of a novel antibiotic, heliquinomycin, on DNA helicase and cell growth. *J. Antibiot.* 51, 480–486. doi: 10.7164/antibiotics.51.480

Cho, S. S., Choi, Y. H., Simkhada, J. R., Mander, P., Park, D. J., and Yoo, J. C. (2012). A newly isolated *Streptomyces* sp. CS392 producing three antimicrobial compounds. *Bioprocess. Biosyst. Eng.* 35, 247–254. doi: 10.1007/s00449-011-0599-7

Choi, H. J., Kim, D. W., Choi, Y. W., Lee, Y. G., Lee, Y. I., Jeong, Y. K., et al. (2012). Broad-spectrum *in vitro* antimicrobial activities of *Streptomyces* sp. strain BCNU 1001. *Biotechnol. Bioprocess Eng.* 17, 576–583. doi: 10.1007/s12257-011-0151-2

Choo, E. J., and Chambers, H. F. (2016). Treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. *Infect. Chemother.* 48, 267–273. doi: 10.3947/ic.2016.48.4.267

CLSI (2015). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically—Tenth Edition: Approved Standard M07-A10*. Wayne, PA: CLSI. Available online at: http://www.facm.ucl.ac.be/intranet/CLSI/CLSI-2015-M07-A10-original.pdf

CLSI (2018). *Performance Standards for Antimicrobial Susceptibility Testing; 28th Informational Supplement. CLSI Document M100*. Wayne, PA: Clinical and Laboratory Standards Institute. Available online at: http://www.facm.ucl.ac.be/intranet/CLSI/CLSI-2018-M100-S28-unlocked.pdf

Conceição, T., De Lencastre, H., and Aires-De-Sousa, M. (2017). Frequent isolation of methicillin resistant *Staphylococcus aureus* (MRSA) ST398 among healthy pigs in Portugal. *PLoS ONE* 12:e0175340. doi: 10.1371/journal.pone.0175340

Corre, C., Song, L., O'rourke, S., Chater, K. F., and Challis, G. L. (2008). 2-Alkyl-4-hydroxymethylfuran-3-carboxylic acids, antibiotic production inducers discovered by *Streptomyces coelicolor* genome mining. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17510–17515. doi: 10.1073/pnas.0805530105

Costerton, J. W., Geesey, G. G., and Cheng, K. J. (1978). How bacteria stick. *Sci. Am.* 238, 86–95. doi: 10.1038/scientificamerican0178-86

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, Í., and Penadés, J. R. (2001). Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J. Bacteriol.* 183, 2888–2896. doi: 10.1128/JB.183.9.2888-2896.2001

Cui, L., Isii, T., Fukuda, M., Ochiai, T., Neoh, H.-M., Da Cunha Camargo, I. L. B., et al. (2010). An *Rpo*B mutation confers dual heteroresistance to daptomycin and vancomycin in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 54, 5222–5233. doi: 10.1128/AAC.00437-10

Cundliffe, E., and Thompson, J. (1981). The mode of action of nosiheptide (multhiomycin) and the mechanism of resistance in the producing organism. *Microbiology* 126, 185–192. doi: 10.1099/00221287-126-1-185

Da Paz Pereira, J. N., Rabelo, M. A., Da Costa Lima, J. L., Neto, A. M. B., De Souza Lopes, A. C., and Maciel, M. V. (2016). Phenotypic and molecular characterization of resistance to macrolides, lincosamides and type

B streptogramin of clinical isolates of *Staphylococcus* spp. of a University Hospital in Recife, Pernambuco, Brazil. *Braz. J. Infect. Dis.* 20, 276–281. doi: 10.1016/j.bjid.2016.03.003

Dabul, A. N. G., Avaca-Crusca, J. S., Van Tyne, D., Gilmore, M. S., and Camargo, I. L. B. C. (2018). Resistance in *in vitro* selected tigecycline-resistant methicillin-resistant *Staphylococcus aureus* sequence type 5 Is driven by mutations in *mep*R and *mep*A genes. *Microb. Drug Resist.* 24, 519–526. doi: 10.1089/mdr.2017.0279

Dailey, H. A., Gerdes, S., Dailey, T. A., Burch, J. S., and Phillips, J. D. (2015). Noncanonical coproporphyrin-dependent bacterial heme biosynthesis pathway that does not use protoporphyrin. *Proc. Natl. Acad. Sci. U.S.A.* 112, 2210–2215. doi: 10.1073/pnas.1416285112

Dalisay, D. S., Williams, D. E., Wang, X. L., Centko, R., Chen, J., and Andersen, R. J. (2013). Marine sediment-derived *Streptomyces* bacteria from British Columbia, Canada are a promising microbiota resource for the discovery of antimicrobial natural products. *PLoS ONE* 8:e77078. doi: 10.1371/journal.pone.0077078

Dantes, R., Mu, Y.,Belflower, R., Aragon, D., Dumyati, G., Harrison, L. H., et al. (2013). National burden of invasive methicillin-resistant *Staphylococcus aureus* infections, United States, 2011. *JAMA Intern. Med.* 173, 1970–1978. doi: 10.1001/jamainternmed.2013.10423

Datta, R., Shah, A., Huang, S. S., Cui, E., Nguyen, V., Welbourne, S. J., et al. (2014). High nasal burden of methicillin-resistant *Staphylococcus aureus* increases risk of invasive disease. *J. Clin. Microbiol.* 52, 312–314. doi: 10.1128/jcm.01606-13

Daum, M., Peintner, I., Linnenbrink, A., Frerich, A., Weber, M., Paululat, T., et al. (2009). Organisation of the biosynthetic gene cluster and tailoring enzymes in the biosynthesis of the tetracyclic quinone glycoside antibiotic polyketomycin. *ChemBioChem* 10, 1073–1083. doi: 10.1002/cbic.200800823

Davoodabadi, F., Mobasherizadeh, S., Mostafavizadeh, K., Shojaei, H., Havaei, S. A., Koushki, A. M., et al. (2016). Nasal colonization in children with community acquired methicillin-resistant *Staphylococcus aureus*. *Adv. Biom. Res.* 5:86. doi: 10.4103/2277-9175.182217

De Dios Caballero, J., Pastor, M. D., Vindel, A., Máiz, L., Yagüe, G., Salvador, C., et al. (2015). Emergence of cfr-mediated linezolid resistance in a methicillin-resistant *Staphylococcus aureus* epidemic clone isolated from patients with cystic fibrosis. *Antimicrob. Agents Chemother.* 02067–02015. doi: 10.1128/AAC.02067-15

De Lima Procópio, R. E., Da Silva, I. R., Martins, M. K., De Azevedo, J. L., and De Araújo, J. M. (2012). Antibiotics produced by *Streptomyces*. *Braz. J. Infect. Dis.* 16, 466–471. doi: 10.1016/j.bjid.2012.08.014

Ding, Z. G., Zhao, J. Y., Li, M. G., Huang, R., Li, Q. M., Cui, X. L., et al. (2012). Griseusins F and G, spiro-naphthoquinones from a tin mine tailings-derived alkalophilic *Nocardiopsis* species. *J. Nat. Prod.* 75, 1994–1998. doi: 10.1021/np3004936

Djinni, I., Defant, A., Kecha, M., and Mancini, I. (2013). Antibacterial polyketides from the marine alga-derived endophitic *Streptomyces sundarbansensis*: a study on hydroxypyrone tautomerism. *Mar. Drugs* 11, 124–135. doi: 10.3390/md11010124

Djinni, I., Defant, A., Kecha, M., and Mancini, I. (2014). Metabolite profile of marine-derived endophytic *Streptomyces sundarbansensis* WR1L1S8 by liquid chromatography-mass spectrometry and evaluation of culture conditions on antibacterial activity and mycelial growth. *J. Appl. Microbiol.* 116, 39–50. doi: 10.1111/jam.12360

Dlugónska, H. (2015). The Nobel Prize 2015 in physiology or medicine for highly effective antiparasitic drugs. *Ann. Parasitol.* 61, 299–301. doi: 10.17420/ap6104.23

Driche, E. H., Belghit, S., Bijani, C., Zitouni, A., Sabaou, N., Mathieu, F., et al. (2015). A new *Streptomyces* strain isolated from Saharan soil produces di-(2-ethylhexyl) phthalate, a metabolite active against methicillin-resistant *Staphylococcus aureus*. *Ann. Microbiol.* 65, 1341–1350. doi: 10.1007/s13213-014-0972-2

Driche, E. H., Sabaou, N., Bijani, C., Zitouni, A., Pont, F., Mathieu, F., et al. (2017). *Streptomyces* sp. AT37 isolated from a Saharan soil produces a furanone derivative active against multidrug-resistant *Staphylococcus aureus*. *World J. Microbiol. Biotechnol.* 33:105. doi: 10.1007/s11274-017-2265-y

Dukic, V. M., Lauderdale, D. S., Wilder, J., Daum, R. S., and David, M. Z. (2013). Epidemics of community-associated methicillin-resistant *Staphylococcus aureus* in the United States: a meta-analysis. *PLoS ONE* 8:e52722. doi: 10.1371/journal.pone.0052722

ECDC (2016). *Surveillance Atlas of Infectious Disease*. Available online at: https://atlas.ecdc.europa.eu/public/index.aspx (Accessed August 15, 2018).

El Aila, N. A., Al Laham, N. A., and Ayesh, B. M. (2017). Nasal carriage of methicillin resistant *Staphylococcus aureus* among health care workers at Al Shifa hospital in Gaza Strip. *BMC Infect. Dis.* 17:28. doi: 10.1186/s12879-016-2139-1

Endo, T., and Yonehara, H. (1978). Identity of multhiomycin with nosiheptide. *J. Antibiot.* 31, 623–625. doi: 10.7164/antibiotics.31.623

Fabret, C., and Hoch, J. A. (1998). A two-component signal transduction system essential for growth of *Bacillus subtilis*: implications for anti-infective therapy. *J. Bacteriol.* 180, 6375–6383.

Feng, C., Ling, H., Du, D., Zhang, J., Niu, G., and Tan, H. (2014). Novel nikkomycin analogues generated by mutasynthesis in *Streptomyces ansochromogenes*. *Microb. Cell Fact.* 13:59. doi: 10.1186/1475-2859-13-59

Ferdowsian, H. R., and Beck, N. (2011). Ethical and scientific considerations regarding animal testing and research. *PLoS ONE* 6:e24059. doi: 10.1371/journal.pone.0024059

Fernandes, P., and Martens, E. (2017). Antibiotics in late clinical development. *Biochem. Pharmacol.* 133, 152–163. doi: 10.1016/j.bcp.2016.09.025

Fishovitz, J., Hermoso, J. A., Chang, M., and Mobashery, S. (2014). Penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. *IUBMB Life* 66, 572–577. doi: 10.1002/iub.1289

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., and Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.* 14:563. doi: 10.1038/nrmicro.2016.94

Floss, H. G. (2006). Combinatorial biosynthesis—potential and problems. *J. Biotechnol.* 124, 242–257. doi: 10.1016/j.jbiotec.2005.12.001

Frankenfeld, C., Mittal, S., Melendez, Y., Mendez-Vigo, L., Lamp, K. C., Keller, K. N., et al. (2018). Daptomycin: a comparison of two intravenous formulations. *Drug Des Devel. Ther.* 12, 1953–1958. doi: 10.2147/DDDT.S167010

Fu, P., Kong, F., Wang, Y., Wang, Y., Liu, P., Zuo, G., et al. (2013). Antibiotic metabolites from the coral-associated actinomycete *Streptomyces* sp. OUCMDZ-1703. *Chin. J. Chem.* 31, 100–104. doi: 10.1002/cjoc.201201062

Fukumoto, A., Kim, Y. P., Hanaki, H., Shiomi, K., Tomoda, H., and Omura, S. (2008). Cyslabdan, a new potentiator of imipenem activity against methicillin-resistant *Staphylococcus aureus*, produced by *Streptomyces* sp. K04-0144: II. Biological activities. *J. Antibiot.* 61, 7–10. doi: 10.1038/ja.2008.102

Furumai, T., Eto, K., Sasaki, T., Higuchi, H., Onaka, H., Saito, N., et al. (2002). TPU-0037-A, B, C and D, novel lydicamycin congeners with anti-MRSA activity from *Streptomyces platensis* TP-A0598. *J. Antibiot.* 55, 873–880. doi: 10.7164/antibiotics.55.873

Fuse, S., Tsukamoto, H., Yuan, Y., Wang, T. S. A., Zhang, Y., Bolla, M., et al. (2010). Functional and structural analysis of a key region of the cell wall inhibitor moenomycin. *ACS Chem. Biol.* 5, 701–711. doi: 10.1021/cb100048q

Galley, N. F., O'reilly, A. M., and Roper, D. I. (2014). Prospects for novel inhibitors of peptidoglycan transglycosylases. *Bioorg. Chem.* 55, 16–26. doi: 10.1016/j.bioorg.2014.05.007

Gerber, N. N., and Lechevalier, M. P. (1976). Prodiginine (prodigiosin-like) pigments from *Streptomyces* and other aerobic *Actinomycetes*. *Can. J. Microbiol.* 22, 658–667. doi: 10.1139/m76-097

Ghadin, N., Zin, N. M., Sabaratnam, V., Badya, N., Basri, D. F., Lian, H. H., et al. (2008). Isolation and characterization of a novel endophytic *Streptomyces* SUK 06 with antimicrobial activity from Malaysian plant. *Asian J. Plant Sci.* 7, 189–194. doi: 10.3923/ajps.2008.189.194

Ghahremani, M., Jazani, N. H., and Sharifi, Y. (2018). Emergence of vancomycin-intermediate and-resistant *Staphylococcus aureus* among methicillin-resistant *S. aureus* isolated from clinical specimens in the northwest of Iran. *J. Glob. Antimicrob. Resist.* 14, 4–9. doi: 10.1016/j.jgar.2018.01.017

Gorwitz, R. J., Jernigan, D. B., and Jernigan, J. A. (2006). *Strategies for Clinical Management of MRSA in the Community; Summary of an Experts' Meeting Convened by the Centers for Disease Control and Prevention*. Available online at: https://www.cdc.gov/mrsa/pdf/MRSA-Strategies-ExpMtgSummary-2006.pdf

Gotoh, Y., Doi, A., Furuta, E., Dubrac, S., Ishizaki, Y., Okada, M., et al. (2010). Novel antibacterial compounds specifically targeting the essential WalR response regulator. *J. Antibiot.* 63, 127–134. doi: 10.1038/ja.2010.4

Gouda, S., Das, G., Sen, S. K., Shin, H.-S., and Patra, J. K. (2016). Endophytes: a treasure house of bioactive compounds of medicinal

importance. *Front. Microbiol.* 7:1538. doi: 10.3389/fmicb.2016.01538

Greule, A., Zhang, S., Paululat, T., and Bechthold, A. (2017). From a natural product to its biosynthetic gene cluster: a demonstration using polyketomycin from *Streptomyces diastatochromogenes* Tü6028. *J. Vis. Exp.* 54952. doi: 10.3791/54952

Grumann, D., Nübel, U., and Bröker, B. M. (2014). *Staphylococcus aureus* toxins–their functions and genetics. *Infect. Genet. Evol.* 21, 583–592. doi: 10.1016/j.meegid.2013.03.013

Gu, J., Gui, Y., Chen, L., Yuan, G., Lu, H. Z., and Xu, X. (2013). Use of natural products as chemical library for drug discovery and network pharmacology. *PLoS ONE* 8:e62839. doi: 10.1371/journal.pone.0062839

Guo, H., Hall, J. W., Yang, J., and Ji, Y. (2017). The SaeRS two-component system controls survival of *Staphylococcus aureus* in human blood through regulation of coagulase. *Front. Cell. Infect. Microbiol.* 7:204. doi: 10.3389/fcimb.2017.00204

Guo, J.-J., Dai, B.-L., Chen, N.-P., Jin, L.-X., Jiang, F.-S., Ding, Z.-S., et al. (2016). The anti-*Staphylococcus aureus* activity of the phenanthrene fraction from fibrous roots of *Bletilla striata*. *BMC Complemen. Altern. Med.* 16:491. doi: 10.1186/s12906-016-1488-z

Haider, S., and Wright, D. (2013). Case report: panton-valentine leukocidin *Staphylococcus* causing fatal necrotising pneumonia in a young boy. *BMJ Case Rep.* 2013:bcr2012007655. doi: 10.1136/bcr-2012-007655

Hashem, R. A., Yassin, A. S., Zedan, H. H., and Amin, M. A. (2013). Fluoroquinolone resistant mechanisms in methicillin-resistant *Staphylococcus aureus* clinical isolates in Cairo, Egypt. *J. Infect. Dev. Ctries.* 7, 796–803. doi: 10.3855/jidc.3105

Hassan, R., Shaaban, M. I., Bar, F. M. A., El-Mahdy, A. M., and Shokralla, S. (2016). Quorum sensing inhibiting activity of *Streptomyces coelicoflavus* isolated from soil. *Front. Microbiol.* 7:659. doi: 10.3389/fmicb.2016.00659

Haste, N. M., Farnaes, L., Perera, V. R., Fenical, W., Nizet, V., and Hensler, M. E. (2011a). Bactericidal kinetics of marine-derived napyradiomycins against contemporary methicillin-resistant *Staphylococcus aureus*. *Mar. Drugs* 9, 680–689. doi: 10.3390/md9040680

Haste, N. M., Hughes, C. C., Tran, D. N., Fenical, W., Jensen, P. R., Nizet, V., et al. (2011b). Pharmacological properties of the marine natural product marinopyrrole A against methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 55, 3305–3312. doi: 10.1128/aac.01211-10

Haste, N. M., Thienphrapa, W., Tran, D. N., Loesgen, S., Sun, P., Nam, S. J., et al. (2012). Activity of the thiopeptide antibiotic nosiheptide against contemporary strains of methicillin-resistant *Staphylococcus aureus*. *J. Antibiot.* 65, 593–598. doi: 10.1038/ja.2012.77

Hibbing, M. E., Fuqua, C., Parsek, M. R., and Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nat. Rev. Microbiol.* 8, 15–25. doi: 10.1038/nrmicro2259

Higginbotham, S. J., and Murphy, C. D. (2010). Identification and characterisation of a *Streptomyces* sp. isolate exhibiting activity against methicillin-resistant *Staphylococcus aureus*. *Microbiol. Res.* 165, 82–86. doi: 10.1016/j.micres.2008.12.004

Hiramatsu, K., Ito, T., Tsubakishita, S., Sasaki, T., Takeuchi, F., Morimoto, Y., et al. (2013). Genomic basis for methicillin resistance in *Staphylococcus aureus*. *Infect. Chemother.* 45, 117–136. doi: 10.3947/ic.2013.45.2.117

Ho, P. L., and Hong Kong intensive care unit antimicrobial resistance study (HK-ICARE) Group (2003). Carriage of methicillin-resistant *Staphylococcus aureus*, ceftazidime-resistant Gram-negative bacilli, and vancomycin-resistant enterococci before and after intensive care unit admission. *Crit. Care Med.* 31, 1175–1182. doi: 10.1097/01.ccm.0000059437.01924.97

Hong, K., Gao, A. H., Xie, Q. Y., Gao, H., Zhuang, L., Lin, H. P., et al. (2009). Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China. *Mar. Drugs* 7, 24–44. doi: 10.3390/md7010024

Hooper, D. C., and Jacoby, G. A. (2016). Topoisomerase inhibitors: fluoroquinolone mechanisms of action and resistance. *Cold Spring Harb. Perspect. Med.* 6:a025320. doi: 10.1101/cshperspect.a025320

Hopp, D. C., Milanowski, D. J., Rhea, J., Jacobsen, D., Rabenstein, J., Smith, C., et al. (2008). Citreamicins with potent gram-positive activity. *J. Nat. Prod.* 71, 2032–2035. doi: 10.1021/np800503z

Horinouchi, S., and Beppu, T. (2007). Hormonal control by A-factor of morphological development and secondary metabolism in *Streptomyces*. *Proc. Jpn. Acad. B* 83, 277–295. doi: 10.2183/pjab.83.277

Howden, B. P., Peleg, A. Y., and Stinear, T. P. (2014). The evolution of vancomycin intermediate *Staphylococcus aureus* (VISA) and heterogenous-VISA. *Infect. Genet. Evol.* 21, 575–582. doi: 10.1016/j.meegid.2013.03.047

Hughes, C. C., Kauffman, C. A., Jensen, P. R., and Fenical, W. (2010). Structures, reactivities, and antibiotic properties of the marinopyrroles A-F. *J. Org. Chem.* 75, 3240–3250. doi: 10.1021/jo1002054

Hughes, C. C., Prieto-Davo, A., Jensen, P. R., and Fenical, W. (2008). The marinopyrroles, antibiotics of an unprecedented structure class from a marine *Streptomyces* sp. *Org. Lett.* 10, 629–631. doi: 10.1021/ol702952n

Igarashi, M., Shida, T., Sasaki, Y., Kinoshita, N., Naganawa, H., Hamada, M., et al. (1999). Vinylamycin, a new depsipeptide antibiotic, from *Streptomyces* sp. *J. Antibiot.* 52, 873–879. doi: 10.7164/antibiotics.52.873

Igarashi, M., Tsuchida, T., Kinoshita, N., Kamijima, M., Sawa, R., Sawa, T., et al. (1998). Cremimycin, a novel 19-membered macrocyclic lactam antibiotic, from *Streptomyces* sp. *J. Antibiot.* 51, 123–129. doi: 10.7164/antibiotics.51.123

Igarashi, M., Watanabe, T., Hashida, T., Umekita, M., Hatano, M., Yanagida, Y., et al. (2013). Waldiomycin, a novel WalK-histidine kinase inhibitor from *Streptomyces* sp. MK844-mF10. *J. Antibiot.* 66, 459–464. doi: 10.1038/ja.2013.33

Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T., et al. (2003). Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nat. Biotechnol.* 21:526. doi: 10.1038/nbt820

Ilic-Tomic, T., Genčić, M. S., Živković, M. Z., Vasiljevic, B., Djokic, L., Nikodinovic-Runic, J., and Radulović, N.S. (2015). Structural diversity and possible functional roles of free fatty acids of the novel soil isolate *Streptomyces* sp. NP10. *Appl. Microbiol. Biotechnol.* 99, 4815–4833. doi: 10.1007/s00253-014-6364-5

Imai, Y., Sato, S., Tanaka, Y., Ochi, K., and Hosaka, T. (2015). Lincomycin at subinhibitory concentrations potentiates secondary metabolite production by *Streptomyces* spp. *Appl. Environ. Microbiol.* 81, 3869–3879. doi: 10.1128/aem.04214-14

Iniyan, A. M., Kannan, R. R., and Vincent, S. G. P. (2017). Characterization of culturable actinomycetes associated with halophytic rhizosphere as potential source of antibiotics. *Proc. Natl. Acad. Sci. India Sect. B Biol. Sci.* 87, 233–242. doi: 10.1007/s40011-015-0601-2

Iniyan, A. M., Mary, T. R. J., Joseph, F. J. R. S., Kannan, R. R., and Vincent, S. G. P. (2016). Cell wall distracting anti-Methicillin-resistant *Staphylococcus aureus* compound PVI331 from a marine sponge associated *Streptomyces*. *J. Appl. Biomed.* 14, 273–283. doi: 10.1016/j.jab.2016.04.003

IPCC (2007). *Working Group II: Impacts, Adaptation and Vulnerability*. Available online at: https://www.ipcc.ch/publications_and_data/ar4/wg2/en/ch3.html (Accessed June 27, 2018).

IWG-SCC (1999). *Currently Identified SCCmec Types in S. aureus Strains*. Available online at: http://www.sccmec.org/Pages/SCC_TypesEN.html [Accessed February 27, 2018).

Jain, S. N., Vishwanatha, T., Reena, V., Divyashree, B., Sampath, A., Siddhalingeshwara, K., et al. (2011). Antibiotic synergy test: chequerboard method on multi drug resistant *Pseudomonas aeruginosa*. *Int. Res. J. Pharm.* 2, 196–198.

Jamal, M., Ahmad, W., Andleeb, S., Jalil, F., Imran, M., Nawaz, M. A., et al. (2017). Bacterial biofilm and associated infections. *J. Chin. Med. Assoc.* 81, 7–11. doi: 10.1016/j.jcma.2017.07.012

Janardhan, A., Kumar, A. P., Viswanath, B., Saigopal, D. V., and Narasimha, G. (2014). Production of bioactive compounds by Actinomycetes and their antioxidant properties. *Biotechnol. Res. Int.* 2014:217030. doi: 10.1155/2014/217030

Jansen Van Rensburg, M. J., Whitelaw, A. C., and Elisha, B. G. (2012). Genetic basis of rifampicin resistance in methicillin-resistant *Staphylococcus aureus* suggests clonal expansion in hospitals in Cape Town, South Africa. *BMC Microbiol.* 12:46. doi: 10.1186/1471-2180-12-46

Jenke-Kodama, H., Sandmann, A., Müller, R., and Dittmann, E. (2005). Evolutionary implications of bacterial polyketide synthases. *Mol. Biol. Evol.* 22, 2027–2039. doi: 10.1093/molbev/msi193

Jevons, M. P. (1961). "Celbenin"-resistant Staphylococci. *BMJ* 1:124. doi: 10.1136/bmj.1.5219.124-a

Ji, Q., Chen, P. J., Qin, G., Deng, X., Hao, Z., Wawrzak, Z., et al. (2016). Structure and mechanism of the essential two-component signal-transduction system WalKR in *Staphylococcus aureus*. *Nat. Commun.* 7:11000. doi: 10.1038/ncomms11000

Ji, Z., Qiao, G., Wei, S., Fan, L., and Wu, W. (2012). Isolation and characterization of two novel antibacterial cyclic hexapeptides from *Streptomyces alboflavus* 313. *Chem. Biodivers.* 9, 1567–1578. doi: 10.1002/cbdv.201100364

Jin, C. Y., Jun, H. K., Jung, W. H., Nae, S. P., Jae, K. S., June, W. L., et al. (2007). Production and biological activity of laidlomycin, anti-MRSA/VRE antibiotic from *Streptomyces* sp. CS684. *J. Microbiol.* 45, 6–10.

Kallifidas, D., Kang, H. S., and Brady, S. F. (2012). Tetarimycin, A., an MRSA-active antibiotic identified through induced expression of environmental DNA gene clusters. *J. Am. Chem. Soc.* 134, 19552–19555. doi: 10.1021/ja3093828

Kannan, R. R., Iniyan, A. M., and Prakash, V. S. (2011). Isolation of a small molecule with anti-MRSA activity from a mangrove symbiont *Streptomyces* sp. PVRK-1 and its biomedical studies in zebrafish embryos. *Asian Pac. J. Trop. Biomed.* 1, 341–347. doi: 10.1016/s2221-1691(11)60077-4

Kannan, R. R., Iniyan, A. M., and Vincent, S. G. (2014). Production of a compound against methicillin resistant *Staphylococcus aureus* (MRSA) from *Streptomyces rubrolavendulae* ICN3 and its evaluation in zebrafish embryos. *Indian J. Med. Res.* 139, 913–920.

Kaur, D. C., and Chate, S. S. (2015). Study of antibiotic resistance pattern in methicillin resistant *Staphylococcus aureus* with special reference to newer antibiotic. *J. Glob. Infect. Dis.* 7:78. doi: 10.4103/0974-777x.157245

Kawada-Matsuo, M., Yoshida, Y., Zendo, T., Nagao, J., Oogai, Y., Nakamura, Y., et al. (2013). Three distinct two-component systems are involved in resistance to the class I bacteriocins, Nukacin ISK-1 and nisin A, in *Staphylococcus aureus*. *PLoS ONE* 8:e69455. doi: 10.1371/journal.pone.0069455

Khanal, R., Sah, P., Lamichhane, P., Lamsal, A., Upadhaya, S., and Pahwa, V. K. (2015). Nasal carriage of methicillin resistant *Staphylococcus aureus* among health care workers at a tertiary care hospital in Western Nepal. *Antimicrob. Resist. Infect. Control* 4:39. doi: 10.1186/s13756-015-0082-3

Kitani, S., Miyamoto, K. T., Takamatsu, S., Herawati, E., Iguchi, H., Nishitomi, K., et al. (2011). Avenolide, a *Streptomyces* hormone controlling antibiotic production in *Streptomyces avermitilis*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16410–16415. doi: 10.1073/pnas.1113908108

Kloss, P., Xiong, L., Shinabarger, D. L., and Mankin, A. S. (1999). Resistance mutations in 23 S rRNA identify the site of action of the protein synthesis inhibitor linezolid in the ribosomal peptidyl transferase center1. *J. Mol. Biol.* 294, 93–101. doi: 10.1006/jmbi.1999.3247

Kojiri, K., Nakajima, S., Suzuki, H., Okura, A., and Suda, H. (1993). A new antitumour substance, BE-18591, produced by a Streptomycete. *J. Antibiot.* 46, 1799–1803. doi: 10.7164/antibiotics.46.1799

Kometani, T., Takeuchi, Y., and Yoshii, E. (1983). Pyranonaphthoquinone antibiotics. 4. Total synthesis of (+)-griseusin A, an enantiomer of the naturally occurring griseusin A. *J. Org. Chem.* 48, 2311–2314. doi: 10.1021/jo00162a002

Koupahi, H., Honarmand Jahromy, S., and Rahbar, M. (2016). Evaluation of different phenotypic and genotypic methods for detection of methicillin resistant *Staphylococcus aureus* (MRSA). *Iran. J. Pathol.* 11:370–376.

Koyama, N., Tokura, Y., Munch, D., Sahl, H. G., Schneider, T., Shibagaki, Y., et al. (2012). The nonantibiotic small molecule cyslabdan enhances the potency of beta-lactams against MRSA by inhibiting pentaglycine interpeptide bridge synthesis. *PLoS ONE* 7:e48981. doi: 10.1371/journal.pone.0048981

Koyama, N., Yotsumoto, M., Onaka, H., and Tomoda, H. (2013). New structural scaffold 14-membered macrocyclic lactone ring for selective inhibitors of cell wall peptidoglycan biosynthesis in *Staphylococcus aureus*. *J. Antibiot.* 66, 303–304. doi: 10.1038/ja.2012.122

Kreiswirth, B., Kornblum, J., Arbeit, R. D., Eisner, W., Maslow, J. N., Mcgeer, A., et al. (1993). Evidence for a clonal origin of methicillin resistance in *Staphylococcus aureus*. *Science* 259, 227–230. doi: 10.1126/science.8093647

Krishnamurthy, A., Nagel, S., and Orlov, D. (2014). Sizing up repo. *J. Finance* 69, 2381–2417. doi: 10.1111/jofi.12168

Kumar, K., and Chopra, S. (2013). New drugs for methicillin-resistant *Staphylococcus aureus:* an update. *J. Antimicrob. Chemother.* 68, 1465–1470. doi: 10.1093/jac/dkt045

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., et al. (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* 390, 249–256. doi: 10.1038/36786

Kwon, H. C., Espindola, A. P., Park, J. S., Prieto-Davo, A., Rose, M., Jensen, P. R., et al. (2010). Nitropyrrolins, A.-E., cytotoxic farnesyl-alpha-nitropyrroles from a marine-derived bacterium within the actinomycete family Streptomycetaceae. *J. Nat. Prod.* 73, 2047–2052. doi: 10.1021/np1006229

Lacret, R., Oves-Costales, D., Gómez, C., Díaz, C., De La Cruz, M., Pérez-Victoria, I., et al. (2015). New ikarugamycin derivatives with antifungal and antibacterial properties from *Streptomyces zhaozhouensis*. *Mar. Drugs* 13, 128–140. doi: 10.3390/md13010128

Lange, R., Wagner, C., De Saizieu, A., Flint, N., Molnos, J., Stieger, M., et al. (1999). Domain organization and molecular characterization of 13 two-component systems identified by genome sequencing of *Streptococcus pneumoniae*. *Gene* 237, 223–234. doi: 10.1016/S0378-1119(99)00266-8

Law, J. W., Ser, H.-L., Khan, T. M., Chuah, L.-H., Pusparajah, P., Chan, K.-G., et al. (2017b). The potential of *Streptomyces* as biocontrol agents against the rice blast fungus, *Magnaporthe oryzae* (*Pyricularia oryzae*). *Front. Microbiol.* 8:3. doi: 10.3389/fmicb.2017.00003

Law, J. W., Ser, H. L., Duangjai, A., Saokaew, S., Bukhari, S. I., Khan, T. M., et al. (2017a). *Streptomyces colonosanans* sp. nov., a novel actinobacterium isolated from Malaysia mangrove soil exhibiting antioxidative activity and cytotoxic potential against human colon cancer cell lines. *Front. Microbiol.* 8:877. doi: 10.3389/fmicb.2017.00877

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K., Wertheim, H. F. L., Sumpradit, N., et al. (2013). Antibiotic resistance—the need for global solutions. *Lancet Infect. Dis.* 13, 1057–1098. doi: 10.1016/S1473-3099(13)70318-9

Lee, A. S., De Lencastre, H., Garau, J., Kluytmans, J., Malhotra-Kumar, S., Peschel, A., et al. (2018). Methicillin-resistant *Staphylococcus aureus*. *Nat. Rev.Dis. Primers* 4:18033. doi: 10.1038/nrdp.2018.33

Lee, E. Y., and Caffrey, A. (2017). Thrombocytopenia with tedizolid and linezolid. *Antimicrob. Agents Chemother.* 62:e01453-17. doi: 10.1128/AAC.01453-17

Lee, H. W., Choi, J. W., Kim, H. W., Han, D. P., Shin, W. S., and Yi, D. H. (1997). A peptide antibiotic AMRSA1 active against multidrug-resistant *Staphylococcus aureus* produced by *Streptomyces* sp. HW-003. *J. Microbiol. Biotechnol.* 7, 402–408.

Lee, L. H., Zainal, N., Azman, A. S., Eng, S. K., Ab Mutalib, N. S., Yin, W. F., et al. (2014a). Streptomyces *pluripotens* sp. nov., A bacteriocin-producing streptomycete that inhibits meticillin-resistant *Staphylococcus aureus*. *Int. J. Syst. Evol. Microbiol.* 64, 3297–3306. doi: 10.1099/ijs.0.065045-0

Lee, L. H., Zainal, N., Azman, A. S., Eng, S. K., Goh, B. H., Yin, W. F., et al. (2014b). Diversity and antimicrobial activities of actinobacteria isolated from tropical mangrove sediments in Malaysia. *Sci. World J.* 2014:698178. doi: 10.1155/2014/698178

León, J., Aponte, J. J., Rojas, R., Cuadra, D., Ayala, N., Tomás, G., et al. (2011). Study of marine actinomycetes isolated from the central coast of Peru and their antibacterial activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*. *Rev. Peru. Med. Exp. Salud Publica* 28, 237–246. doi: 10.1590/S1726-46342011000200010

Li, R. (2016). Marinopyrroles: unique drug discoveries based on marine natural products. *Med. Res. Rev.* 36, 169–189. doi: 10.1002/med.21359

Lim, K. T., Hanifah, Y. A., Yusof, M., and Thong, K. L. (2012). ermA, ermC, tetM and tetK are essential for erythromycin and tetracycline resistance among methicillin-resistant *Staphylococcus aureus* strains isolated from a tertiary hospital in Malaysia. *Indian J. Med. Microbiol.* 30:203. doi: 10.4103/0255-0857.96693

Liu, L. L., Xu, Y., Han, Z., Li, Y. X., Lu, L., Lai, P. Y., et al. (2012). Four new antibacterial xanthones from the marine-derived actinomycetes *Streptomyces caelestis*. *Mar. Drugs* 10, 2571–2583. doi: 10.3390/md10112571

Liu, S., Guo, H., Zhang, T., Han, L., Yao, P., Zhang, Y., et al. (2015). Structure-based mechanistic insights into terminal amide synthase in nosiheptide-represented thiopeptides biosynthesis. *Sci. Rep.* 5:12744. doi: 10.1038/srep12744

Liu, Y., Haste, N. M., Thienphrapa, W., Li, J., Nizet, V., Hensler, M., et al. (2014). Marinopyrrole derivatives as potential antibiotic agents against methicillin-resistant *Staphylococcus aureus* (III). *Mar. Drugs* 12, 2458–2470. doi: 10.3390/md12052458

Loncaric, I., Brunthaler, R., and Spergser, J. (2013). Suspected goat to human transmission of methicillin-resistant *Staphylococcus aureus*

sequence type 398. *J. Clin. Microbiol.* 51, 1625–1626. doi: 10.1128/JCM.03052-12

Long, S. W., Olsen, R. J., Mehta, S. C., Palzkill, T., Cernoch, P. L., Perez, K. K., et al. (2014). PBP2a mutations causing high-level ceftaroline resistance in clinical methicillin-resistant *Staphylococcus aureus* isolates. *Antimicrob. Agents Chemother.* 58, 6668–6674. doi: 10.1128/AAC.03622-14

Lovering, A. L., Safadi, S. S., and Strynadka, N. C. (2012). Structural perspective of peptidoglycan biosynthesis and assembly. *Annu. Rev. Biochem.* 81, 451–478. doi: 10.1146/annurev-biochem-061809-112742

LPSN (2018). *Genus Streptomyces*. Available online at: http://www.bacterio.net/streptomyces.html (Accessed April 02, 2018).

Luo, J., Li, J., Yang, H., Yu, J., and Wei, H. (2017). Accurate detection of methicillin-resistant *Staphylococcus aureus* in mixtures utilizing single bacterial duplex droplet digital PCR. *J. Clin. Microbiol.* 55, 2946–2955. doi: 10.1128/JCM.00716-17

Ma, D. S. L., Tan, L. T., Chan, K. G., Yap, W. H., Pusparajah, P., Chuah, L. H., et al. (2018). Resvertrol – potential antibacterial agent against foodborne pathogens. *Front. Pharmacol.* 9:102. doi: 10.3389/fphar.2018.00102

Mahdiyoun, S. M., Kazemian, H., Ahanjan, M., Houri, H., and Goudarzi, M. (2016). Frequency of aminoglycoside-resistance genes in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from hospitalized patients. *Jundishapur J. Microbiol.* 9:e35052. doi: 10.5812/jjm.35052

Mai-Prochnow, A., Clauson, M., Hong, J., and Murphy, A. B. (2016). Gram positive and Gram negative bacteria differ in their sensitivity to cold plasma. *Sci. Rep.* 6:38610. doi: 10.1038/srep38610

Malik, H., Sur, B., Singhal, N., and Bihari, V. (2008). Antimicrobial protein from *Streptomyces fulvissimus* inhibitory to methicillin resistant *Staphylococcus aureus*. *Indian J. Exp. Biol.* 46, 254–257.

Martinez-Klimova, E., Rodríguez-Peña, K., and Sánchez, S. (2017). Endophytes as sources of antibiotics. *Biochem. Pharmacol.* 134, 1–17. doi: 10.1016/j.bcp.2016.10.010

Matsumoto, N., Tsuchida, T., Maruyama, M., Kinoshita, N., Homma, Y., Iinuma, H., et al. (1999). Lactonamycin, a new antimicrobial antibiotic produced by *Streptomyces rishiriensis* MJ773-88K4. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activities. *J. Antibiot.* 52, 269–275. doi: 10.7164/antibiotics.52.269

McCarthy, H., Rudkin, J. K., Black, N. S., Gallagher, L., O'neill, E., and O'gara, J. P. (2015). Methicillin resistance and the biofilm phenotype in *Staphylococcus aureus*. *Front. Cell. Infect. Microbiol.* 5:1. doi: 10.3389/fcimb.2015.00001

McCourt, J., O'halloran, D. P., Mccarthy, H., O'gara, J. P., and Geoghegan, J. A. (2014). Fibronectin-binding proteins are required for biofilm formation by community-associated methicillin-resistant *Staphylococcus aureus* strain LAC. *FEMS Microbiol. Lett.* 353, 157–164. doi: 10.1111/1574-6968.12424

McGuinness, W. A., Malachowa, N., and Deleo, F. R. (2017). Focus: infectious diseases: vancomycin resistance in *Staphylococcus aureus*. *Yale J. Biol. Med.* 90, 269–281.

Miao, L., Xu, J., Yao, Z., Jiang, Y., Zhou, H., Jiang, W., et al. (2017). The anti-quorum sensing activity and bioactive substance of a marine derived *Streptomyces*. *Biotechnol. Biotechnol. Equip.* 31, 1007–1015. doi: 10.1080/13102818.2017.1348253

Mirzaee, M., Najar Peerayeh, S., and Ghasemian, A.-M. (2014). Detection of icaABCD genes and biofilm formation in clinical isolates of methicillin resistant *Staphylococcus aureus*. *Iran. J. Pathol.* 9, 257–262.

Mkrtchyan, H. V., Xu, Z., and Cutler, R. R. (2015). Diversity of *SCC*mec elements in Staphylococci isolated from public washrooms. *BMC Microbiol.* 15:120. doi: 10.1186/s12866-015-0451-3

Mohammadipanah, F., Kazemi Shariat Panahi, H., Imanparast, F., and Hamedi, J. (2016). Development of a reversed-phase liquid chromatographic assay for the quantification of total persipeptides in fermentation broth. *Chromatographia* 79, 1325–1332. doi: 10.1007/s10337-016-3140-y

Mohandas, S. P., Ravikumar, S., Menachery, S. J., Suseelan, G., Narayanan, S. S., Nandanwar, H., et al. (2012). Bioactives of microbes isolated from Western Ghat belt of Kerala show β-lactamase inhibition along with wide spectrum antimicrobial activity. *Appl. Biochem. Biotechnol.* 167, 1753–1762. doi: 10.1007/s12010-012-9596-9

Momose, I., Chen, W., Kinoshita, N., Iinuma, H., Hamada, M., and Takeuchi, T. (1998). Polyketomycin, a new antibiotic from *Streptomyces* sp. MK277-AF1. I. Taxonomy, production, isolation, physico-chemical properties and biological activities. *J. Antibiot.* 51, 21-25. doi: 10.7164/antibiotics.51.21

Mora, C., Tittensor, D. P., Adl, S., Simpson, A. G., and Worm, B. (2011). How many species are there on Earth and in the ocean? *PLoS Biol.* 9:e1001127. doi: 10.1371/journal.pbio.1001127

Morakchi, H., Ayari, A., Taok, M., Kirane, D., and Cochet, N. (2009). Characterization of *Streptomyces* strain SLO-105 isolated from Lake Oubeira sediments in North-East of Algeria. *Afr. J. Biotechnol.* 8, 6332–6336. doi: 10.5897/ajb09.1096

Morales, G., Picazo, J. J., Baos, E., Candel, F. J., Arribi, A., Peláez, B., et al. (2010). Resistance to linezolid is mediated by the cfr gene in the first report of an outbreak of linezolid-resistant *Staphylococcus aureus*. *Clin. Infect. Dis.* 50, 821–825. doi: 10.1086/650574

Moyo, S. J., Nkinda, L., Majigo, M., Mmbaga, E., Mbembati, N., Aboud, S., et al. (2017). Prevalence of methicillin-resistant *Staphylococcus aureus* carriage on admission among patients attending regional hospitals in Dar es Salaam, Tanzania. *BMC Res. Notes* 10, 417. doi: 10.1186/s13104-017-2668-8

Nakajima, S., Kojiri, K., and Suda, H. (1993). A new antitumor substance, BE-18591, produced by a Streptomycete. *J. Antibiot.* 46, 1894–1896. doi: 10.7164/antibiotics.46.1894

Nelson, R. E., Jones, M., Liu, C.-F., Samore, M. H., Evans, M. E., Graves, N., et al. (2015). The impact of healthcare-associated methicillin-resistant *Staphylococcus aureus* infections on post-discharge healthcare costs and utilization. *Infect. Control Hosp. Epidemiol.* 36, 534–542. doi: 10.1017/ice.2015.22

Nesme, J., and Simonet, P. (2015). The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. *Environ. Microbiol.* 17, 913–930. doi: 10.1111/1462-2920.12631

Newman, D. J., and Cragg, G. M. (2016). Natural products as sources of new drugs from 1981 to 2014. *J. Nat. Prod.* 79, 629–661. doi: 10.1021/acs.jnatprod.5b01055

Nicolaou, K., Simmons, N. L., Chen, J. S., Haste, N. M., and Nizet, V. (2011). Total synthesis and biological evaluation of marinopyrrole A and analogs. *Tetrahedron Lett.* 52, 2041–2043. doi: 10.1016/j.tetlet.2010.09.059

NOAA (2018). *Oceans and Coasts*. Available online at: http://www.noaa.gov/oceans-coasts (Accessed November 26, 2017).

Okada, A., Igarashi, M., Okajima, T., Kinoshita, N., Umekita, M., Sawa, R., et al. (2010). Walkmycin B targets WalK (YycG), a histidine kinase essential for bacterial cell growth. *J. Antibiot.* 63, 89–94. doi: 10.1038/ja.2009.128

Ong, M. H.-L., Ho, W.-Y., Ng, W.-W., and Chew, C.-H. (2017). High prevalence of tetM as compared to tetK amongst methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from hospitals in Perak, Malaysia. *Jundishapur J. Microbiol.* 10:e13935. doi: 10.5812/jjm.13935

Oskay, M. (2009). Antifungal and antibacterial compounds from *Streptomyces* strains. *Afr. J. Biotechnol.* 8, 3007–3017.

Osman, M., Al Nasbeh, A., Rafei, R., Mallat, H., Achkar, M., Dabboussi, F., et al. (2015). Characterization of resistance genes to macrolides, lincosamides and streptogramins (MLS) among clinical isolates of *Staphylococcus aureus* in North Lebanon. *Int. Arab. J. Antimicrob. Agents* 5:3. doi: 10.3823/780

Otoguro, K., Ui, H., Ishiyama, A., Arai, N., Kobayashi, M., Takahashi, Y., et al. (2003). *In vitro* antimalarial activities of the microbial metabolites. *J. Antibiot.* 56, 322–324. doi: 10.7164/antibiotics.56.322

Otto, M. (2013). Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity. *Annu. Rev. Med.* 64, 175–188. doi: 10.1146/annurev-med-042711-140023

Otto, M. (2014). *Staphylococcus aureus* toxins. *Curr. Opin. Microbiol.* 17, 32–37. doi: 10.1016/j.mib.2013.11.004

Özakin, S., Davis, R. W., Umile, T. P., Pirinccioglu, N., Kizil, M., Celik, G., et al. Minbiole, K.P.C., and Ince, E. (2016). The isolation of tetrangomycin from terrestrial *Streptomyces* sp. CAH29: evaluation of antioxidant, anticancer, and anti-MRSA activity. *Med. Chem. Res.* 25, 2872–2881. doi: 10.1007/s00044-016-1708-6

Panton, P., and Valentine, F. (1932). Staphylococcal toxin. *Lancet* 219, 506–508.

Park, H. B., Lee, J. K., Lee, K. R., and Kwon, H. C. (2014). Angumycinones, A., and B, two new angucyclic quinones from *Streptomyces* sp. KMC004 isolated from acidic mine drainage. *Tetrahedron Lett.* 55, 63–66. doi: 10.1016/j.tetlet.2013.10.112

Pathare, N. A., Asogan, H., Tejani, S., Al Mahruqi, G., Al Fakhri, S., Zafarulla, R., et al. (2016). Prevalence of methicillin resistant *Staphylococcus aureus* [MRSA] colonization or carriage among health-care workers. *J. Infect. Public Health* 9, 571–576. doi: 10.1016/j.jiph.2015.12.004

Paululat, T., Zeeck, A., Gutterer, J. M., and Fiedler, H.-P. (1999). Biosynthesis of polyketomycin produced by *Streptomyces diastatochromogenes* Tü 6028. *J. Antibiot.* 52, 96–101.

Peacock, S. J., and Paterson, G. K. (2015). Mechanisms of methicillin resistance in *Staphylococcus aureus. Annu. Rev. Biochem.* 84, 577–601. doi: 10.1146/annurev-biochem-060614-034516

Peoples, A. J., Zhang, Q., Millett, W. P., Rothfeder, M. T., Pescatore, B. C., Madden, A. A., et al. (2008). Neocitreamicins, I., and II, novel antibiotics with activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant Enterococci. *J. Antibiot.* 61, 457–463. doi: 10.1038/ja.2008.62

Pistella, E., Falcone, M., Baiocchi, P., Pompeo, M. E., Pierciaccante, A., Penni, A., et al. (2005). *In vitro* activity of fosfomycin in combination with vancomycin or teicoplanin against *Staphylococcus aureus* isolated from device-associated infections unresponsive to glycopeptide therapy. *Infez. Med.* 13, 97–102.

Porrero, M. C., Mentaberre, G., Sánchez, S., Fernández-Llario, P., Gómez-Barrero, S., Navarro-Gonzalez, N., et al. (2013). Methicillin resistant *Staphylococcus aureus* (MRSA) carriage in different free-living wild animal species in Spain. *Vet. J.* 198, 127–130. doi: 10.1016/j.tvjl.2013.06.004

Quiles-Melero, I., Gómez-Gil, R., Romero-Gómez, M. P., Sánchez-Díaz, A. M., De Pablos, M., García-Rodriguez, J., et al. (2012). Mechanisms of linezolid-resistance among Staphylococci in a tertiary hospital. *J. Clin. Miicrobiol.* 51, 998–1001. doi: 10.1128/JCM.01598-12

Rachman, A. R. A., Suhaili, Z., and Desa, M. N. M. (2017). "The evolution and dissemination of methicillin resistance determinant in *Staphylococcus aureus*," in *The Rise of Virulence and Antibiotic Resistance in Staphylococcus aureus*, eds S. Enany and L. E. Crotty Alexander (Croatia: InTech), 1–214. doi: 10.5772/65514

Raja, A., Labonte, J., Lebbos, J., and Kirkpatrick, P. (2003). Daptomycin. *Nat. Rev. Drug Discov.* 2, 943–944. doi: 10.1038/nrd1258

Rajan, B. M., and Kannabiran, K. (2013a). Antagonistic activity of marine *Streptomyces* sp. VITBRK1 on drug resistant gram positive cocci. *Pharm. Lett.* 5, 185–191.

Rajan, B. M., and Kannabiran, K. (2013b). Antimicrobial activity of *Streptomyces albofaciens* against methicillin resistant *Staphylococcus aureus* and vancomycin resistant *Enterococcus* multi-drug resistant species. *Res. J. Pharm. Biol. Chem. Sci.* 4, 1248–1257.

Rajan, B. M., and Kannabiran, K. (2014). Extraction and identification of antibacterial secondary metabolites from marine *Streptomyces* sp. VITBRK2. *Int. J. Mol. Cell. Med.* 3, 130–137.

Rajan, B. M., and Kannabiran, K. (2015). Antibiotic potency of 2,4-dichloro-5-sulfamoyl benzoic acid extracted from marine bacterium *Streptomyces* sp. VITBRK3 against methicillin resistant *Staphylococcus aureus. Pharm. Lett.* 7, 244–252.

Rateb, M. E., Houssen, W. E., Arnold, M., Abdelrahman, M. H., Deng, H., Harrison, W. T., et al. (2011). Chaxamycins A - D, bioactive ansamycins from a hyper-arid desert *Streptomyces* sp. *J. Nat. Prod.* 74, 1491–1499. doi: 10.1021/np200320u

Reusser, F. (1969). Mode of action of albocycline, an inhibitor of nicotinate biosynthesis. *J. Bacteriol.* 100, 11–13.

Rho, J. R., Subramaniam, G., Choi, H., Kim, E. H., Ng, S. P., Yoganathan, K., et al. (2015). Gargantulide, A., a complex 52-membered macrolactone showing antibacterial activity from *Streptomyces* sp. *Org. Lett.* 17, 1377–1380. doi: 10.1021/acs.orglett.5b00068

Rigali, S., Titgemeyer, F., Barends, S., Mulder, S., Thomae, A. W., Hopwood, D. A., et al. (2008). Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*. *EMBO Rep.* 9, 670–675. doi: 10.1038/embor.2008.83

Roch, M., Gagetti, P., Davis, J., Ceriana, P., Errecalde, L., Corso, A., et al. (2017). Daptomycin resistance in clinical MRSA strains is associated with a high biological fitness cost. *Front. Microbiol.* 8:2303. doi: 10.3389/fmicb.2017.02303

Rolo, J., Worning, P., Nielsen, J. B., Bowden, R., Bouchami, O., Damborg, P., et al. (2017). Evolutionary origin of the Staphylococcal cassette chromosome mec (SCCmec). *Antimicrob. Agents. Chemother.* 16:e02302-16. doi: 10.1128/AAC.02302-16

Rosenthal, N., and Brown, S. (2007). The mouse ascending: perspectives for human-disease models. *Nat. Cell Biol.* 9:993. doi: 10.1038/ncb437

Rutherford, S. T., and Bassler, B. L. (2012). Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb. Perspect. Med.* 2:a012427. doi: 10.1101/cshperspect.a012427

Saravolatz, L. D., Markowitz, N., Arking, L., Pohlod, D., and Fisher, E. (1982). Methicillin-resistant *Staphylococcus aureus*: epidemiologic observations during a community-acquired outbreak. *Ann. Intern. Med.* 96, 11–16. doi: 10.7326/0003-4819-96-1-11

Saravolatz, L. D., Stein, G. E., and Johnson, L. B. (2011). Ceftaroline: a novel cephalosporin with activity against methicillin-resistant *Staphylococcus aureus*. *Clin. Infect. Dis.* 52, 1156–1163. doi: 10.1093/cid/cir147

Sawa, R., Takahashi, Y., Itoh, S., Shimanaka, K., Kinoshita, N., Homma, Y., et al. (1994). Aldecalmycin, a new antimicrobial antibiotic from *Streptomyces*. I. Taxonomy, fermentation, isolation, physico-chemical and biological properties. *J. Antibiot.* 47, 1266–1272. doi: 10.7164/antibiotics.47.1266

Schriever, C. A., Fernández, C., Rodvold, K. A., and Danziger, L. H. (2005). Daptomycin: a novel cyclic lipopeptide antimicrobial. *Am. J. Health Syst. Pharm.* 62, 1145–1158.

Ser, H.-L., Palanisamy, U. D., Yin, W.-F., Abd Malek, S. N., Chan, K.-G., Goh, B.-H., et al. (2015c). Presence of antioxidative agent, Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-in newly isolated *Streptomyces mangrovisoli* sp. nov. *Front. Microbiol.* 6:854. doi: 10.3389/fmicb.2015.00854

Ser, H.-L., Tan, W.-S., Ab Mutalib, N.-S., Yin, W.-F., Chan, K.-G., Goh, B.-H., et al. (2016c). Draft genome sequence of mangrove-derived *Streptomyces* sp. MUSC 125 with antioxidant potential. *Front. Microbiol.* 7:1470. doi: 10.3389/fmicb.2016.01470

Ser, H.-L., Tan, W.-S., Ab Mutalib, N.-S., Yin, W.-F., Chan, K.-G., Goh, B.-H., et al. (2017). Genome sequence of *Streptomyces mangrovisoli* MUSC 149T isolated from intertidal sediments. *Braz. J. Microbiol.* 49, 13–15. doi: 10.1016/j.bjm.2017.01.013

Ser, H. L., Ab Mutablib, N. S., Yin, W. F., Goh, B. H., Lee, L. H., and Chan, K. G. (2018a). Genome sequence of *Streptomyces antioxidans* MUSC164^{T} isolated from mangrove forest. *Prog. Microbes Mol. Biol.* 1, 1–3.

Ser, H. L., Ab Mutalib, N. S., Yin, W. F., Chan, K. G., Goh, B. H., and Lee, L. H. (2015b). Evaluation of antioxidative and cytotoxic activities of *Streptomyces pluripotens* MUSC 137 isolated from mangrove soil in Malaysia. *Front. Microbiol.* 6:1398. doi: 10.3389/fmicb.2015.01398

Ser, H. L., Law, J. W., Chaiyakunapruk, N., Jacob, S. A., Palanisamy, U. D., Chan, K. G., et al. (2016a). Fermentation conditions that affect clavulanic acid production in *Streptomyces clavuligerus*: a systematic review. *Front. Microbiol.* 7:522. doi: 10.3389/fmicb.2016.00522

Ser, H. L., Tan, L. T., Palanisamy, U. D., Abd Malek, S. N., Yin, W. F., Chan, K. G., et al. (2016b). *Streptomyces antioxidans* sp. nov., a novel mangrove soil actinobacterium with antioxidative and neuroprotective potentials. *Front. Microbiol.* 7:899. doi: 10.3389/fmicb.2016.00899

Ser, H. L., Tan, W. S., Ab Mutalib, N. S., Cheng, H. J., Yin, W. F., Chan, K. G., et al. (2015a). Genome sequence of *Streptomyces pluripotens* MUSC 135T exhibiting antibacterial and antioxidant activity. *Mar. Genomics* 24, 281–283. doi: 10.1016/j.margen.2015.09.010

Ser, H. L., Yin, W. F., Chan, K. G., Khan, T. M., Goh, G. H., and Lee, L. H. (2018b). Antioxidant and cytotoxic potentials of *Streptomyces gilvigriseus* MUSC26^{T} isolated from mangrove soil in Malaysia. *Prog. Microbes Mol. Biol.* 1, 1–12.

Sharma, D., Kaur, T., Chadha, B. S., and Manhas, R. K. (2011). Antimicrobial activity of actinomycetes against multidrug resistant *Staphylococcus aureus*, *E. coli* and various other pathogens. *Trop. J. Pharm. Res.* 10, 801–808. doi: 10.4314/tjpr.v10i6.14

Sharma, D., Mandal, S. M., and Manhas, R. K. (2014). Purification and characterization of a novel lipopeptide from *Streptomyces amritsarensis* sp. nov. active against methicillin-resistant *Staphylococcus aureus. AMB Express* 4, 1–9. doi: 10.1186/s13568-014-0050-y

Shi, J., Mao, N.-F., Wang, L., Zhang, H.-B., Chen, Q., Liu, H., et al. (2014). Efficacy of combined vancomycin and fosfomycin against methicillin-resistant *Staphylococcus aureus* in biofilms *in vivo*. *PLoS ONE* 9:e113133. doi: 10.1371/journal.pone.0113133

Shibabaw, A., Abebe, T., and Mihret, A. (2013). Nasal carriage rate of methicillin resistant *Staphylococcus aureus* among Dessie Referral Hospital health care

workers; Dessie, Northeast Ethiopia. *Antimicrob. Resist. Infect. Control* 2:25. doi: 10.1186/2047-2994-2-25

Shin, H. J., Lee, H. S., and Lee, D. S. (2010). The synergistic antibacterial activity of 1-acetyl-beta-carboline and beta-lactams against methicillin-resistant *Staphylococcus aureus* (MRSA). *J. Microbiol. Biotechnol.* 20, 501–505.

Sit, P. S., Teh, C. S., Idris, N., Sam, I.-C., Omar, S. F. S., Sulaiman, H., et al. (2017). Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) infection and the molecular characteristics of MRSA bacteraemia over a two-year period in a tertiary teaching hospital in Malaysia. *BMC Infect. Dis.* 17:274. doi: 10.1186/s12879-017-2384-y

Siti Junaidah, A., Suhaini, S., Mohd Sidek, H., Basri, D. F., and Zin, N. M. (2015). Anti-methicillin resistant *Staphylococcus aureus* activity and optimal culture condition of *Streptomyces* sp. SUK 25. *Jundishapur J. Microbiol.* 8, 1–7. doi: 10.5812/jjm.16784

Smith, J. M. B. (2018). "Desert," in *Encyclopedia Britannica*. Available online at: https://www.britannica.com/science/desert (Accessed June 21, 2018).

Socha, A. M., Garcia, D., Sheffer, R., and Rowley, D. C. (2006a). Antibiotic bisanthraquinones produced by a streptomycete isolated from a cyanobacterium associated with *Ecteinascidia turbinata*. *J. Nat. Prod.* 69, 1070–1073. doi: 10.1021/np050449b

Socha, A. M., Laplante, K. L., and Rowley, D. C. (2006b). New bisanthraquinone antibiotics and semi-synthetic derivatives with potent activity against clinical *Staphylococcus aureus* and *Enterococcus faecium* isolates. *Bioorg. Med. Chem.* 14, 8446–8454. doi: 10.1016/j.bmc.2006.08.038

Songia, S., Mortellaro, A., Taverna, S., Fornasiero, C., Scheiber, E. A., Erba, E., et al. (1997). Characterization of the new immunosuppressive drug undecylprodigiosin in human lymphocytes: retinoblastoma protein, cyclin-dependent kinase-2, and cyclin-dependent kinase-4 as molecular targets. *J. Immunol.* 158, 3987–3995.

Srinu, M., Murali Krishna Kumar, M., and Girija Shankar, G. (2013). Actinomycin "D" from marine sediment associated *Streptomyces capillispiralis* MTCC10471. *Asian J. Pharmaceut. Res. Health Care* 5, 16–23.

Stegger, M., Wirth, T., Andersen, P. S., Skov, R. L., De Grassi, A., Simões, P. M., et al. (2014). Origin and evolution of European community-acquired methicillin-resistant *Staphylococcus aureus*. *MBio.* 5:e01044-14. doi: 10.1128/mBio.01044-14

Stein, C., Makarewicz, O., Bohnert, J. A., Pfeifer, Y., Kesselmeier, M., Hagel, S., et al. (2015). Three dimensional checkerboard synergy analysis of colistin, meropenem, tigecycline against multidrug-resistant clinical *Klebsiella pneumonia* isolates. *PLoS ONE* 10:e0126479. doi: 10.1371/journal.pone.0126479

Strieker, M., Tanović, A., and Marahiel, M. A. (2010). Nonribosomal peptide synthetases: structures and dynamics. *Curr. Opin. Struct. Biol.* 20, 234–240. doi: 10.1016/j.sbi.2010.01.009

Sujatha, P., Bapi Raju, K. V., and Ramana, T. (2005). Studies on a new marine streptomycete BT-408 producing polyketide antibiotic SBR-22 effective against methicillin resistant *Staphylococcus aureus*. *Microbiol. Res.* 160, 119–126. doi: 10.1016/j.micres.2004.10.006

Sun, P., Maloney, K. N., Nam, S. J., Haste, N. M., Raju, R., Aalbersberg, W., et al. (2011). Fijimycins AC, three antibacterial etamycin-class depsipeptides from a marine-derived *Streptomyces* sp. *Bioorg. Med. Chem.* 19, 6557–6562. doi: 10.1016/j.bmc.2011.06.053

Sung, A. A., Gromek, S. M., and Balunas, M. J. (2017). Upregulation and identification of antibiotic activity of a marine-derived S*treptomyces* sp. via co-cultures with human pathogens. *Mar. Drugs* 15:250. doi: 10.3390/md15080250

Suzuki, N., Ohtaguro, N., Yoshida, Y., Hirai, M., Matsuo, H., Yamada, Y., et al. (2015). A compound inhibits biofilm formation of *Staphylococcus aureus* from *Streptomyces*. *Biol. Pharm. Bull.* 38, 889–892. doi: 10.1248/bpb.b15-00053

Takano, E. (2006). γ-Butyrolactones: *Streptomyces* signalling molecules regulating antibiotic production and differentiation. *Curr. Opin. Microbiol.* 9, 287–294. doi: 10.1016/j.mib.2006.04.003

Tan, L. T., Chan, K.-G., Khan, T. M., Bukhari, S. I., Saokaew, S., Duangjai, A., et al. (2017). *Streptomyces* sp. MUM212 as a source of antioxidants with radical scavenging and metal chelating properties. *Front. Pharmacol.* 8:276. doi: 10.3389/fphar.2017.00276

Tan, L. T., Chan, K. G., Chan, C. K., Khan, T. M., Lee, L. H., and Goh, B. H. (2018). Antioxidative potential of a *Streptomyces* sp. MUM292 isolated from mangrove soil. *Biomed. Res. Int.* 2018, 1–13. doi: 10.1155/2018/4823126

Tan, L. T., Ser, H.-L., Yin, W.-F., Chan, K.-G., Lee, L.-H., and Goh, B.-H. (2015). Investigation of antioxidative and anticancer potentials of *Streptomyces* sp. MUM256 isolated from Malaysia mangrove soil. *Front. Microbiol.* 6:1316. doi: 10.3389/fmicb.2015.01316

Tan, L. T.-H., Chan, K.-G., Lee, L.-H., and Goh, B.-H. (2016). *Streptomyces* bacteria as potential probiotics in aquaculture. *Front. Microbiol.* 7:79. doi: 10.3389/fmicb.2016.00079

Tanaka, T., Endo, T., Shimazu, A., Yoshida, R., Suzuki, Y., Otake, N., et al. (1970). A new antibiotic, multhiomycin. *J. Antibiot.* 23, 231–237. doi: 10.7164/antibiotics.23.231

Tang, C., Hoo, P. C. X., Tan, L. T. H., Pusparajah, P., Khan, T. M., Lee, L. H., et al. (2016). Golden needle mushroom: a culinary medicine with evidence-based biological activities and health promoting properties. *Front. Pharmacol.* 7:474. doi: 10.3389/fphar.2016.00474

Tavares, D. A., Sá-Leão, R., Miragaia, M., and De Lencastre, H. (2010). Large screening of CA-MRSA among *Staphylococcus aureus* colonizing healthy young children living in two areas (urban and rural) of Portugal. *BMC Infect. Dis.* 10:110. doi: 10.1186/1471-2334-10-110

Taylor, G., Kirkland, T., Kowalewska-Grochowska, K., and Wang, Y. (1990). A multistrain cluster of methicillin-resistant *Staphylococcus aureus* based in a native community. *Can. J. Infect. Dis.Med. Microbiol.* 1, 121–126. doi: 10.1155/1990/618630

Toh, S. M., Xiong, L., Arias, C. A., Villegas, M. V., Lolans, K., Quinn, J., et al. (2007). Acquisition of a natural resistance gene renders a clinical strain of methicillin-resistant *Staphylococcus aureus* resistant to the synthetic antibiotic linezolid. *Mol. Microbiol.* 64, 1506–1514. doi: 10.1111/j.1365-2958.2007.05744.x

Tsubakishita, S., Kuwahara-Arai, K., Sasaki, T., and Hiramatsu, K. (2010). Origin and molecular evolution of the determinant of methicillin resistance in Staphylococci. *Antimicrob. Agents Chemother.* 54, 4352–4359. doi: 10.1128/aac.00356-10

Tsuchida, T., Iinuma, H., Nishida, C., Kinoshita, N., Sawa, T., Hamada, M., et al. (1995). Tetrodecamycin and dihydrotetrodecamycin, new antimicrobial antibiotics against *Pasteurella piscicida* produced by *Streptomyces nashvillensis* MJ885-mF8: I. Taxonomy, fermentation, isolation, characterization and biological activities. *J. Antibiot.* 48, 1104–1109. doi: 10.7164/antibiotics.48.1104

Tsuji, N., Kobayashi, M., Terui, Y., and Tori, K. (1976). The structures of griseusins A and B, new isochromanquinone antibiotics. *Tetrahedron* 32, 2207–2210. doi: 10.1016/0040-4020(76)85133-2

Uchida, R., Hanaki, H., Matsui, H., Hamamoto, H., Sekimizu, K., Iwatsuki, M., et al. (2014). *In vitro* and *in vivo* anti-MRSA activities of nosokomycins. *Drug Discov. Ther.* 8, 249–254. doi: 10.5582/ddt.2014.01050

Uchida, R., Iwatsuki, M., Kim, Y. P., Ohte, S., Mura, S., and Tomoda, H. (2010). Nosokomycins, new antibiotics discovered in an *in vivo*-mimic infection model using silkworm larvae. I: fermentation, isolation and biological properties. *J. Antibiot.* 63, 151–155. doi: 10.1038/ja.2010.9

Udo, E. E., and Boswihi, S. S. (2017). Antibiotic resistance trends in methicillin-resistant *Staphylococcus aureus* isolated in Kuwait Hospitals: 2011-2015. *Med. Princ. Pract.* 26, 485–490. doi: 10.1159/000481944

Udo, E. E., Pearman, J. W., and Grubb, W. B. (1993). Genetic analysis of community isolates of methicillin-resistant *Staphylococcus aureus* in Western Australia. *J. Hosp. Infect.* 25, 97–108. doi: 10.1016/0195-6701(93)90100-e

Uematsu, H., Yamashita, K., Kunisawa, S., Fushimi, K., and Imanaka, Y. (2017). Estimating the disease burden of methicillin-resistant *Staphylococcus aureus* in Japan: retrospective database study of Japanese hospitals. *PLoS ONE* 12:e0179767. doi: 10.1371/journal.pone.0179767

Ullah, F., Malik, S. A., Ahmed, J., Shah, S., Ayaz, M., Hussain, S., et al. (2012). Investigation of the genetic basis of tetracycline resistance in *Staphylococcus aureus* from Pakistan. *Trop. J. Pharm. Res.* 11, 925–931. doi: 10.4314/tjpr.v11i6.8

Urgen, M., Kocabaş, F., Nalbantsoy, A., Kocabas, E. H., Uzel, A., and Bedir, E. (2010). Bioassay-guided isolation of antibacterial and cytotoxic compounds from the mesophilic actinomycete M-33-5. *Nat. Prod. Commun.* 5, 249–252.

Utsumi, R. (2017). Bacterial signal transduction networks via connectors and development of the inhibitors as alternative antibiotics. *Biosci. Biotechnol. Biochem.* 81, 1663–1669. doi: 10.1080/09168451.2017.1350565

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., et al. (2015). Global trends in antimicrobial use in food animals. *Proc. Natl. Acad. Sci. U.S.A.* 112, 5649–5654. doi: 10.1073/pnas.1503141112

Van Duijkeren, E., Hengeveld, P., Zomer, T. P., Landman, F., Bosch, T., Haenen, A., et al. (2015). Transmission of MRSA between humans and animals on duck and turkey farms. *J. Antimicrob. Chemother.* 71, 58–62. doi: 10.1093/jac/dkv313

Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *Pharm. Ther.* 40, 277–283.

Vijaya Kumar, E. K., Kenia, J., Mukhopadhyay, T., and Nadkarni, S. R. (1999). Methylsulfomycin, I., a new cyclic peptide antibiotic from a *Streptomyces* sp. HIL Y-9420704. *J. Nat. Prod.* 62, 1562–1564. doi: 10.1021/np990088y

Waksman, S. A., and Henrici, A. T. (1943). The nomenclature and classification of the actinomycetes. *J. Bacteriol.* 46, 337–341.

Wang, Q., Zhang, Y., Wang, M., Tan, Y., Hu, X., He, H., et al. (2017). Neo-actinomycins A and B, natural actinomycins bearing the 5H-oxazolo[4,5-b]phenoxazine chromophore, from the marine-derived *Streptomyces* sp. IMB094. *Sci. Rep.* 7:3591. doi: 10.1038/s41598-017-03769-8

Wardyn, S. E., Kauffman, L. K., and Smith, T. C. (2012). Methicillin-resistant *Staphylococcus aureus* in central Iowa wildlife. *J. Widl. Dis.* 48, 1069–1073. doi: 10.7589/2011-10-295

Wei, S., Fan, L., Wu, W., and Ji, Z. (2012). Two piperazic acid-containing cyclic hexapeptides from *Streptomyces alboflavus* 313. *Amino Acids* 43, 2191–2198. doi: 10.1007/s00726-012-1303-1

WHO (2017). *WHO Publishes List of Bacteria for Which New Antibiotics Are Urgently Needed*. WHO. Available online at: http://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

Wojtas, K. P., Riedrich, M., Lu, J. Y., Winter, P., Winkler, T., Walter, S., et al. (2016). Total Synthesis of Nosiheptide. *Angew. Chem. Int. Ed.* 55, 9772–9776. doi: 10.1002/anie.201603140

Woodruff, H. B. (2014). Selman, A. Waksman, winner of the 1952 Nobel Prize for physiology or medicine. *Appl. Envion. Microbiol.* 80, 2–8. doi: 10.1128/aem.01143-13

Wu, H., Moser, C., Wang, H. Z., Høiby, N., and Song, Z. J. (2015). Strategies for combating bacterial biofilm infections. *Int. J. Oral Sci.* 7:1. doi: 10.1038/ijos.2014.65

Xu, J., Wang, Y., Xie, S. J., Xu, J., Xiao, J., and Ruan, J. S. (2009). *Streptomyces xiamenensis* sp. nov., isolated from mangrove sediment. *Int. J. Syst. Evol. Microbiol.* 59, 472–476. doi: 10.1099/ijs.0.000497-0

Yamanaka, K., Ryan, K. S., Gulder, T. A., Hughes, C. C., and Moore, B. S. (2012). Flavoenzyme-catalyzed atropo-selective N, C-bipyrrole homocoupling in marinopyrrole biosynthesis. *J. Am. Chem. Soc.* 134, 12434–12437. doi: 10.1021/ja305670f

Yang, D., Teng, Q., Dong, L.-B., CrnovcIc, I., Huang, T., Ge, H et al.. (2017). Genome mining of *Streptomyces mobaraensis* DSM40847 as a bleomycin producer providing a biotechnology platform to engineer designer bleomycin analogues. *Org. Lett.* 19, 1386–1389. doi: 10.1021/acs.orglett.7b00283

Yang, J. C., Hwang, H.-S., Lee, H. J., Jung, S. Y., Ji, S. J., Oh, S.-H., et al. (2014). Distribution of vascular plants along the altitudinal gradient of Gyebangsan (Mt.) in Korea. *J. Asia-Pac. Biodivers.* 7, e40–e71. doi: 10.1016/j.japb.2014.03.008

Yu, L., Dai, H. F., Zhao, Y. X., Zuo, W. J., Dong, W. H., Mei, W. L., et al. (2013). Two new sesquiterpene derivatives from soil actinomycete *Streptomyces albospinus* 15-4-2. *Phytochem. Lett.* 6, 110–112. doi: 10.1016/j.phytol.2012.11.009

Yu, T. W., Bibb, M. J., Revill, W. P., and Hopwood, D. A. (1994). Cloning, sequencing, and analysis of the griseusin polyketide synthase gene cluster from *Streptomyces griseus*. *J. Bacteriol.* 176, 2627–2634. doi: 10.1128/jb.176.9.2627-2634.1994

Yuan, G. J., Li, P. B., Yang, J., Pang, H. Z., and Pei, Y. (2014). Anti-methicillin-resistant *Staphylococcus aureus* assay of azalomycin F5a and its derivatives. *Chin. J. Nat. Med.* 12, 309–313. doi: 10.1016/s1875-5364(14)60061-3

Yücel, S., and Yamaç, M. (2010). Selection of *Streptomyces* isolates from Turkish karstic caves against antibiotic resistant microorganisms. *Pak. J. Pharm. Sci.* 23, 1–6.

Zainal, N., Ser, H.-L., Yin, W.-F., Tee, K.-K., Lee, L.-H., and Chan, K.-G. (2016). *Streptomyces humi* sp. nov., an actinobacterium isolated from soil of a mangrove forest. *Antonie van Leeuwenhoek* 109, 467–474. doi: 10.1007/s10482-016-0653-1

Zhang, C., Guo, Y., and Chu, X. (2018). *In vitro* generation of Panton-Valentine leukocidin (PVL) in clinical methicillin-resistant *Staphylococcus aureus* (MRSA) and its correlation with PVL variant, clonal complex, infection type. *Sci. Rep.* 8:7696. doi: 10.1038/s41598-018-26034-y

Zhao, H., Lim, Y. H., Wong, F. T., Yeo, W. L., Ching, K. C., Lim, Y. W., et al. (2018). Auroramycin, a potent antibiotic from *Streptomyces roseosporus* by CRISPR-Cas9 activation. *ChemBioChem* 19, 1716–1719. doi: 10.1002/cbic.201800266

Zheng, L., Yan, M., Fan, F., and Ji, Y. (2015). The Essential WalK histidine Kinase and WalR regulator differentially mediate autolysis of *Staphylococcus aureus* RN4220. *J. Nat. Sci.* 1:e111.

Zhou, Z., Sun, N., Wu, S., Li, Y.-Q., and Wang, Y. (2016). Genomic data mining reveals a rich repertoire of transport proteins in *Streptomyces*. *BMC Genomics* 17:510. doi: 10.1186/s12864-016-2899-4

Zhu, H., Zhou, X., Lei, X., and Zhou, Q. (2013). Antagonist of MRSA by *Streptomyces* sp. SJY056 from soil of the three-river headwater region in China. *J. Food Agric. Environ.* 11, 420–422. doi: 10.1234/4.2013.4674

Zuma, A. V. P., Lima, D. F., Assef, A. P. D., Marques, E. A., and Leão, R. S. (2017). Molecular characterization of methicillin-resistant *Staphylococcus aureus* isolated from blood in Rio de Janeiro displaying susceptibility profiles to non-β-lactam antibiotics. *Braz. J. Microbiol.* 48, 237–241. doi: 10.1016/j.bjm.2016.09.016

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Genome Mining of *Streptomyces* sp. YIM 130001 Isolated From Lichen Affords New Thiopeptide Antibiotic

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Streptomyces bacteria are recognized as an important source for antibiotics with broad applications in human medicine and animal health. Here, we report the isolation of a new lichen-associating *Streptomyces* sp. YIM 130001 from the tropical rainforest in Xishuangbanna (Yunnan, China), which displayed antibacterial activity against *Bacillus subtilis*. The draft genome sequence of this isolate strain revealed 18 putative biosynthetic gene clusters (BGCs) for secondary metabolites, which is an unusually low number compared to a typical streptomycete. Inactivation of a lantibiotic dehydrogenase-encoding gene from the BGC presumed to govern biosynthesis of a thiopeptide resulted in the loss of bioactivity. Using comparative HPLC analysis, two peaks in the chromatogram were identified in the extract from the wild-type strain, which were missing in the extract from the mutant. The compounds corresponding to the identified peaks were purified, and structure of one compound was elucidated using NMR. The compound, designated geninthiocin B, showed high similarity to several 35-membered macrocyclic thiopeptides geninthiocin, Val-geninthiocin and berninamycin A. Bioinformatics analysis of the geninthiocin B BGC revealed its close homology to that of berninamycins.

Keywords: new *Streptomyces* sp. from lichen, antibacterial activity, genome mining, new thiopeptide antibiotic, berninamycins

INTRODUCTION

The successful use of any therapeutic agent is compromised by the potential development of tolerance or resistance to that compound from the time it is first deployed. This concerns all agents used in the treatment of bacterial, fungal, parasitic and viral infections, as well as cancer (Davies and Davies, 2010). Infectious diseases caused by bacteria are among the top causes of mortality in the world, and if appropriate actions are not implemented, it is estimated that by year 2050 up to 10 million people could die each year because of infections caused by antibiotic resistant pathogens (de Kraker et al., 2016). A successful curative treatment of such common, yet frequently deadly illnesses, as pneumonia and tuberculosis, could be soon history, if measures to tackle the antibiotic resistance

problems are not implemented. Apart from the reduction of overuse and misuse of antimicrobial agents, identification of new antibiotics still plays an essential role in controlling bacterial infections.

The majority of antibiotics used in medicine, veterinary practice and agriculture originate from actinomycete bacteria, predominantly from those belonging to the genus *Streptomyces* (Barka et al., 2015). *Streptomyces* are Gram-positive bacteria with genomes of high GC content, widely distributed in terrestrial as well as in aquatic ecosystems, and have a complex life cycle, with a multicellular mycelial growth (Labeda et al., 2012). Their life cycle starts with the germination of a spore that grows out to form vegetative hyphae, and further differentiates from vegetative mycelium into the aerial mycelium. Morphological differentiation in *Streptomyces* is an intricately regulated process, which typically correlates with production of secondary metabolites (SMs), such as antibiotics (van Wezel and McDowall, 2011).

Thiopeptide antibiotics are a prominent class of antimicrobials with potent activity against Gram-positive bacteria, produced primarily by *Streptomyces* species (Bagley et al., 2005). The interest in this family of antibiotics was recently renewed, since many members of this class show activity against various drug-resistant pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae* (PRSP), and vancomycin-resistant enterococci (VRE) (Bagley et al., 2005). Recent success in generation of new thiopeptide analogs with significantly improved pharmacological properties may pave the way to the introduction of these types of compounds in drug development pipelines aimed at antibiotic resistant pathogens (Just-Baringo et al., 2014).

Thiopeptides belong to the group of ribosomally synthesized and post-translationally modified peptides (RiPPs) possessing unique thiazole rings and a six-membered tri-or tetra-substituted nitrogen heterocycle that can be present in one of the three oxidation states: a piperidine, dehydropiperidine, or pyridine. Biosynthesis of thiopeptides follows a relatively straightforward pathway: a precursor peptide consisting of an N-terminal leader and a C-terminal core peptide, encoded by a single gene, is synthesized and undergoes post-translational modifications. All thiopeptide biosynthetic gene clusters (BGC) contain, in addition to the gene encoding the precursor peptide, a set of at least five genes that encode enzymes required for heterocyclization, dehydration, and the formation of the central six-membered nitrogen heterocycle (Morris et al., 2009; Burkhart et al., 2017).

Advances in genetics, genomics and computer science have changed the way of natural product discovery. The complete genome sequencing of *Streptomyces coelicolor A3(2)* and *Streptomyces avermitilis* in early 2000s revealed a yet unexplored biosynthetic potential of the members belonging to this genus (Bentley et al., 2002; Ikeda et al., 2003) and led to the idea of genome-guided prediction and isolation of SMs, the approach known as genome mining (Du and van Wezel, 2018). Comparison of BGCs encoded in newly sequenced genomes using software such as antiSMASH (Blin et al., 2017) helps to identify conserved genes for enzymes involved in biosynthetic pathways for known classes of compounds, and thus to predict types of SMs they specify. In some cases, even the structure of the target SM can be predicted to a certain extent (Winter et al., 2011). To make the production, isolation and identification of SM faster and easier, the genome mining approach is combined with comparative metabolic profiling (Winter et al., 2011). Many different techniques contributed to the successful characterization of new compounds, such as heterologous expression (Yamada et al., 2015), regulatory genes overexpression or inactivation (Jiang et al., 2015; Chen et al., 2017), as well as knock-out of core scaffold-biosynthetic genes (Tian et al., 2016).

Here, we present the results of a genome mining linked to the metabolite profiling that led to identification of a new analog of the thiopeptide antibiotic geninthiocin, geninthiocin B, and its BGC from a *Streptomyces* sp. isolated from a lichen. By combining the classical bioassay-based screening with genome mining we could rapidly connect the thiopeptide BGC with the molecule it specifies.

MATERIALS AND METHODS

Isolation and Identification of *Streptomyces* sp. YIM 13001

Streptomyces sp. YIM 130001 was isolated from a lichen *Lepidostroma yunnana* sp. nov. sample collected format the tropical rainy forest in Xishuangbanna (Yunnan, China) using YIM 212 medium (Raffinose 5.0 g, histidine 1.0 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, Agar 15 g, water to 1 L, pH 7.2∼7.4) and incubation temperature of 28°C. Genomic DNA of YIM 130001 was isolated as described below, and used as a template to PCR-amplify 16S rRNA gene fragment with previously described primers and protocol (Bredholdt et al., 2007). The resulting DNA fragment was sequenced, and the sequence deposited in the GenBank under accession number MH532527. Phylogenetic analysis of the 16S rDNA sequence was performed using software MEGA 7.0 (Kumar et al., 2016).

Genome Sequencing and Analyses

The genomic DNA isolation was done from a culture grown in 50 mL of 3% Tryptone Soya Broth medium (TSB, OXOID, United Kingdom) inoculated with 50 μL of freshly prepared spore suspension of YIM 130001 (20% glycerol, v/v) and incubated in 250 mL baffled flasks at 28°C, 250 rpm overnight. The genomic DNA was isolated using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, United States) as described previously (Schaffert et al., 2016). For the genome sequencing, chromosomal DNA was used to generate a TruSeq PCR-free library that was sequenced on an Illumina MiSeq system in a 2 × 300 nt run. A total of 348.8 Mbp sequence data (43.5 × coverage) were assembled using NEWBLER version 2.8 (Roche), resulting in 59 scaffolds containing 80 contigs. Gene prediction and annotation were performed with PROKKA SOFTWARE (Seemann, 2014), the relevant genome features are listed in **Supplementary Table S1**.

This Whole Genome Shotgun project has been deposited at the DDBJ/ENA/GenBank under the accession number QODG00000000. The version described in this paper is version QODG01000000.

Generation of Recombinant Bacterial Strains, Plasmids, and General Growth Conditions

All routine DNA standard techniques, cloning methods, and plasmid transformation into *Escherichia coli* were performed as described in Sambrook et al. (1989). PCR fragment amplifications were done with Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, United States) using oligonucleotides listed in **Supplementary Table S2**. Plasmids and bacterial strains used or constructed during this study are represented in **Supplementary Table S3**.

Escherichia coli strains were grown in Luria–Bertani (LB) broth or on LB agar, supplemented with chloramphenicol (25 $\mu g\ mL^{-1}$), apramycin (100 $\mu g\ mL^{-1}$), kanamycin (25 $\mu g\ mL^{-1}$). XL1-blue strain was used for general cloning, ET12567 (pUZ8002) was used for intergeneric conjugative transfer of plasmids to *Streptomyces* as described before (Flett et al., 1997).

To inactivate the gene *genB*, a 1055 bps internal fragment from *genB* gene was amplified with primers genB_*Hind*III/genB_*Eco*RI primer pair from YIM 130001 genomic DNA and cloned into the 3.1 kb *Eco*RI/*Hind*III fragment of the vector pSOK201 (**Supplementary Table S3**). The generated plasmid pC1_KN was transferred into the YIM 130001 strain via conjugation and resulting *genB*-distruption mutant (YIM 130001/KN) was verified by PCR using genB_fwd/genB_rev primer pair (**Supplementary Figure S1C**). Wild-type strain YIM 130001 harboring empty vector pSOK804 was used as a control.

Strain Fermentation and Extraction on a Small-Scale

The production of secondary metabolites by YIM 130001 was tested in the following liquid media: 5010 (g/L: sucrose 30.0, $NaNO_3$ 2.0, KH_2PO_4 1.0, $MgSO_4 \times 7\ H_2O$ 0.5, KCl 0.5, $FeSO_4 \times 7\ H_2O$ 0.01, pH 7.8): 5288 (g/L: glycerol 15.0, soy meal 10.0, NaCl 5.0, $CaCO_3$ 1.0, $CoCl_2 \times 6\ H_2O$ 0.001, pH 7.8); 5321 (g/L: peptone 10.0, glucose 20.0, $CaCO_3$ 2.0, $CoCl_2 \times 6\ H_2O$ 0.001, pH 7.8); 5333 (g/L: yeast extract 4.0, soluble starch 15.0, K_2HPO_4 1.0, $MgSO_4 \times 7\ H_2O$ 0.5, pH 7.8); and SM17 (Zettler et al., 2014). Based on the results from bioassay and HPLC, SM17 medium was chosen as most suitable for the production of antibacterial compound.

The pre-culture of strains YIM 130001, YIM 130001/pSOK806, and YIM 130001/KN were prepared from inoculation of 10 mL TSB medium, containing the 50 $\mu g\ mL^{-1}$ apramycin for recombinant strains, with 50 μL of spore-suspension in 250 mL baffled flasks and cultivation for 24 h at 250 rpm, 30°C. Fifty milliliter of SM17 medium without apramycin was inoculated with 5% of pre-culture and was cultivated in 250 mL-baffled flasks for 5 days, 250 rpm at 30°C. The fermented broth was extracted with 50 mL of butanol (100%) by 250 rpm, at 30°C for 2 h. The separation of organic phase was done via centrifugation at 7000 rpm for 20 min Next, butanol was removed from the extract by rotor-evaporation at 45°C, yielding oily crude extract which was dissolved in 1 mL methanol (100%), filtrated through 0.22°μm sterile syringe filter (ThermoFisher Scientific) and used for bioassay and HPLC analysis.

Bioassay of Antimicrobial Activity

The antibacterial activity of crude extract was tested by disk diffusion assay. Petri dishes containing 25 mL of nutrient agar media, depending on the bacterial species, were seeded with bacterial suspensions from glycerol stock (20% glycerol, v/v). The sterile Geade AA disk of Whatmann filter (6 mm in diameter) was impregnated with 15 μL of crude extract in methanol, dried for 15 min in the sterile bench and placed on the surface of the seeded agar plate. After 18 h of incubation the inhibition zone around the disk was measured. The antimicrobial properties of extracts were tested against *Bacillus subtilis*, *E. coli* XL1 Blue, and *Candida albicans* ATCC 10231. The strains *B. subtilis* and *E. coli* XL1 Blue, were grown in LB-medium for preparation of stock solutions (20% glycerol, v/v). The strain *C. albicans* ATCC 10231 was grown at 30°C for 18 h in M19 liquid medium (for glycerol stock solution, 20 % glycerol, v/v), or on M19 agar plates for bioassays (Hakvåg et al., 2008).

Analytical Metabolomics Profiling of Extracts

Analytical RP HPLC was carried out with an Agilent 1290 HPLC system equipped with a diode-array UV detector (DAD) at 192–600 nm, and with the analytical column ZORBAX Eclipse XDB-C18 4.6 × 150 mm, 5 μm (Agilent). Elution was carried out with water containing formic acid (0.1%, v/v) as solvent A and acetonitrile as solvent B, with flow rate of 1 mL/min. The elution program used was: 0–47.5 min: linear gradient from 5 to 95% of solvent B, 47.5–50 min: isocratic 100% solvent B, 50–65 min: isocratic 5% solvent B.

Production of Antimicrobial Compounds in a Bioreactor

Batch cultivation of YIM 130001 wild type strain was performed in 3 L bioreactor (BioFlo/CelliGen 115 Fermentor, New Brunswick Scientific) with a 1.5 L-working volume. The pre-culture was prepared by inoculation of 10 mL TSB medium with 50 μL of spore-suspension in 250 mL baffled flasks and cultivation for 24 h at 250 rpm, 30°C. Next, two 250 mL baffled flasks containing 50 mL TSB medium were inoculated with 5% of pre-culture and cultivated for 48 h at 250 rpm at 30°C to obtain the pre-culture for bioreactor inoculation. One point five liter of SM17 medium in bioreactor were inoculated with 75 mL of pre-culture and 1 mL of 10% silicone antifoam (stock solution: 30% in H_2O, Sigma Aldrich) was added at the start of the fermentation. The dissolved oxygen level (DO) was kept above 40% using a stirring cascade from 300 to 1000 rpm, aeration rate from bioreactor was 0.75 vvm. The batch cultivation was

carried out for 7 days at 30°C, without controlling pH value and foam formation in bioreactor. To prevent the foam formation, additional 1 mL of 10% silicone antifoam solution was added manually on fermentation days 3 and 5.

Extraction and Purification of Antimicrobial Compounds

After 7 days of fermentation approximately 1.5 L of whole fermentation broth with the dry cell mass 58 g L^{-1} were extracted with 100% butanol (1:1 v/v), at 30°C and 250 rpm for 2 h. The separation of organic phase was done by centrifugation at 9000 rpm for 20 min. The oily crude extract (14.06 g) was obtained after removing the solvent in the rotavapor at 45°C. To remove the oily substances from the sample, to the crude extract was supplemented with 200 mL of 100% *n*-pentane, the mixture was well stirred and after 2 h of sedimentation at room temperature, the *n*-pentane phase was decanted and the pellet air-dried, yielding 8.06 g of air-dried material. Next, the pellet was dissolved in 50 mL of 100% methanol. The sample was purified using flash chromatography by gravity flow with silica gel 60 (0.063–0.200 mm, 70–230 mesh ASTM, Merck) as stationary phase. Mobile phases used were: fraction 1, ethyl acetate (100%), fraction 2, ethyl acetate/methanol (9:1, v/v), and fraction 3, ethyl acetate/methanol (8:2). All extractions and purifications steps were tested for bioactivity against *B. subtilis* and analytical RP HPLC as describe above. The fraction 2 (ca. 500 mL) showed the activity in the bioassay and the presence of expected peaks at 22 and 25 min in RP HPLC. The organic solvents from the fraction 2 were removed in rotavapor at 45°C, yielding 125 mg of material, which was dissolved in 1 mL of 100% methanol and subjected to preparative HPLC.

Preparative HPLC was carried out with an Agilent 1260 HPLC system equipped with a DAD and binary pump. Five hundred microliter of injected sample was separated on preparative column ZORBAX XDB-C18 21.2 × 150 mm 5 μm (Agilent). Elution was carried out with water containing formic acid (0.1%, v/v) as solvent A and acetonitrile as solvent B, with flow rate of 20 mL/min. The elution program used was: 0–47.5 min: linear gradient from 5 to 95% of solvent B, 47.5–50 min: isocratic 100% solvent B, 50–65 min: isocratic 5% solvent B. The fractions on retention time from 21.8 to 22.2 min (for compound 1) and 25.4–25.8 min (compound 2) were collected and freeze dried. Before freeze drying, the 15 μL of each sample was tested for bioactivity against *B. subtilis*.

Flow Injection Analysis – qTOF Mass Spectrometry

Analyses were performed with an ACQUITY I-class UHPLC system, operating in flow injection analysis mode (FIA), coupled to a Synapt G2Si HDMS mass spectrometer (Waters, Milford, MA, United States) equipped with an ESI source operating in negative or positive mode.

Flow injection analysis analysis was performed by operating the UHPLC in bypass, in order to direct the flow passed the column compartment, and directly to the mass spectrometer. A mobile phase consisting of 100% methanol was used, and a linear flow gradient was programmed. The flow rate was constant at 0.150 mL min^{-1} for 0.10 min, then reduced to 0.030 min until 1.50 min, then increased to 0.200 mL min^{-1} until 1.60 min, and finally increased to 0.800 mL min^{-1} until 1.85 min. The system was equilibrated for additionally 0.25 min at 0.150 mL/min. The total run time was 2.0 min. The injection volume was set to 2 μL, and needle wash solvent consisted of 10% water in pure methanol. Sample compartment was cooled to 10°C before introducing the sample to the holder.

Mass spectrometric (MS) analyses were performed under constant ESI conditions. The capillary voltage, cone voltage and source offset voltage in negative and positive mode were set at −2.5 kV/3.0 kV, −30 V/30 V, and −40 V/40 V, respectively. The source temperature was maintained at 120°C, desolvation gas temperature 200°C, and desolvation gas flow rate was set at 800 L h^{-1}. The cone gas flow rate was fixed at 50 L h^{-1} and the nebulizer gas flow maintained at 6.0 bar. The mass spectrometer was operated in scan mode. The resolution mode was set to continual high resolution. Scan time was 0.5 s, and inter-scan delay 0.015 s. Mass range acquired was 50–2000 Da, the same range as the valid calibration performed with Na-formate immediately before analysis using IntelliStart.

During the FIA analysis, a lockmass flow of 10 μL min^{-1} of leucine enkephalin (1 ng mL^{-1}) was infused into the ion source to correct the mass axis on the fly. The lockmass flows from a separate capillary, and the capillary voltage of this capillary was set to 2.5 kV (negative or positive, depending on the operation mode).

UHPLC/FIA–qTOF data were acquired and processed using MassLynx software (v4.1). Elemental composition and isotope model features in MassLynx were used for identification.

NMR-Based Structure Elucidation

The sample was prepared by dissolving 1.5 mg of compound in 160 μL DMSO-d6 (99.9% d) and transferred to a 3 mm NMR tube. All homo-and heteronuclear NMR spectra were recorded on a Bruker Ascend 800 MHz Avance III HD NMR spectrometer (Bruker BioSpin AG, Fälladen, Switzerland) equipped with 5 mm cryogenic CP-TCI probe. All NMR spectra were recorded at 25°C. Shifts were determined relative to TMS, using the residual DMSO signals for spectra calibration. For chemical shift assignment of compound, the following spectra were recorded: 1D 13C, 2D double quantum filtered correlation spectroscopy (DQF-COSY), 2D rotating-frame nuclear Overhauser effect correlation spectroscopy (ROESY) with 250 ms mixing time, 2D ^{13}C heteronuclear single quantum coherence (HSQC) with multiplicity editing, 2D ^{13}C HSQC-[^{1}H,^{1}H]TOCSY with 70 ms mixing time on protons, 2D heteronuclear multiple bond correlation (HMBC) with BIRD filter to suppress first order correlations and 2D ^{15}N HSQC. The spectra were recorded, processed, and analyzed using TopSpin 3.5 software (Bruker BioSpin AG, Fälladen, Switzerland). Details on NMR-based

structure elucidation and recorded NMR spectra are given in **Supplementary Material**.

RESULTS AND DISCUSSION

Isolation and Preliminary Characterization of *Streptomyces* sp. YIM 130001

An actinomycete strain YIM 130001 was isolated from the lichen *Lepidostroma yunnana* sp. nov., collected at the rainy forest environment in Xishuangbann, province Yunnan in China. Based on the phylogenetic analysis analysis, this isolate was identified as *Streptomyces* sp. YIM 130001, with closest 16S rDNA sequence identity (98%) to that of the type strain *Streptomyces malachitospinus* NBRC 101004 (**Figure 1**). The best database match to the non-type strain was to the endophytic *Streptomyces* sp. KLBMP1330 isolated, among other actinomycetes, from the plant *Dendranthema indicum* (Linn.) Des Moul collected in East China (Xing et al., 2014).

The ability of YIM 130001 to produce antimicrobial compounds was tested by cultivating it in different types of media and testing culture extracts in bioassays (see "Materials and Methods" for details). It was shown that the *n*-butanol extract from the culture grown in SM17 medium exhibits bioactivity against *B. subtilis*, while no effect was shown on the growth of *E. coli* and *C. albicans*.

Analysis of the *Streptomyces* sp. YIM 130001 Genome

Due to the fact that *Streptomyces* sp. YIM 130001 appeared to be phylogenetically diverse from the closest type strain and was bioactive, we decided to obtain its genome sequence in order to reveal secondary metabolite biosynthesis potential of this strain. The high-quality draft genome sequence of *Streptomyces* sp. YIM 130001 revealed a single linear chromosome of 8,025,327 bp, with no plasmids, and a G+C content of 70.75% (see **Supplementary Table S1**, **Supplemental Material**, for additional genome features). Analysis of the YIM 130001 genome sequence using antiSMASH 4.1.0 software (Blin et al., 2017) revealed 17 putative BGCs for the biosynthesis of secondary metabolites (**Table 1**), which is an unusually low number compared to a typical streptomycete with a 8-Mb genome. Whole genome sequencing of multiple *Streptomyces* species typically reveals between 25 and 50 BGCs per genome (Katz and Baltz, 2016).

Seven of the clusters identified in YIM 130001 genome contain genes encoding modular enzymes, such as polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPs). Cluster 17, a PKS type I BGC, was predicted to govern biosynthesis of a putative enediyene (**Table 1**). Up to now, only 12 natural enediyenes were characterized and two of them are approved as anticancer drugs and at least other four are in various stages of drug development, making this compound class attractive for drug discovery (Gredičak and Jerić, 2007). Cluster 10 was predicted to contain NRPS-encoding genes, which putative product is a griseobactin/bacillibactin-like siderophore. Four clusters were annotated as hybrid BGCs: the putative products from clusters 9 and 11 could not be predicted, while the BGC 12 has a homology to the cluster specifying streptogramins biosynthesis. Streptogramin-like antibiotics occur as two structurally different compounds that act synergistically to inhibit ribosomal peptidyl transfer during bacterial protein biosynthesis, while separately they exhibit only moderate antibacterial activity (Di Giambattista et al., 1989). antiSMASH initially predicted cluster 14 as being of terpene-NRPS hybrid type. However, closer inspection using BLAST and genetic context suggested that this locus is likely to comprise two separate BGCs, 14a for a carotenoid, and for 14b non-ribosomally synthesized peptide (**Table 1**).

In addition to the PKS- and NRPS- containing gene clusters, three BGCs for terpenoid biosynthesis were identified, one of them presumably responsible for hopanoids synthesis (**Table 1**). Hopanoids have a condensing effect on biological membranes due to their rigid ring structures, which help to stabilize the membranes once integrated. In *S. coelicolor* hopanoids are synthesized during formation of aerial hyphae to alleviate stress

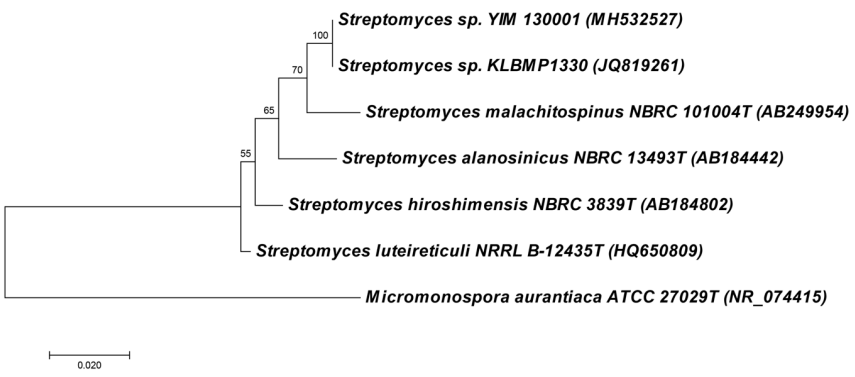

FIGURE 1 | Molecular phylogenetic analysis of 16S rRNA gene fragments of *Streptomyces* sp. YIM 130001 and related species by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. Evolutionary analyses were conducted in MEGA7 (19).

TABLE 1 | Secondary metabolite BGCs in *Streptomyces* sp. YIM 130001 predicted with antiSMASH 4.1.0 followed by manual curation with Protein BLAST and MIBiG algorithms.

Cluster no.	Cluster type	Best database cluster hit	Putative product
1	Thiopeptide	*Streptomyces atroolivaceus* NRRL ISP-5137	Geninthiocin B
2	T3pks	no plausible hits	Polyketide
3	Terpene	no plausible hits	Terpenoid
4	Terpene	*Streptomyces mobaraensis* NBRC 13819	Geosmin
5	Ectoine	*Streptomyces anulatus*	Ectoine
6	Bacteriocin	*Streptomyces turgidiscabies* Car8	Bacteriocin-like
7	Bacteriocin	no plausible hits	Bacteriocin-like
8	Bacteriocin	no plausible hits	Likely false
9	T1pks-Otherks	*Streptomyces albus* BK3-25	Polyketide, glycosylated
10	Nrps	Many *Streptomyces* spp.	Griseobactin/ Bacillibactin-likesiderophore
11	Nrps-Linaridin	no plausible hits	NRPS-PKS hybrid product
12	T3pks-Transatpks-Terpene-Otherks-Nrps	*Streptomyces* sp. CB01635	Streptogramins
13	Terpene	*Streptomyces* sp. Root55	Hopene
14a 14b	Terpene Nrps	*Streptomyces phaeoluteigriseus* *Streptomyces* sp. Wb2n-11	Isorenieratene NRS peptide
15	Siderophore	Many *Streptomyces* spp.	Acinetoferrins
16	Lassopeptide	Many *Streptomyces* spp.	Putative Class II lasso peptide
17	T1pks	*Streptomyces sp.* CB02414	Enediyne

in aerial mycelium by diminishing water permeability across the membrane (Poralla et al., 2000). Cluster 16 was predicted to encode a putative lassopeptide, whereby manual inspection with Protein BLAST and MIBiG algorithms showed similarity to BGCs in many *Streptomyces* spp. The genes from cluster 15 showed high similarity (59–93%) to the genes from many other *Streptomyces* spp. that encode putative siderophore actinoferrin involved in iron acquisition (Sandy and Butler, 2009). A likely product from cluster 5 was predicted as ectoine, its BGC has high similarity with its counterpart in *Streptomyces anulatus*. Ectoines are most commonly found osmolytes in *Streptomyces* and help microorganisms to cope with osmotic stress (Sadeghi et al., 2014). Three BGCs were predicted to encode bacteriocins-like compounds. These clusters showed high similarity to other conserved clusters, the metabolic products of which are not known. Cluster 8, due to sequence gaps within the genome, was likely falsely annotated as a BGC (**Table 1**). The cluster 1 was predicted by antiSMASH software as a thiopeptide BGC, and after the manual Protein BLAST search, its high homology to the berninamycin gene cluster (Malcolmson et al., 2013) was noticed.

Analysis of Thiopeptide BGC and Identification of the Cognate Bioactivity

Among the clusters identified in the *Streptomyces* sp. YIM 130001 genome, cluster 1 was predicted by antiSMASH to specify biosynthesis of a thiopeptide, and manual inspection of encoded gene products using BLAST predicted that it could yield a berninamycin-like analog (**Table 1**). Due to the fact that the members of thiopeptide family are known for their antimicrobial activities against Gram-positive bacteria (Just-Baringo et al., 2014), we assumed that the product of cluster 1 might be responsible for the activity against *B. subtilis* in crude extract. In order to investigate this, *genB* gene encoding a putative lantibiotic dehydratase (**Table 2**) was inactivated via insertion of the pC1_KN vector into its coding region, yielding recombinant strain YIM 130001/KN. The correct insertion of the knock-out vector was subsequently confirmed by PCR (**Supplementary Figure S1**, **Supplemental Material**). The crude butanol extract from recombinant strain YIM 130001/KN was tested for antibacterial activity against *B. subtilis* and its metabolite profile was analyzed with analytical RP-HPLC in comparison with a butanol extract from the wild-type strain (**Figure 2**). YIM 130001/KN-extract, in contrast to that from the wild-type strain, failed to exhibit antibacterial activity against *B. subtilis*. Furthermore, using comparative analytical RP-HPLC it was shown that in the extract of YIM 130001/KN strain two chromatographic peaks at ca 22 and 25 min disappeared (**Figure 2**), indicating that those two peaks might correlate with both the antibacterial activity and the cluster 1 product.

Purification and Structure Elucidation of Bioactive Compounds Produced by *Streptomyces* sp. YIM 130001

An up-scaled fermentation of YIM 130001 in medium SM17, followed by multiple steps of silica gel and preparative C-18 chromatography (see "Materials and Methods" for details), afforded two compounds, compound 1 (1.5 mg) and compound 2 (0.1 mg), both with antibacterial activities against *B. subtilis* (data not shown). During this study, the optimal conditions to elicit production of compound 2 were not developed, and a sufficient amount could not be purified to perform a proper MS/MS analyses or structure elucidation via NMR. The molecular masses of compounds 1 and 2 were determined on the basis of qTOF mass spectrometry, that afforded the masse-to-charge ratios of 1062.2965 *m/z* and 1046.3030 *m/z* (1061.2965 *m/z* and 1045.3030 *m/z* in negative mode, **Supplementary Figure S2**), respectively.

The NMR structure elucidation of compound 1 showed clearly that the molecule contained a free primary amido-group (N-56, **Supplementary Figure S3**) and eight peptide bonds (**Supplementary Table S4**). In addition, five terminal double bonds indicated five didehydroalanine residues. Threonine residue, didehydrobutyrine and hydroxy valine were also easily

TABLE 2 | Deduced functions of ORFs in and around the geninthiocin B biosynthetic gene cluster.

Gene no.	Size of protein[a]	Protein homolog and its source	Accessions number	Identity (%)	Proposed function
orf 1	1304	β'-subunit DNA-directed RNA polymerase; *Streptomyces scopuliridis*	WP_030351424.1	97	Transcription of DNA into RNA
orf 2	149	*N*-acetyltransferase; *Streptomyces eurocidicus*	WP_102917203.1	71	Transfer of acetyl groups from acetyl-CoA
orf 3	123	S12; Many *Streptomyces* sp.	WP_007265893.1	99	30S ribosomal protein
orf 4	156	S7; *Streptomyces tsukubensis*	WP_077968976.1	99	30S ribosomal protein
orf 5	706	EF-G; *Streptomyces rapamycinicus* NRRL 5491	AGP55834.1	93	Elongation factor
orf 6	397	EF-Tu; *Streptomyces tsukubensis*	WP_077968978.1	98	Elongation factor
genE1	234	hypothetical protein SAMN05444921_112167; *Streptomyces wuyuanensis*	SDM72766.1	76	Thiazoline dehydrogenase
genE2	543	TpaE; *Streptomyces wuyuanensis*	WP_093656415.1	71	Thiazoline dehydrogenase
genG1	212	hypothetical protein; *Streptomyces atroolivaceus*	WP_051709133.1	71	Bacteriocin biosynthesis cyclodehydratase domain
genG2	448	hypothetical protein; *Streptomyces atroolivaceus*	WP_051709136.1	82	Thiazole-forming peptide maturase
genD	357	BerD; *Streptomyces bernensis*	AGN11669.1	61	Putative pyridine-forming enzyme
genA	46	thiocillin/thiostrepton family thiazolyl peptide; *Streptomyces wuyuanensis*	WP_094030676.1	86	Geninthiocin B structural gene
genB	885	hypothetical protein; *Streptomyces atroolivaceus*	WP_051709141.1	78	Lanthibiotic dehydratase N-terminus
genC	320	hypothetical protein; *Streptomyces atroolivaceus*	WP_078597759.1	76	Lanthibiotic dehydratase C-teminus
genH	400	cytochrome P450; *Streptomyces atroolivaceus*	WP_033297595.1	83	Cytochrome P450
genI	114	hypothetical protein; *Streptomyces wuyuanensis*	WP_093656403.1	86	C-terminal amide-forming enzyme
orf 7	102	S10; Many *Streptomyces* sp.	WP_014054162.1	100	30S ribosomal protein
orf 8	214	L3; *Streptomyces varsoviensis*	WP_030890763.1	98	50S ribosomal protein
orf 9	215	L4; *Streptomyces indicus*	WP_093613746.1	94	50S ribosomal protein
orf 10	106	L23; *Streptomyces indicus*	WP_093613748.1	96	50S ribosomal protein

[a]*Numbers refer to amino acid residues.*

identifiable. Identification of cyclic structures of thiazol, oxazol, and pyridine rings were somewhat more challenging because of relatively large number of non-protonated carbons/heteroatoms and distant protons. By combining data from DQF-COSY, HMBC, HSQC, ROESY, and ^{15}N-HSQC, the connectivity between the previously identified fragments was established. Protons from two OH groups could not be detected and assigned, probably because of their strong involvement in H-bonding. The shift assignments of ^{1}H, ^{13}C, and ^{15}N (protonated) are shown in **Supplementary Table S4**. Based on NMR results, the molecular formula of compound 1 was determined as $C_{47}H_{46}N_{14}O_{14}S$, and the elucidated chemical structure indicated it closely resembles thiopeptide geninthiocin (Yun et al., 1994), albeit differing from the latter at the C-terminal part (**Figure 3**). Consequently, compound 1 was designated as geninthiocin B. The scaffolds from geninthiocin-like thiopeptides (geninthiocin and val-geninthiocin) are very similar to berninamycins (e.g., berninamycin A), which differ by one additional methyl-group on the oxazol ring (**Figure 3**). At the same time, geninthiocin B differs from two up to now characterized geninthiocins (Yun et al., 1994; Sajid et al., 2008) in lacking one Dha-residue on its C-terminus tail region. The macrocycle of newly discovered geninthiocin B is 35-membered, and because of its tri-substituted pyridine ring, it belongs to the series *d* thiopeptides (Just-Baringo et al., 2014).

Analysis of the Geninthiocin B Biosynthesis Gene Cluster

The geninthiocin B biosynthesis gene cluster spans ca. 23.4 kb and contains 20 open reading frames (**Table 2**, **Figure 4**). BLAST analysis of this thiopeptide BGC showed similarity to *Streptomyces atroolivaceus* and *Streptomyces wuyuanensis* uncharacterized clusters. Its genes, especially their organization, were predicted to have a similarity to the BGC for berninamycin biosynthesis (**Figure 4**, Malcolmson et al., 2013). However, the comparison of the GenA core peptide sequence showed a closer similarity to geninthiocin isolated from *Streptomyces* sp. DD84 (Yun et al., 1994) and val-geninthiocin, originated from *Streptomyces* sp. RSF18 (Sajid et al., 2008). Up to now, only a few thiopeptides with 35-membered macrocycles have been described: geninthiocin, berninamycin, sulfomycin

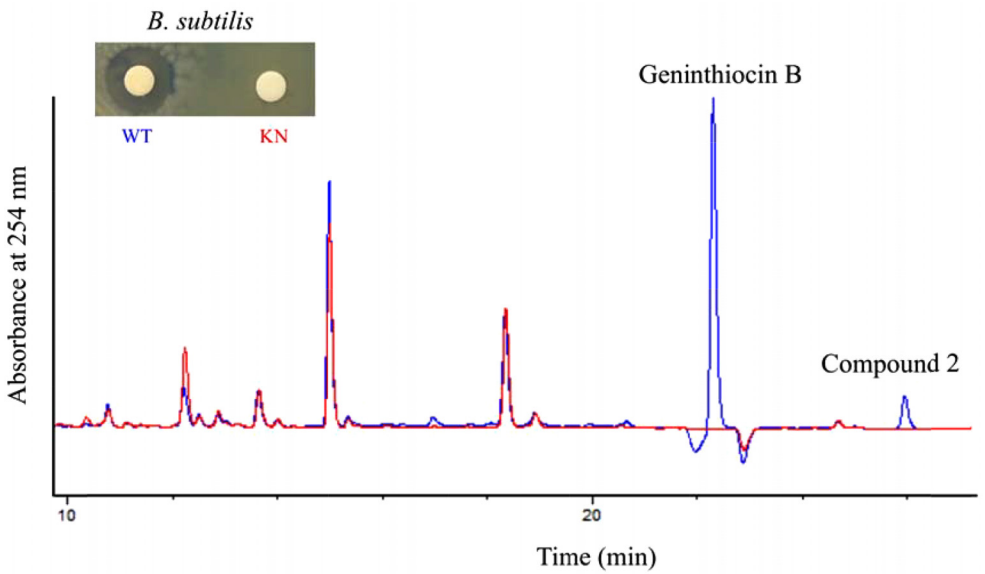

FIGURE 2 | Comparative RP-HPLC chromatogram at 254 nm of the crude extracts from *Streptomyces* sp. YIM 130001 wild type (WT, in blue) and the strain YIM 130001/KN with deactivated *genB* gene (KN, in red), with corresponding bioassay against *B. subtilis*.

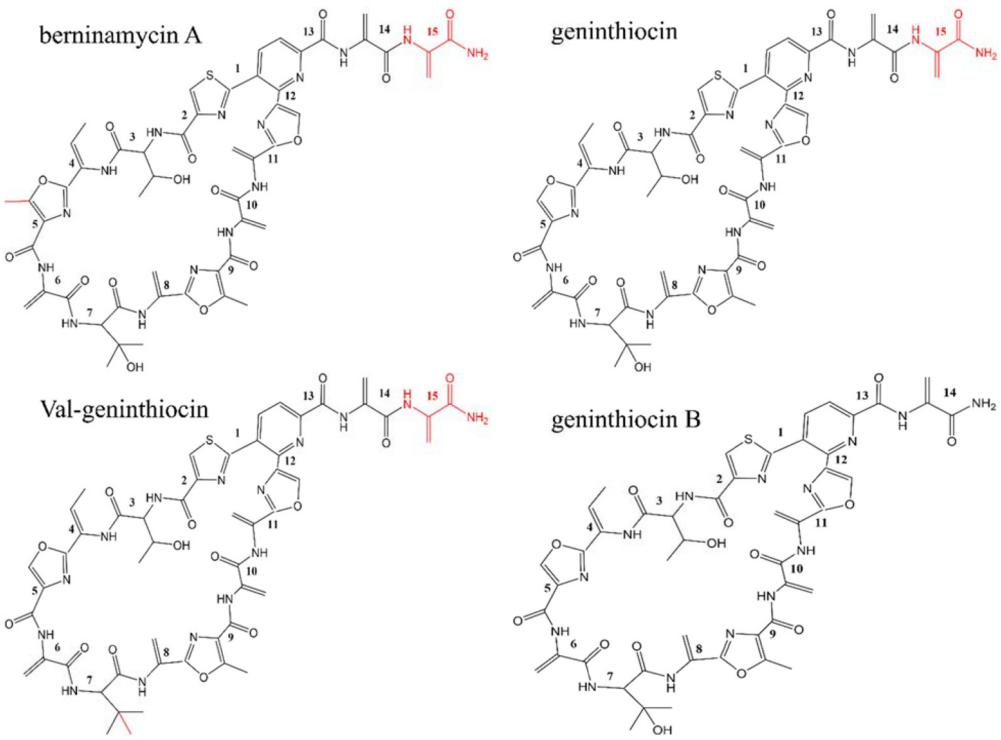

FIGURE 3 | Chemical structures of the newly isolated geninthiocin B and its analogs berninamycin A, geninthiocin, and val-geninthiocin.

(Abe et al., 1988), thioplabin (Ohyama et al., 2002), and TP-1161 (Engelhardt et al., 2010a), and respective BGCs were reported for TP-1161 and berninamycin (Engelhardt et al., 2010b; Malcolmson et al., 2013). The newly isolated geninthiocin B is another member of this thiopeptide group and its identified BGC helps to understand its biosynthesis based on the homology to that of berninamycin (**Figure 4**).

Burkhart et al. (2017) suggested that due to the structural similarity between geninthiocin and berninamycin, the biosynthesis of those two compounds is likely to be very

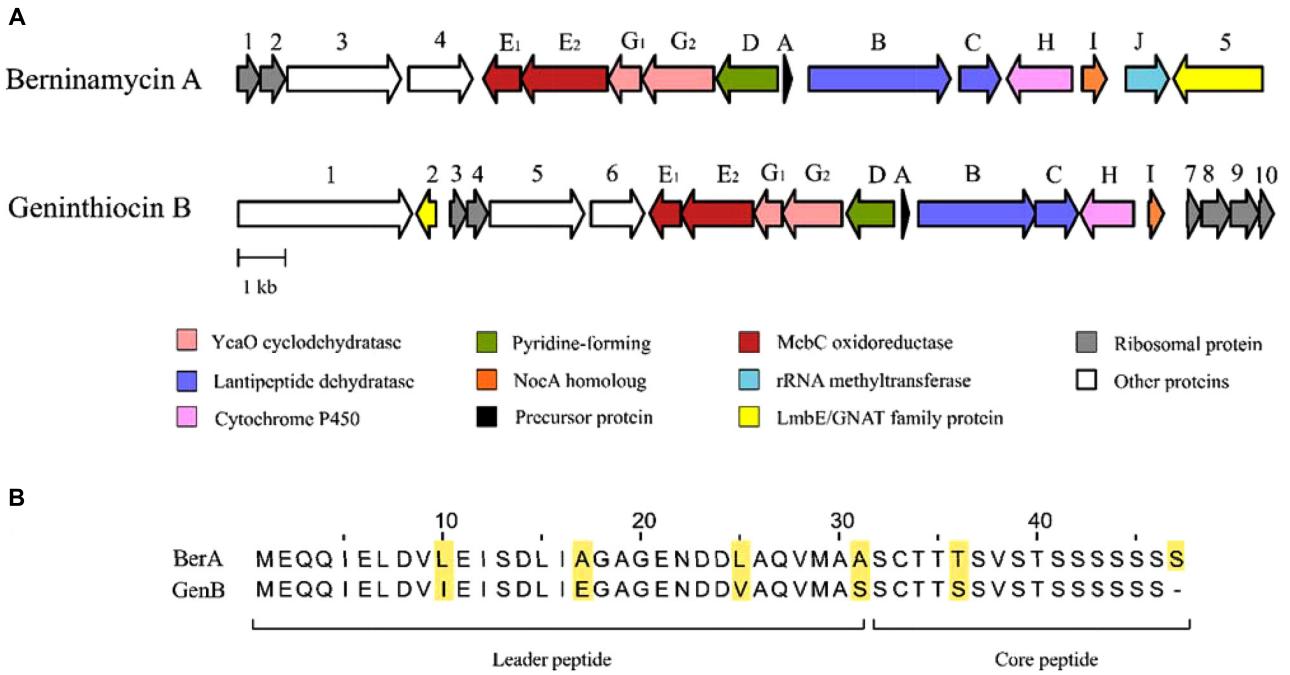

FIGURE 4 | The biosynthetic gene clusters for berninamycin A and the newly isolated geninthiocin B **(A)**, with their precursor peptide sequences **(B)**, encoded by the structural genes. In the precursor peptide sequence, the core peptide appears in the mature thiopeptide, while the leader peptide sequence is removed during posttranslational modification.

similar. *In silico* prediction of enzymatic functions of individual gene products from genenthiocin B BGC allowed us to propose the biosynthesis of geninthiocin B (**Supplementary Figure S4**). The geninthiocin B structural gene (*genA*) encodes a 31-aa leader peptide linked to a C-terminal 15-aa core (**Figure 4**). Ten amino acids of the core peptide are represented by serine, whereby Ser_{15} is absent in the mature scaffold (**Figure 3**). The cleavage of Ser_{15} is needed to afford the C-terminus amide, and is presumably catalyzed by GenI, which is homologous to the NocA and NosA enzymes from nocathiacin and noshipeptide biosynthesis. It has been shown that NosA acts on an intermediate bearing a bis-dehydroalanine tail in noshipeptide and catalyzes an enamide dealkylation to remove the acrylate unit originating from the extended serine residue (Yu et al., 2010; Wang et al., 2015).

As in the berninamycin BGC, the unique feature of geninthiocin B cluster is that its genes for cyclodehydratase and dehydrogenase (most likely responsible for the formation of thiazole/oxazole rings), are split in a non-traditional way (**Figure 4**). The geninthiocin B biosynthesis YcaO "*G*" protein is split into GenG1/GenG2 and the protein "*E*" is split into GenE1/GenE2. The protein GenE2 probably has the partner function of the so-called E1-like proteins, which interact with a side chain of the leader peptide and promote GenG1 and GenG2 to perform the *d* cyclodehydration. In earlier studies it was observed that E1-like partner protein is required for substrate guiding and recognition by YcaO protein (Burkhart et al., 2015), as well as playing a crucial role in allosteric activation of the latter (Dunbar et al., 2012, 2014). This partner protein can either be represented by a discrete polypeptide or fused to the YcaO (Burkhart et al., 2017). Similarly to the berninamycin BGC, unusual is an appearance of the canonical E1-like protein fused to a dehydrogenase (GenE2), as well as the presence of another dehydrogenase component (GenE1) encoded by the gene near *genE2*. Due to this atypical genetic organization, it is unclear how the active azole-forming proteins function with each other. Burkhart et al. (2017) suggested that these proteins might catalyze the formation of a large 35-member macrocycle, since the spacing of azole/azoline heterocycles in the macrocycle region of berninamycin is nearly identical to that of the thiocillin-like thiopeptides, which have a 26-membered macrocycle after maturation.

Proteins GenB and GenC (**Table 2**) showed high similarity to lanthipeptide-like dehydratases and most likely catalyze the formation of the dehydroalanine (Dha) and dehydrobutyrine (Dhb) functional groups. Reconstitution studies using purified enzymes have established both the nature and the timing of the modifications installed during the biosynthesis of the thiopeptide antibiotic thiomuracin in *Thermobispora bispora* (Zhang et al., 2016). It was shown that after formation of the Dha residues, the central six-membered nitrogenous heterocycle is assembled via a formal [4+2] cycloaddition between the two Dha groups (Zhang et al., 2016). We suggest that in geninthiocin B biosynthesis the serine Ser_1 and Ser_{13} are the key amino acids in the final macrocycle formation (**Figure 4**). GenD protein showing high degree of similarity to BerD from berninamycin pathway, which was predicted to catalyze the formation of characteristic for thiopeptides series *d* tri-substituted pyridine

ring, is most probably also involved in the macrocycle formation (Malcolmson et al., 2013).

The gene *genH* (**Figure 4**, **Table 2**) encodes a homolog of BerH from berninamycin BGC, a putative P450 hydroxylase family protein that is most probably involved in valine hydroxylation (Malcolmson et al., 2013). The characterization of two P450 cytochrome-like proteins of nosiheptide (NosB and NosC) in *Streptomyces actuosus* ATCC 25421 indicated that those are responsible for hydroxylation occurring most likely at the tailoring stage, after the main scaffold is formed (Liu et al., 2013). During this work we could detect two compounds: geninthiocin B and an uncharacterized compound 2, both products from the geninthiocin B gene cluster. Based on the high-resolution LC-MS analysis, the compound 2 differs from geninthiocin B by one oxygen atom (**Supplementary Figure S2**). We suggest that compound 2 has similar structure to geninthiocin B, but lacks a hydroxyl group on Val_7-residue. The appearance of a thiopeptide intermediate is not surprising and was described before for berninamycin (berninamycins A and B) (Lau and Rinehart, 1994). Moreover, a desoxygeninthiocin (val-geninthiocin) was isolated from *Streptomyces* sp. RSF18, which has a similar structure as geninthiocin, but lacks a hydroxyl-group on Val_7-residue. If our assumption for structural features of compound 2 is correct, it can be produced as a major metabolite from the geninthiocin B cluster upon inactivation of the *genH* gene. The overall yield of geninthiocin B-related compounds must be improved to allow for more detailed studies on these molecules. The latter can be achieved either via heterologous expression of the cluster in an engineered *Streptomyces* host (Myronovskyi et al., 2018), or re-factoring of the cluster in YIM 130001 via promoter replacement (Horbal et al., 2018).

Geninthiocin B gene cluster is flanked at both ends by the genes encoding ribosomal and translation-associated proteins (**Figure 4**, **Table 2**), which are presumably not involved in geninthiocin B and compound 2 biosynthesises, but may play a key role in self-resistance of *Streptomyces* sp. YIM 130001. Berninamycin, which like genintiocin B also possesses a 35-membered macrocycle, is known to target 50S ribosomal subunit by binding to the 23S/L11 complex, thereby inhibiting protein synthesis (Reusser, 1969; Thompson et al., 1982). The mechanisms of self-resistance to 35-membered macrocyclic thiopeptides are still not completely understood (Just-Baringo et al., 2014), but for the berninamycin-producing *Streptomyces bernensis* it was shown that its respective BGC contains a gene encoding ribosomal RNA methylase. These proteins catalyze specific methylation of ribose on the 23S ribosomal RNA, preventing binding of thiopeptides to the modified ribosomes (Thompson et al., 1982). No genes encoding putative ribosomal RNA methylase could be identified in the geninthiocin B gene cluster or its flanking regions, suggesting that self-resistance in YIM 130001 may be achieved via different mechanism. This may also imply that the molecular target of geninthiocin B is different from that of berninamycin, despite very close similarity between their chemical structures and the same size of the macrocycle.

It has been speculated that some thiopeptides, including thiostreptone commonly used in veterinary medicine and research, may also serve at sub-inhibitory concentrations as signal molecules for physiological development or quorum-sensing in bacteria (Holmes et al., 1993). Keeping in mind the fact that thiopeptides at higher concentration have strong antibacterial activity (Bagley et al., 2005), a likely other ecological function of geninthiocin B could be defense against predatory bacteria and/or means of competition for nutritional sources.

CONCLUSION

Streptomycetes are characterized by a complex metabolism and their ability to produce diverse types of antibiotics. In this work we describe the identification of new thiopeptide antibiotic, geninthiocin B, from *Streptomyces* sp. YIM 130001 with bioactivity against *B. subtilis*. Combined analysis of the genome sequencing data and metabolite profiling led to identification of geninthiocin B gene cluster, confirming the power of genome mining approach in the natural product discovery process.

AUTHOR CONTRIBUTIONS

LW, YJ, and C-LJ isolated and characterized *Streptomyces* sp. YIM 130001. SZ designed the experiments and supervised the study. OS, NS, FA, RL, and KK performed the wetlab experiments. CR and JK sequenced, annotated and analyzed the genome.

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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.2018.03139/full#supplementary-material

REFERENCES

Abe, H., Kushida, K., Shiobara, Y., and Kodama, M. (1988). The structures of sulfomycin I and berninamycin A. *Tetrahedron Lett.* 29, 1401–1404. doi: 10.1016/S0040-4039(00)80308-1

Bagley, M. C., Dale, J. W., Merritt, E. A., and Xiong, X. (2005). Thiopeptide antibiotics. *Chem. Rev.* 105, 685–714. doi: 10.1021/cr0300441

Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.-P., et al. (2015). Taxonomy, physiology, and natural products of Actinobacteria. *Microbiol. Mol. Biol. Rev.* 80, 1–43. doi: 10.1128/MMBR.00019-15

Bentley, S. D., Chater, K. F., Cerdeno-Tarraga, A.-M., Challis, G. L., Thomson, N., James, K. D., et al. (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3 (2). *Nature* 417, 141–147. doi: 10.1038/417141a

Blin, K., Wolf, T., Chevrette, M. G., Lu, X., Schwalen, C. J., Kautsar, S. A., et al. (2017). antiSMASH 4.0—improvements in chemistry prediction and gene cluster boundary identification. *Nucl. Acids Res.* 45, 36–41. doi: 10.1093/nar/gkx319

Bredholdt, H., Galatenko, O. A., Engelhardt, K., Fjaervik, E., Terekhova, L. P., and Zotchev, S. B. (2007). Rare actinomycete bacteria from the shallow water sediments of the Trondheim fjord, Norway: isolation, diversity and biological activity. *Environ. Microbiol.* 9, 2756–2764. doi: 10.1111/j.1462-2920.2007.01387.x

Burkhart, B. J., Hudson, G. A., Dunbar, K. L., and Mitchell, D. A. (2015). A prevalent peptide-binding domain guides ribosomal natural product biosynthesis. *Nat. Chem. Biol.* 11, 564–570. doi: 10.1038/nchembio.1856

Burkhart, B. J., Schwalen, C. J., Mann, G., Naismith, J. H., and Mitchell, D. A. (2017). YcaO-dependent posttranslational amide activation: biosynthesis, structure, and function. *Chem. Rev.* 117, 5389–5456. doi: 10.1021/acs.chemrev.6b00623

Chen, R., Zhang, Q., Tan, B., Zheng, L., Li, H., Zhu, Y., et al. (2017). Genome mining and activation of a silent PKS/NRPS gene cluster direct the production of totopotensamides. *Org. Lett.* 19, 5697–5700. doi: 10.1021/acs.orglett.7b02878

Davies, J., and Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* 74, 417–433. doi: 10.1128/MMBR.00016-10

de Kraker, M. E., Stewardson, A. J., and Harbarth, S. (2016). Will 10 million people die a year due to antimicrobial resistance by 2050? *PLoS Med.* 13:e1002184. doi: 10.1371/journal.pmed.1002184

Di Giambattista, M., Chinali, G., and Cocito, C. (1989). The molecular basis of the inhibitory activities of type A and type B synergimycins and related antibiotics on ribosomes. *J. Antimicrob. Chemother.* 24, 485–507. doi: 10.1093/jac/24.s been speculated that s4.485

Du, C., and van Wezel, G. P. (2018). Mining for microbial gems: integrating proteomics in the postgenomic natural product discovery pipeline. *Proteomics* 30:e1700332. doi: 10.1002/pmic.201700332

Dunbar, K. L., Chekan, J. R., Cox, C. L., Burkhart, B. J., Nair, S. K., and Mitchell, D. A. (2014). Discovery of a new ATP-binding motif involved in peptidic azoline biosynthesis. *Nat. Chem. Biol.* 10, 823–829. doi: 10.1038/nchembio.1608

Dunbar, K. L., Melby, J. O., and Mitchell, D. A. (2012). YcaO domains use ATP to activate amide backbones during peptide cyclodehydrations. *Nat. Chem. Biol.* 8, 569–575. doi: 10.1038/nchembio.944

Engelhardt, K., Degnes, K. F., Kemmler, M., Bredholt, H., Fjaervik, E., Klinkenberg, G., et al. (2010a). Production of a new thiopeptide antibiotic, TP-1161, by a marine *Nocardiopsis* species. *Appl. Environ. Microbiol.* 76, 4969–4976. doi: 10.1128/AEM.00741-10

Engelhardt, K., Degnes, K. F., and Zotchev, S. B. (2010b). Isolation and characterization of the gene cluster for biosynthesis of the thiopeptide antibiotic TP-1161. *Appl. Environ. Microbiol.* 76, 7093–7101. doi: 10.1128/AEM.01442-10

Flett, F., Mersinias, V., and Smith, C. P. (1997). High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes. *FEMS Microbiol. Lett.* 155, 223–229. doi: 10.1111/j.1574-6968.1997.tb13882.x

Gredičak, M., and Jerić, I. (2007). Enediyne compounds-new promises in anticancer therapy. *Acta. Pharm.* 57, 133–150. doi: 10.2478/v10007-007-0011-y

Hakvåg, S., Fjærvik, E., Josefsen, K. D., Ian, E., Ellingsen, T. E., and Zotchev, S. B. (2008). Characterization of *Streptomyces* spp. isolated from the sea surface microlayer in the Trondheim Fjord, Norway. *Mar. Drugs* 6, 620–635. doi: 10.3390/md6040620

Holmes, D., Caso, J., and Thompson, C. (1993). Autogenous transcriptional activation of a thiostrepton-induced gene in Streptomyces lividans. *EMBO J.* 12, 3183–3191. doi: 10.1002/j.1460-2075.1993.tb05987.x

Horbal, L., Marques, F., Nadmid, S., Mendes, M. V., and Luzhetskyy, A. (2018). Secondary metabolites overproduction through transcriptional gene cluster refactoring. *Metab. Eng.* 49, 299–315. doi: 10.1016/j.ymben.2018.09.010

Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T., et al. (2003). Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nat. Biotechnol.* 21:526. doi: 10.1038/nbt820

Jiang, L., Wang, L., Zhang, J., Liu, H., Hong, B., Tan, H., et al. (2015). Identification of novel mureidomycin analogues via rational activation of a cryptic gene cluster in *Streptomyces roseosporus* NRRL 15998. *Sci. Rep.* 5:14111. doi: 10.1038/srep14111

Just-Baringo, X., Albericio, F., and Álvarez, M. (2014). Thiopeptide engineering: a multidisciplinary effort towards future drugs. *Angew. Chem. Int. Ed. Engl.* 53, 6602–6616. doi: 10.1002/anie.201307288

Katz, L., and Baltz, R. H. (2016). Natural product discovery: past, present, and future. *J. Ind. Microbiol. Biotechnol.* 43, 155–176. doi: 10.1007/s10295-015-1723-5

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Labeda, D., Goodfellow, M., Brown, R., Ward, A., Lanoot, B., Vanncanneyt, M., et al. (2012). Phylogenetic study of the species within the family *Streptomycetaceae*. *Antonie Van Leeuwenhoek* 101, 73–104. doi: 10.1007/s10482-011-9656-0

Lau, R. C., and Rinehart, K. L. (1994). Berninamycins B, C, and D, minor metabolites from *Streptomyces bernensis*. *J. Antibiot.* 47, 1466–1472. doi: 10.7164/antibiotics.47.1466

Liu, W., Xue, Y., Ma, M., Wang, S., Liu, N., and Chen, Y. (2013). Multiple oxidative routes towards the maturation of nosiheptide. *Chem. Bio. Chem.* 14, 1544–1547. doi: 10.1002/cbic.201300427

Malcolmson, S. J., Young, T. S., Ruby, J. G., Skewes-Cox, P., and Walsh, C. T. (2013). The posttranslational modification cascade to the thiopeptide berninamycin generates linear forms and altered macrocyclic scaffolds. *Proc. Natl. Acad. Sci. U.S.A.* 110, 8483–8488. doi: 10.1073/pnas.1307111110

Morris, R. P., Leeds, J. A., Naegeli, H. U., Oberer, L., Memmert, K., Weber, E., et al. (2009). Ribosomally synthesized thiopeptide antibiotics targeting elongation factor Tu. *J. Am. Chem. Soc.* 131, 5946–5955. doi: 10.1021/ja900488a

Myronovskyi, M., Rosenkränzer, B., Nadmid, S., Pujic, P., Normand, P., and Luzhetskyy, A. (2018). Generation of a cluster-free *Streptomyces albus* chassis strains for improved heterologous expression of secondary metabolite clusters. *Metab. Eng.* 49, 316–324. doi: 10.1016/j.ymben.2018.09.004

Ohyama, S., Wada, Y., and Hasumi, K. (2002). Antibiotic A10255 (thioplabin) enhances fibrin binding and activation of plasminogen. *J. Antibiot.* 55, 83–91. doi: 10.7164/antibiotics.55.83

Poralla, K., Muth, G., and Härtner, T. (2000). Hopanoids are formed during transition from substrate to aerial hyphae in *Streptomyces coelicolor* A3 (2). *FEMS Microbiol. Lett.* 189, 93–95. doi: 10.1111/j.1574-6968.2000.tb09212.x

Reusser, F. (1969). Mode of action of berninamycin. An inhibitor of protein biosynthesis. *Biochemistry* 8, 3303–3308. doi: 10.1021/bi00836a026

Sadeghi, A., Soltani, B. M., Nekouei, M. K., Jouzani, G. S., Mirzaei, H. H., and Sadeghizadeh, M. (2014). Diversity of the ectoines biosynthesis genes in the salt tolerant *Streptomyces* and evidence for inductive effect of ectoines on their accumulation. *Microbiol. Res.* 169, 699–708. doi: 10.1016/j.micres.2014.02.005

Sajid, I., Shaaban, K. A., Frauendorf, H., Hasnain, S., and Laatscha, H. (2008). Val-Geninthiocin: structure elucidation and msn fragmentation of thiopeptide antibiotics produced by *Streptomyces* sp. RSF18. *Z. Naturforsch. B* 63, 1223–1230. doi: 10.1515/znb-2008-1014

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 3rd Edn. Cold Spring Harbor, NY: CSHL Press.

Sandy, M., and Butler, A. (2009). Microbial iron acquisition: marine and terrestrial siderophores. *Chem. Rev.* 109, 4580–4595. doi: 10.1021/cr9002787

Schaffert, L., Albersmeier, A., Winkler, A., Kalinowski, J., Zotchev, S. B., and Ruckert, C. (2016). Complete genome sequence of the actinomycete *Actinoalloteichus hymeniacidonis* type strain HPA 177T isolated from a marine sponge. *Stand. Genomic. Sci.* 11:91. doi: 10.1186/s40793-016-0213-3

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Thompson, J., Cundliffe, E., and Stark, M. J. (1982). The mode of action of berninamycin and the mechanism of resistance in the producing organism, *Streptomyces bernensis*. *Microbiology* 128, 875–884. doi: 10.1099/00221287-128-4-875

Tian, J., Chen, H., Guo, Z., Liu, N., Li, J., Huang, Y., et al. (2016). Discovery of pentangular polyphenols hexaricins A–C from marine *Streptosporangium* sp. CGMCC 4.7309 by genome mining. *Appl. Microbiol. Biotechnol.* 100, 4189–4199. doi: 10.1007/s00253-015-7248-z

van Wezel, G. P., and McDowall, K. J. (2011). The regulation of the secondary metabolism of *Streptomyces*: new links and experimental advances. *Nat. Prod. Rep.* 28, 1311–1333. doi: 10.1039/c1np00003a

Wang, Y., Liu, S., Yao, P., Yu, Y., Zhang, Y., Lan, W., et al. (2015). Crystallographic analysis of NosA, which catalyzes terminal amide formation in the biosynthesis of nosiheptide. *Acta Crystallogr. F Struct. Biol. Commun.* 71, 1033–1037. doi: 10.1107/S2053230X15011085

Winter, J. M., Behnken, S., and Hertweck, C. (2011). Genomics-inspired discovery of natural products. *Curr. Opin. Chem. Biol.* 15, 22–31. doi: 10.1016/j.cbpa.2010.10.020

Xing, K., Qin, S., Zhang, W. D., Cao, C. L., Ruan, J. S., Huang, Y., et al. (2014). Glycomyces phytohabitans sp. nov., a novel endophytic actinomycete isolated from the coastal halophyte in Jiangsu, East China. *J. Antibiot.* 67, 559–563. doi: 10.1038/ja.2014.40

Yamada, Y., Arima, S., Nagamitsu, T., Johmoto, K., Uekusa, H., Eguchi, T., et al. (2015). Novel terpenes generated by heterologous expression of bacterial terpene synthase genes in an engineered *Streptomyces* host. *J. Antibiot.* 68, 385–394. doi: 10.1038/ja.2014.171

Yu, Y., Guo, H., Zhang, Q., Duan, L., Ding, Y., Liao, R., et al. (2010). NosA catalyzing carboxyl-terminal amide formation in nosiheptide maturation via an enamine dealkylation on the serine-extended precursor peptide. *J. Am. Chem. Soc.* 132, 16324–16326. doi: 10.1021/ja106571g

Yun, B.-S., Hidaka, T., Furihata, K., and Seto, H. (1994). Microbial metabolites with tipA promoter inducing activity. III. Thioxamycin and its novel derivative, thioactin, two thiopeptides produced by *Streptomyces* sp. DP94. *J. Antibiot.* 47, 1541–1545. doi: 10.7164/antibiotics.47.1541

Zettler, J., Xia, H., Burkard, N., Kulik, A., Grond, S., Heide, L., et al. (2014). New aminocoumarins from the rare actinomycete catenulispora acidiphila DSM 44928: identification, structure elucidation, and heterologous production. *Chembiochem* 15, 612–621. doi: 10.1002/cbic.201300712

Zhang, Z., Hudson, G. A., Mahanta, N., Tietz, J. I., van der Donk, W. A., and Mitchell, D. A. (2016). Biosynthetic timing and substrate specificity for the thiopeptide thiomuracin. *J. Am. Chem. Soc.* 138, 15511–15514. doi: 10.1021/jacs.6b08987

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Cave Actinobacteria as Producers of Bioactive Metabolites

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Recently, there is an urgent need for new drugs due to the emergence of drug resistant pathogenic microorganisms and new infectious diseases. Members of phylum Actinobacteria are promising source of bioactive compounds notably antibiotics. The search for such new compounds has shifted to extreme or underexplored environments to increase the possibility of discovery. Cave ecosystems have attracted interest of the research community because of their unique characteristics and the microbiome residing inside including actinobacteria. At the time of writing, 47 species in 30 genera of actinobacteria were reported from cave and cave related habitats. Novel and promising bioactive compounds have been isolated and characterized. This mini-review focuses on the diversity of cultivable actinobacteria in cave and cave-related environments, and their bioactive metabolites from 1999 to 2018.

Keywords: actinobacteria, cave, karst, bioactive compounds, diversity, antimicrobial, anticancer, natural products

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INTRODUCTION

Caves are generally regarded as any natural underground chamber that is large enough for human entrance. They can be classified based on type of rock and formation method. The most common types of caves are limestone and other calcareous rocks (Northup and Lavoie, 2001). Though caves have been studied for hundreds of years, their microbiome are generally underexplored and overlooked. Caves are attracting the interests of microbiologists, in terms of microbial diversity, during the past decade (Laiz et al., 1999; Barton et al., 2004; Barton, 2006). It is believed that microbes collected from pristine sites that are unexplored or rarely visited by humans are likely to be novel taxa or strains which produce unique beneficial chemical compounds. Market demand for new drugs is on the rise due to the emergence of new diseases and drug resistant pathogens (Genilloud, 2017; Kemung et al., 2018; Takahashi and Nakashima, 2018). With a combination of unique conditions including high humidity, relatively low and stable temperature, and low nutrients, caves are expected to harbor novel microorganisms with biotechnological benefits. Members of actinobacteria are reported to be a dominant microbial population in several cave ecosystems (Groth and Saiz-Jimenez, 1999; Cheeptham et al., 2013; Tomczyk-Zak and Zielenkiewicz, 2016; Ghosh et al., 2017).

Actinobacteria are large group of high G+C Gram positive bacteria (Barka et al., 2016). They are regarded as the most prolific source of bioactive compounds in particular commercially available antibiotics. Actinobacteria produce approximately two-thirds of all know antibiotics in

the market, most of these are from members of the genus *Streptomyces* (Barka et al., 2016). Several members of diverse actinobacterial taxa were also found to produce wide range of other biologically active compounds, for examples antibacterial, anticancer, or antifungal drugs (Barka et al., 2016; Genilloud, 2017; Castro et al., 2018; Takahashi and Nakashima, 2018). Isolation of actinobacteria from unique natural habitats is of interest to avoid re-isolation of strains that produce known bioactive metabolites and usually lead to highly diverse actinobacterial communities. The present mini-review provides evidence that actinobacteria from caves are expected to be a good source for drug discovery (Yücel and Yamac, 2010; Cheeptham et al., 2013; Kay et al., 2013; Ghosh et al., 2017; Riquelme et al., 2017).

SELECTIVE ISOLATION OF CAVE ACTINOBACTERIA

In the past decade, there are many reports on the discovery of novel actinobacteria in cave habitats. Successful isolation of actinobacteria from caves depend largely on factors of (1) media composition (Kim et al., 1998) (2) culture condition, and (3) pretreatment methods (Kim et al., 1998; Nakaew et al., 2009a,b; Duangmal et al., 2012; Niyomvong et al., 2012; Velikonja et al., 2014; Fang et al., 2017b; Adam et al., 2018). Media used for the isolation of cave actinobacteria range from routine cultivation media such as International *Streptomyces* Project medium 2 (yeast malt extract agar, ISP2) or tryptic soy agar (TSA) to selective media including humic acid vitamin agar (HV), starch casein agar (SC), starch casein nitrate agar (SCN), peptone-yeast extract/brain-heart infusion medium (PY-BHI), R2A medium, actinomycete isolation agar (AI), and Gauze's medium No.1. Moreover, isolation media that mimic the conditions of low concentration nutrients in caves such as tap water agar, 1/100 ISP2 and oligotrophic medium (M5) were also successfully used for the isolation of actinobacteria. (Lee et al., 2000b; Velikonja et al., 2014; Covington et al., 2018; Passari et al., 2018). High concentration of nutrients in standard cultivation media were reported to cause cell death in cave-associated bacteria due to osmotic stress (Barton, 2006; Ghosh et al., 2017).

Two important culture conditions for actinobacteria isolation are incubation temperature and incubation time. Four incubation temperatures (5°, 13°, 20°, and 28°C) were used for the isolation of soil bacteria including actinobacteria from three caves in Northern Spain (Laiz et al., 2003). The incubation temperature of 5°C was used to represent cave temperature and target psychrotrophs, 28°C as laboratory incubation temperature and 20°C as intermediate temperature between cave and laboratory conditions. The highest number of actinobacterial isolates (mostly sporoactinomycetes) was obtained at 28°C followed by 13°, 20° and 5°C, respectively. However, a higher diversity was observed from 13°C than 28°C. Therefore, these authors concluded that the isolation of actinobacteria is a temperature-dependent process. In addition, longer incubation time was successfully used to promote the recovery of slow-growing actinobacteria (Laiz et al., 2003).

Pretreatment, both chemical and physical methods are generally useful for isolation of various actinobacterial species. Physical pretreatments involve the use of air drying, moist heat, dry heat and electromagnetic wave. Moist heating (water bath at 50°C for 5–6 min) is useful for eliminating of fast growing bacteria (Niyomvong et al., 2012; Velikonja et al., 2014). Dry heating at 120°C for 1 h is effective in reducing number of unwanted bacteria and found to be an effective method for isolation of members of the genera *Dactylosporangium*, *Streptosporangium* and *Microbispora*, while growth of streptomycetes was limited (Jiang et al., 2016). In addition, dry heating with or without phenol treatment resulted in a reduction of bacteria and heat-labile *Streptomyces*, thus heat resistant rare actinobacteria were readily isolated (Kim et al., 1998; Nakaew et al., 2009a; Niyomvong et al., 2012). However, these treatments also affect the number of viable actinobacteria (Niyomvong et al., 2012). Pretreatment using microwave irradiation was effective for the isolation of rare actinobacteria (Niyomvong et al., 2012) and capable of inducing spore germination in some species of *Streptomyces*, *Nocardia*, *Streptosporangium*, *Lentzea*, *Micromonospora,* and *Micropolyspora* (currently transferred to *Nocardia*) (Bulina et al., 1997; Wang et al., 2013; Velikonja et al., 2014). For chemical pretreatment, the type and concentration of calcium salts are important for the isolation of actinobacteria (Fang et al., 2017b). Selective media supplemented with $CaCO_3$ yield higher actinobacterial count than those supplemented with $CaCl_2$ and $(CH_3COO)_2Ca$. The concentration of these three salts, at low concentration (0.1 and 0.01% (w/v) yield higher CFU of actinobacteria than in its absence or at high concentration. Calcium is important for environmental stress tolerance in actinobacteria because calcium forms a compound with dipicolinic acid as calcium dipicolinate and acts as secondary stabilizing agent for spore against environmental stress (Moir and Smith, 1990).

NOVEL ACTINOBACTERIAL TAXA

Several novel actinobacterial taxa isolated from caves and cave related habitats during the period of 20 years from 1999 to 2018 were summarized in **Table 1**. In total, 47 species within 30 genera were described including 7 novel genera. The highest number of novel species was from genus *Streptomyces* (5) followed by *Amycolatopsis* (4) and *Nocardia* (4). The majority of these novel actinobacteria were isolated from cave soils including 6 novel genera, *Antricoccus, Beutenbergia*, *Knoellia, Lysinibacter Spelaeicoccus* and *Sphaerimonospora*. Only the genus *Hoyosella* was recovered from complex biofilm on the ceiling and wall of Altamira cave, Spain. The extreme conditions within the caves are expected to create stress for the inhabitant microorganisms at the genetic level, paving the way for the evolution of new species and their novel metabolites (Tawari and Gupta, 2013). Therefore, caves are considered as an attractive source for the isolation of novel actinobacterial taxa.

Most species were isolated from selective media that were designed for the isolation of actinobacteria such as humic acid vitamin agar, starch casein agar, starch casein nitrate

TABLE 1 | Novel actinobacterial taxa isolated from cave and related habitats between 1999 and 2018.

Family	Genus	Species	Sources	Media	References
Brevibacteriaceae	*Spelaeicoccus*	*Spelaeicoccus albus*	Soil from natural cave in Jeju, Korea	Starch casein agar	Lee, 2013b
Conexibacteraceae	*Conexibacter*	*Conexibacter stalactiti*	Pieces of stalactites from Yongcheon cave in Jeju, Korea	Starch casein agar	Lee, 2017
Glycomycetaceae	*Stackebrandtia*	*Stackebrandtia cavernae*	Rocks from karst cave, Guizhou, south-west China	R2A agar with 0.1% $CaCO_3$	Zhang et al., 2016
Intrasporangiaceae	*Fodinibacter*	*Fodinibacter luteus*	Sample from wall of a salt mine in Yunnan, China	Marine agar 2216	Wang et al., 2009
	Knoellia	*Knoellia sinensis*	Soils from the Reed Flute cave near Guilin, Guangxi, China	Casein mineral medium	Groth et al., 2002
		Knoellia subterranea	Soils from the Reed Flute cave near Guilin, Guangxi, China	Peptone/yeast extract/brain- heart infusion medium (PY-BHI)	Groth et al., 2002
	Ornithinimicrobium	*Ornithinimicrobium cavernae*	Stalagmites from karst cave in Luoyang country, Henan, northern China	ISP 2 with nystatin and nalidixic acid	Zhang et al., 2018
Kineosporiaceae	*Augustibacter*	*Augustibacter spluncae*	Pieces of stalactites from Yongcheon cave in Jeju, Korea	Starch casein agar	Ko and Lee, 2017
Microbacteriaceae	*Agromyces*	*Agromyces subbeticus*	Cyanobacterial biofilm from Cave of Bats, near Zuheros, Cordoba, southern Spain	Peptone/yeast extract/ brain- heart infusion medium (PY-BHI)	Jurado et al., 2005
	Humibacter	*Humibacter antri*	Clay soils from natural cave in Jeju, Korea	Starch casein agar	Lee, 2013a
	Lysinibacter	*Lysinibacter cavernae*	Soils from wild karst cave in the Wulong region, Chongqing, China	FA (fulvic acid) agar	Tuo et al., 2015
Micrococcaceae	*Arthrobacter*	*Arthrobacter psychrophenolicus*	Carbonate-rich deposit from Alpine ice cave, Salzburg, Austria	Soil-extract agar	Margesin et al., 2004
	Beutenbergia	*Beutenbergia cavernae*	Soils from the Reed Flute cave near Guilin, Guangxi, China	Casein mineral medium and peptone/yeast extract/brain-heart infusion medium	Groth et al., 1999
Micromonosporaceae	*Catellatospora*	*Catellatospora koreensis*	Soils from gold-mine cave in Kongju, Korea	Yeast extract; glucose; K_2HPO_4;Na_2HPO_4;KNO_3; NaCl; $MgSO_4.7H_2O$; $CaCl_2.2H_2O$ and trace mineral solution	Lee et al., 2000a
	Micromonospora	*Micromonospora kangleipakensisi*	Sample from limestone quarry at Hundung, Manipur, India	Gauze's medium	Nimaichand et al., 2013c
Mycobacteriaceae	*Hoyosella*	*Hoyosella altamirensis*	Complex bioflim on the cave ceiling and walls from Altamira cave, Cantabria, Spain	Starch casein agar	Jurado et al., 2009
Nocardioidaceae	*Jiangella*	*Jiangella alkaliphila*	Soils from natural cave on Jeju island, Korea	Starch casein agar	Lee, 2008
	Nocardioides	*Nocardioides cavernae*	Soils from karst cave in Xingyi county, Guizhou, south-western China	R2A agar with cycloheximide and nalidixic acid	Han et al., 2017
	Tenggerimyces	*Tenggerimyces flavus*	Soil from Shenxian cave, Henan, China	R2A agar with cycloheximide, nalidixic acid and potassium dichromate	Li et al., 2016
Norcardiaceae	*Nocardia*	*Nocardia altamirensis*	Complex microbial community forming a gray-colored colonization on the walls from Altamira cave, Cantabria, Spain	Tryptose soy agar	Jurado et al., 2008
		Nocardia cavernae	Soil from karst cave in Xingyi county, Guizhou, south-western China	Humic acid-vitamin agar with cycloheximide and nalidixic acid	Li et al., 2017
		Nocardia jejuensis	Soil from natural cave on Jeju island, Korea	Starch casein agar	Lee, 2006c

(Continued)

TABLE 1 | Continued

Family	Genus	Species	Sources	Media	References
		Nocardia speluncae	Soil from natural cave on Jeju island, Korea	Starch casein agar	Seo et al., 2007
	Rhodococcus	*Rhodococcus antrifimi*	Dried bat dung from natural cave in Jeju, Korea	Starch casein agar	Ko et al., 2015
		Rhodococcus canchipurensis	Soil from limestone quarry at Hundung, Manipur, India	Starch casein nitrate agar	Nimaichand et al., 2013a
Propionibacteriaceae	*Microlunatus*	*Microlunatus cavernae*	Soil from Alu ancient cave, Yunnan, south-west China	R2A medium	Cheng et al., 2013a
Pseudonocardiaceae	*Amycolatopsis*	*Amycolatopsis halotolerans*	Soil from natural cave in Jeju island, Korea	Starch/casein agar	Lee, 2006b
		Amycolatopsis jejuensis	Dried bat dung from natural cave in Jeju island, Korea	Starch casein agar	Lee, 2006b
		Amycolatopsis jiguanensis	Soil from Ji Guan cave, Henan, middle-eastern China	ISP2	Huang et al., 2016
		Amycolatopsis xuchangensis	Soil from Ji Guan cave, Henan, middle-eastern China	ISP2	Huang et al., 2016
	Lentzea	*Lentzea cavernae*	Limestone from karst cave in Xingyi county, Guizhou, south-western China	Humic acid-vitamin agar with cycloheximide and nalidixic acid	Fang et al., 2017a
		Lentzea guizhouensis	Limestone from Puding karst ecosystem research station of the Chinese Academy of Sciences in Guizhou Province, south-west china	Modified ATCC-172 medium at 1/10 concentration	Cao et al., 2015
	Saccharothrix	*Saccharothrix albidocapillata*	Soil from gold mine cave in Kongju, Korea	Tap water agar and oligotrophic (M5) medium	Lee et al., 2000b
		Saccharothrix violacea	Soil from gold mine cave in Kongju, Korea	Tap water agar and oligotrophic (M5) medium	Lee et al., 2000b
	Saccharopolyspora	*Saccharopolyspora cavernae*	Swallow cave, Yunnan, south-west China	Improved DSMZ medium 405	Cheng et al., 2013b
Streptomycataceae	*Streptomyces*	*Streptomyces boninensis*	Soil from limestone cave, Ogasawa islands, Tokyo, Japan	Humic acid-vitamin agar with benlate and nalidixic acid	Také et al., 2018
		Streptomyces canchipurensis	Soil from limestone quarry at Hundung, Manipur, India	Gauze's medium No. 1	Li et al., 2014
		Streptomyces hundungensis	Soil from limestone quarry at Hundung, Manipur, India	Starch casein nitrate agar	Nimaichand et al., 2013b
		Streptomyces lunaelactis	Moonmilk deposit from Grotte des Collemboles cave in Comblain-au-Pont, Belgium	ISP and starch nitrate (SN) medium with nalidixic acid and nystatin	Maciejewska et al., 2015
		Streptomyces manipurensis	Soil from limestone quarry at Hundung, Manipur, India	Starch casein Nitrate agar	Nimaichand et al., 2012
Streptosporangiaceae	*Sphaerimonospora*	*Sphaerimonospora thailandensis* (formerly *Microbispora thailandensis*)	Soil from tropical limestone cave (Khao No-Khao Kaeo), Nakorn Sawan, Thailand	Humic acid-vitamin agar with nalidixic acid and ketoconazole	Duangmal et al., 2012
		Sphaerimonospora cavernae	Soil from tropical limestone cave (Khao No-Khao Kaeo), Nakorn Sawan, Thailand	-	Mingma et al., 2016
	Nonomuraea	*Nonomuraea monospora*	Soil from Pha Tup cave forest park, Nan, Thailand	Humic acid-vitamin agar with nystatin and cycloheximide	Nakaew et al., 2012
		Nonomuraea indica	Soil from limestone open pit mine from Gulbarga region, Karnataka, India	Starch casein agar	Quadri et al., 2015
Thermomonosporaceae	*Actinocorallia*	*Actinocorallia cavernae*	Natural cave in Jeju, Korea	Starch/casein agar	Lee, 2006a
Not assigned to family (Suborder *Frankineae*)	*Antricoccus*	*Antricoccus suffuscus*	Soil from natural cave in Jeju, Korea	Starch casein agar	Lee, 2015

agar. However, some novel species were isolated using general cultivation media such as ISP2 media (*Amycolatopsis jiguanensis* and *A. xuchangensis*) and TSA (*Nocardia altamirensis*). In addition, low nutrient media (tap water agar and oligotrophic M5 media) were preferable for the isolation of *Saccharothrix violacea* and *S. albidocapillata*. Most novel species were incubated at 28°–30°C for 1–6 weeks. However, *Arthrobacter psychrophenolicus* was isolated at 4°C, this may be because this species originated from Alpine ice cave in Salzburg, Austria (Margesin et al., 2004). *Lysinibacter cavernae* was isolated at 15°C from soil in a wild karst cave in the Wulong region, Chongqing, China (Tuo et al., 2015). *Streptomyces lunaelactis* was isolated at 17°C from a moonmilk deposit in the Grotte des Collemboles cave in Belgium (Maciejewska et al., 2015).

Pretreatment procedures were also useful for isolation of some novel species. For example, *Microbispora thailandensis* was isolated from soil pretreated with microwave radiation at a frequency of 2460 MHz and power setting of 100 W for 45 s (Duangmal et al., 2012). *Nonomuraea monospora* was isolated from soil treated with phenol (Nakaew et al., 2012). *Streptomyces manipurensis* was isolated from soil supplemented with 0.1 g of $CaCO_3$ for 1 day to prevent the growth of fast growing bacteria (Nimaichand et al., 2012).

BIOACTIVE COMPOUNDS FROM CAVE ACTINOBACTERIA

Caves are extreme habitats with low nutrient, temperature and light intensity but have high humidity (Schabereiter-Gurtner et al., 2002). These unique characteristics may promote the production of bioactive substances in particular antibiotics by actinobacteria (Nakaew et al., 2009a). Bioactive metabolites from cave associated actinobacteria have been purified, their structure elucidated and reported in recent years (**Table 2**). These compounds mostly displayed anti-bacterial and/or anti-cancer activities. The most prolific producer is members of the genus *Streptomyces*.

Cervimycin A, B, C, and D were produced from *Streptomyces tendae* strain HKI 0179, isolated from a rock wall in an ancient cave, the Grotta dei Cervi in Italy. Cervimycins A and B are novel polyketide glycosides. However, cervimycin C and D have the same structure as known compounds A2121-3 and A2121-2. Cervimycins A-D are highly active against Gram positive bacteria (*B. subtilis* and *S. aureus*) and multi-drug-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE) and efflux-resistant *S. aureus* EfS4 (Herold et al., 2005).

Xiakemycin A is a novel pyranonaphthoquinone (PNQ) antibiotics produced by *Streptomyces* sp. CC8-201 from remote karst soil in China. Xiakemycin A showed strong inhibitory activities against Gram positive bacteria (*S. aureus, S. epidermidis, E. faecalis, and E. faecium*) and cytotoxic against numerous cancer cell lines (human lung cancer A549 cells, breast cancer MCF-7 cells, hepatoma HepG-2 cell, cervical cancer HeLa cells, colon carcinoma HCT-116 cell p53 wt cells, neuroblastoma SH-SY5Y cells, and human prostate cancer PC-3) (Jiang et al., 2015).

Hypogeamicins A, B, C, and D were produced by *Nonomuraea specus* isolated from Hardin's cave system in Tennessee, USA. Hypogeamicin A showed cytotoxicity to colon cancer cell line TCT-1 while hypogeamicin B-D were active against *B. subtilis* with no cytotoxicity to TCT-1. However, hypogeamicin B-D are not as potent as erythromycin and gentamicin in terms of antimicrobial activity against *B. subtilis* (Derewacz et al., 2014).

Huanglongmycin A, B, and C are aromatic polyketides from *Streptomyces* sp. CB09001, isolated from karstic cave soil of Xiangxi, China. Huanglongmycin A showed a weak anti-Gram negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) and moderate cytotoxicity against A549 lung cancer cell line. Huanglongmycin B has weak antibacterial activity against *S. aureus* and multi-drug-resistant *S. aureus* (MRSA). Huanglongmycin C showed neither antibacterial nor anticancer activities (Jiang et al., 2018). Undecylprodigiosin was produced by *Streptomyces* sp. JS520 isolated from sediments in cave in the mountain Miroc, Serbia. Undecylprodigiosin is a deep red pigment with antibacterial activity against *Micrococcus luteus*, *B. subtilis,* and *C. albicans*. Moreover, undecylprodigiosin also showed antioxidative and UV-protective properties (Stankovic et al., 2012).

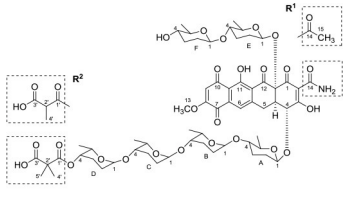
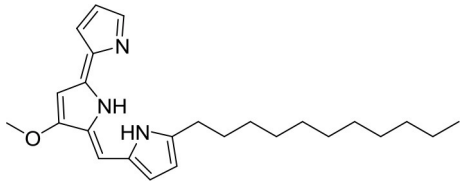
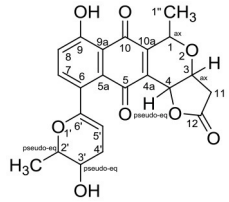
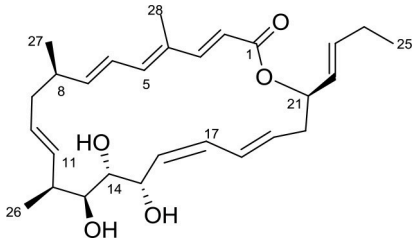
Four known compounds with bioactivity (cyclodysidin D, chaxalactin B, stylissazole B, and gyrophoric acid) were reported to produce by *Streptomyces* sp. IB 2014/I/ 78-8 from moonmilk speleothem of Bolshaya Oreshnaya cave in Siberia (Axenov-Gibanov et al., 2016). Cyclodysidin D is previously reported in marine sponge, *Dysidea tupha* associated *Streptomyces* sp. RV 15. This compound showed no activity against bacteria, fungi and parasites (Abdelmohsen et al., 2014). Chaxalactin B was produced from *Streptomyces* sp. C34 from a hyper-arid soil samples collected from the Atacama Desert, Chile. This compound has strong activity against Gram positive bacteria (Castro et al., 2018). Stylissazole B was isolated from the marine sponge *Stylissa carteri* collected in the Solomon islands but no report on bioactivity (Patel et al., 2010). Gyrophoric acid isolated from *Humicola* sp. FO-2942 is an inhibitor of diacylglycerol acyltransferase and a lipid-lowering agent (Inokoshi et al., 2010).

BIOACTIVITY OF UNCHARACTERIZED COMPOUNDS

Several cave actinobacteria have been screened for their biological activity such as antibacterial, anticancer and antifungal. However, no pure compound and their structure were reported in these studies. The screening of only bioactivity without the structure elucidation of bioactive metabolites may not useful for the discovery of new antibiotics (Hug et al., 2018). Nevertheless, these findings provide evidence which supports the potential of cave actinobacteria to be exploited for novel bioactive compounds.

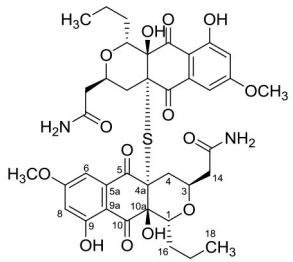
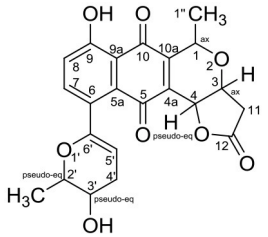
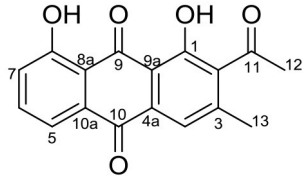
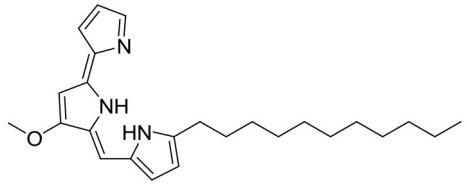
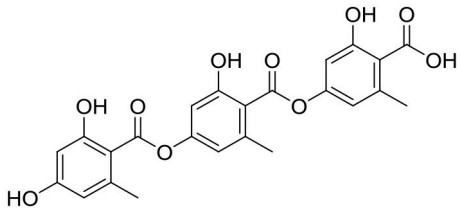
Turkish karstic caves were reported to harbor actinobacteria, for which 62% of the isolates, were active against several microbial pathogens (Gram positive bacteria, Gram negative bacteria, yeast, and filamentous fungi). *Streptomyces* sp. 1492 had strong activity against clinical strains of MRSA,

TABLE 2 | Bioactive metabolites from cave actinobacteria.

Bioactivity	Compounds	Structure	Producing strain	Source of strain	References
Antibacterial	Cervimycins A, B, C, and D	 Cervimycin A: $R^1 = COCH_3$, R^2 = dimethylmalonyl Cervimycin B: $R^1 = COCH_3$, R^2 = monomethylmalonyl Cervimycin C: $R^1 = CONH_2$, R^2 = dimethylmalonyl Cervimycin D: $R^1 = CONH_2$, R^2 = monomethylmalonyl	*Streptomyces tendae* strain HKI 0179	Rock wall from Ancient cave, The Grotta dei Cervi, Italy	Herold et al., 2005
	Undecylprodigiosin		*Streptomyces* sp. JS520	Cave on mountain Miroc, Serbia	Stankovic et al., 2012
	Xiakemycin A		*Streptomyces* sp. CC8-201	Soil from karst cave,Chongqing city, China	Jiang et al., 2015
	Chaxalactin B		*Streptomyces* sp. IB 2014/I/ 78-8	Bolshaya Oreshnaya cave in the Mansk area of the Krasnoyaesk, Siberia, Russia	Axenov-Gibanov et al., 2016

(Continued)

TABLE 2 | Continued

Bioactivity	Compounds	Structure	Producing strain	Source of strain	References
Anticancer	Hypogeamicins A		*Nonomuraea specus*	Hardin's cave system located close to Ashland City, Tennessee	Derewacz et al., 2014
	Xiakemycin A		*Streptomyces* sp. CC8-201	Soil from karst cave,Chongqing city, China	Jiang et al., 2015
	Huanglongmycin (HLM) A,		*Streptomyces* sp. CB09001	Soil from karstic cave in Xiangxi, China	Jiang et al., 2018
Antioxidative activity	Undecylprodigiosin		*Streptomyces* sp. JS520	Cave on mountain Miroc, Serbia	Stankovic et al., 2012
Inhibitory activity against lipid metabolism	Gyrophoric acid		*Streptomyces* sp. IB 2014/I/78-8	Bolshaya Oreshnaya cave in the Mansk area of the Krasnoyaesk, Siberia, Russia	Axenov-Gibanov et al., 2016 Tomoda and Omura, 2001

VRE, and *Acinetobacter baumanii* (Yücel and Yamac, 2010). *Streptomyces* E9 isolated from Helmcken Falls cave in British Columbia could inhibit the growth of *Paenibacillus larvae*, a causative agent of American foulbrood disease in honeybees (Kay et al., 2013). A moonmilk speleothems of limestone caves, Grotte des Collemboles in Belgium were investigated for antimicrobial producing cultivable actinobacteria. A collection of obtained *Streptomyces* displayed strong inhibitory activity against Gram positive and Gram negative bacteria (Maciejewska et al., 2016). In a study of cultivable actinobacteria from Azores volcanic caves in Portugal, 18.1% of 148 actinobacterial isolates have antibacterial activity against at least one of the following bacteria: *Salmonella typhimurium*, *E. coli*, *P. aeruginosa*, *Proteus* sp., *Listeria monocytogenes*, *L. innocua,* and *S. aureus*. Most of the active isolates belong to the genus *Streptomyces* (*S. nojiriensis*, *S. spiroverticillatus*, *S. avidinii*, and *S. mauvecolor*) followed by *Arthrobacter* (Riquelme et al., 2017). A total of 40 taxa belonging to the genera *Agromyces, Amycolatopsis, Kocuria, Micrococcus, Micromonospora, Nocardia, Streptomyces,* and *Rhodococcus* were recovered from moonmilk deposits inside the Grotte des Collemboles, Belgium. Antimicrobial activity was found in isolated strains against Gram positive bacteria (87%) and Gram negative bacteria (59%) (Adam et al., 2018). Sixteen isolates of *Streptomyces* spp. from Chaabe cave in Algeria were screened for their antimicrobial activity using agar cylinder method. All of them showed strong anti-Gram positive (*S. aureus*, *M. luteus, L. monocytogenes,* and *B. subtilis)* activity (Belyagoubi et al., 2018).

For anticancer activity, a rare actinobacterium *Spirillospora albida* strain CMU-PNK470 was isolated from Phanangkhoi cave in northern Thailand (Nakaew et al., 2009a). This bacterium showed activity against human small lung cancer cell (NCI-H1870) with an IC_{50} value of 10.18 μg/ml. Similarly, *Nonomurea roseola* strain PT708 isolated from Phatup cave forest park in northern Thailand was tested positive for anticancer activity against human oral cavity cancer (KB) and human small lung cancer cells (NCI-H187) (Nakaew et al., 2009b). Moreover, these two strains are also active against some Gram positive pathogenic bacteria (*B. cereus*, MRSA, and *Paenibacillus larvae*).

Some examples of antifungal activity from cave actinobacteria have been reported. Antagonistic *Streptomyces*, *Micromonospora*, *Streptosporangium,* and *Dactylosporangium* were isolated from five caves (Cheondong, Kosoo, Nadong, Seonglyu, and Ssangyong) in Korea (Kim et al., 1998). They showed activity against at least one of plant pathogenic fungi (*Alternaria solani*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* f.sp. *lycopersici*, *Magnaporthe grisea*, *Phytophthora capsici,* and *Rhizoctonia solani*). Similarly, members of genera *Streptomyces* and *Janibacter* isolated from limestone deposit sites in Hundung, Manipur, India were reported to show anticandidal and biocontrol activities against rice fungal pathogens (*Curvularia oryzae, F. oxysporum, Helminthosporum oryzae, Pyricularia oryzae, R. pryzae-sativae,* and *R. solani*) as well as antibacterial activity (Nimaichand et al., 2015). However, *Amycolatopsis*, *Rhodococcus,* and *Pseudonocardia* isolates showed only biocontrol activity against rice fungal pathogen. Recently, five *Streptomyces* spp. from Chaabe cave in Algeria was reported to produce non-polyenic antifungal substances active against *C. albicans* (Belyagoubi et al., 2018).

CONCLUSION AND FUTURE PERSPECTIVES

Emerging and re-emerging infectious diseases are threatening human society at an alarming rate. It is a call of emergency to find an effective cure for these pathogens. Actinobacteria are proving again to be prolific producers of promising bioactive compounds with widely application. Cave and karst environments are underexplored microbiologically and should not be overlooked for the search and discovery of novel actinobacteria and their chemical diversity of useful compounds. It is evident from this mini-review that cave environments harbor novel and diverse actinobacteria (**Table 1**). These actinobacteria offer a rich source of bioactive compounds as exemplified in **Table 2**. We opine that in order to explore cave actinobacteria to their full potential, 2 major research area must be addressed. The first area of research should deal with the ability to isolate and cultivate actinobacteria of interest. It is well-accepted that most microorganisms could not be cultivated in laboratory. The isolation and cultivation of bioactive producing actinobacteria under laboratory conditions represent the first challenge. Currently, the isolation strategy specifically for cave actinobacteria is lacking. There is still an urgent need for an improved selective isolation to target specific actinobacterial taxa of interest and extended our ability to tap into the majority of these uncultivable bacteria. Modification of growth conditions and use of new culturing methods were proposed for cultivation of previously uncultivable microorganisms (Pham and Kim, 2012). A combination of enrichment techniques including heat-pretreatments of samples, adjusting media pH and calcium salts supplements were effectively applied to isolate rare actinobacteria from karstic caves (Fang et al., 2017b).

The advancement of next generation sequencing and accumulation of high quality whole genome data provide a powerful tool and useful information to support the search for novel bioactive metabolites for drug development. Currently, these genome data of actinobacteria reveal the presence of several biosynthetic gene clusters of secondary metabolites and reaffirm status of actinobacteria as prolific producers of bioactive compounds. However, these gene clusters are not normally expressed under laboratory conditions. Many secondary metabolites encoded by these gene clusters remain unidentified in fermentation broth (Scherlach and Hertweck, 2009; Ren et al., 2017). Therefore, the second challenge lies in our ability to activate these silent gene clusters. Recently, specific biological and chemical stimuli namely exposure to antibiotics, metals and mixed microbial culture, were successfully employed to activate secondary metabolites production in cave actinobacteria (Covington et al., 2018). Evidently, the study on cave actinobacteria and their bioactive compounds is still at an early stage. There still remains room for further study to

guarantee cave actinobacteria as producers of new bioactive compounds for the benefit of human well-being.

AUTHOR CONTRIBUTIONS

PR contributed data for selective isolation, novel taxa, bioactive metabolites and **Tables 1**, **2**. WP conceived the idea, wrote, and revised the whole manuscript.

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REFERENCES

Abdelmohsen, U. R., Bayar, K., and Hentschel, U. (2014). Diversity, abundance and natural products of marine sponge-associatd actinomycetes. *Nat. Prod. Rep.* 31, 381–399. doi: 10.1039/C3NP70111E

Adam, D., Maciejewska, M., Naôm, é, A., Martinet, L., Coppieters, W., Karim, L., et al. (2018). Isolation, characterization, and antibacterial activity of hard-to-culture actinobacteria from cave moonmilk deposits. *Antibiotics* 7:28. doi: 10.3390/antibiotics7020028

Axenov-Gibanov, D., Voytsekhovskaya, I. V., Tokovenko, B. T., Protasov, E. S., Gamaiunov, S. V., Rabets, Y. V., et al. (2016). Actinobacteria isolated from an underground lake moonmilk speleothem from the biggest conglomeratic karstic cave in Siberia as sources of novel biologically active compounds. *PLoS ONE* 11:e0149216. doi: 10.1371/journal.pone.0149216

Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.-P., et al. (2016). Taxonomy, physiology, and natural products of actinobacteria. *Microbiol. Mol. Biol. Rev.* 80, 1–43. doi: 10.1128/MMBR.00019-15

Barton, A. H., Taylor, M. R., and Pace, N. R. (2004). Molecular phylogenetic analysis of a bacterial community in an oligotrophic cave environment. *Geomicrobiol. J.* 21, 11–20. doi: 10.1080/01490450490253428

Barton, H. A. (2006). Introduction to cave microbiology: a review for the non-specialist. *J. Cave Karst Stud.* 68, 43–54.

Belyagoubi, L., Belyagoubi-Benhammou, N., Jurada, V., Dupont, J., Lacost, S., Djebbah, F., et al. (2018). Antimicrobial activities of culturable microorganisms (actinomycetes and fungi) isolated from Chaabe cave, Algeria. *Int. J. Speleol.* 47, 189–199. doi: 10.5038/1827-806X.47.2.2148

Bulina, T. I., Alferova, I. V., and Terkhova, L. P. (1997). A novel approach to isolation of actinomycetes involving irradiation of soil samples with microwaves. *Microbiology* 66, 231–234.

Cao, C. L., Zhou, X. Q., Qin, S., Tao, F. X., Jiang, J. H., and Lian, B. (2015). *Lentzea guizhouensis* sp. nov., a novel lithophilous actinobacterium isolated from limestone from the karst area, Guizhou, China. *Antonie van Leeuwenhoek* 108, 1365–1372. doi: 10.1007/s10482-015-0589-x

Castro, J. F., Razmilic, V., Gomez-Escribano, J. P., Andrews, B., Asenjo, J., and Bibb, M. (2018). The 'gifted' actinomycetes *Streptomyces leeuwenhoekii*. *Antonie van Leeuwenhoek* 111, 1433–1488. doi: 10.1007/s10482-018-1034-8

Cheeptham, N., Sadoway, T., Rule, D., Watson, K., Moote, P., Soliman, L. C., et al. (2013). Cure from the cave: volcanic cave actinomycetes and their potential in drug discovery. *Int. J. Speleol.* 42, 35–47. doi: 10.5038/1827-806X.42.1.5

Cheng, J., Chen, W., Zhang, B. H., Nimaichand, S., Zhou, E. M., Lu, X. H., et al. (2013a). *Microlunatus cavernae* sp. nov., a novel actinobacterium isolated from Alu ancient cave, Yunnan, south-west China. *Antonie van Leeuwenhoek* 104, 95–101. doi: 10.1007/s10482-013-9929-x

Cheng, J., Zhang, Y. G., Chen, W., Li, L., Zhang, D. F., Wang, H. F., et al. (2013b). *Saccharopolyspora cavernae* sp. nov., a novel actinomycete isolated from the Swallow cave in Yunnan, south-west China. *Antonie van Leeuwenhoek* 104, 837–843. doi: 10.1007/s10482-013-9996-z

Covington, B. C., Spraggins, J. M., Ynigez-Gutierrez, A. E., Hylton, Z. B., and Bachmann, B. O. (2018). Response of secondary metabolism of hypogean actinobacterial genera to chemical and biological stimuli. *Appl. Environ. Microbiol.* 84, e01125–e01118. doi: 10.1128/AEM.01125-18

Derewacz, D. K., McNees, C. R., Scalmani, G., Covington, C. L., Shanmugam, G., Marnett, L. J., et al. (2014). Structure and stereochemical determination of hypogeamicins from a cave-derived actinomycete. *J. Nat. Prod.* 77, 1759–1763. doi: 10.1021/np400742p

Duangmal, K., Mingma, R., Pathom-aree, W., Niyomvong, N., Inahashi, Y., Matsumoto, A., et al. (2012). *Microbispora thailandensis* sp. nov., an actinomycete isolate from cave soil. *J. Antibiot.* 65, 491–494. doi: 10.1038/ja.2012.57

Fang, B. Z., Han, M. X., Liu, L., Zhang, Z. T., Liu, W. L., Shen, J. T., et al. (2017a). *Lentzea covernae* sp. nov., an actinobacterium isolated from a karst cave sample, and emended description of the genus *Lentzea*. *Int. J. Syst. Evol. Microbiol.* 67, 2357–2362. doi: 10.1099/ijsem.0.002364

Fang, B. Z., Nimaichand, S., Han, M. X., Jiao, J. Y., Cheng, J., Wei, D. Q., et al. (2017b). Insights on the effects of heat pretreatment, pH, and calcium salts on isolation of rare *Actinobacteria* from karstic caves. *Front. Microbiol.* 8:1535. doi: 10.3389/fmicb.2017.01535

Genilloud, O. (2017). Actinomycetes: still a source of novel antibiotics. *Nat. Prod. Rep.* 34, 1203–1232. doi: 10.1039/C7NP00026J

Ghosh, S., Kuisiene, N., and Cheeptham, N. (2017). The cave microbiome as a source for drug discovery: reality or pipe dream? *Biochem. Pharmacol.* 134, 18–34. doi: 10.1016/j.bcp.2016.11.018

Groth, I., and Saiz-Jimenez, C. (1999). Actinomycetes in hypogean environments. *Geomicrobiol. J.* 16, 1–8. doi: 10.1080/014904599270703

Groth, I., Schumann, P., Schuetze, B., Augsten, K., Kramer, I., and Stackebrandt, E. (1999). *Beutenbergia cavernae* gen. *nov.,* sp. nov., an L-lysine-containing actinomycete isolated from a cave. *Int. J. Syst. Bacteriol.* 49, 1733–1740. doi: 10.1099/00207713-49-4-1733

Groth, I., Schumann, P., Schutze, B., Augsten, K., and Stackebrandt, E. (2002). *Knoellia sinensis* gen. nov., sp. nov. and *Knoellia subterranea* sp. nov., two novel actinocacteria isolated from a cave. *Int. J. Syst. Evol. Microbiol.* 52, 77–84. doi: 10.1099/00207713-52-1-77

Han, M. X., Fang, B. Z., Tian, Y., Zhang, W. Q., Jiao, J. Y., Liu, L., et al. (2017). *Nocardioides cavernae* sp. nov., an actinobacterium isolated from a karst cave. *Int. J. Syst. Evol. Microbiol.* 67, 633–639. doi: 10.1099/ijsem.0.001676

Herold, K., Gollmick, F. A., Groth, I., Roth, M., Menzel, K. D., Mollmann, U., et al. (2005). Cervimycin A-D: a polyketide glycoside complex from a cave bacterium can defeat vancomycin resistance. *Chem. Eur. J.* 11, 5523–5530. doi: 10.1002/chem.200500320

Huang, J. R., Ming, H., Li, S., Zhao, Z. L., Meng, X. L., Zhang, J. X., et al. (2016). *Amycolatopsis xuchangensis* sp. nov. and *Amycolatopsis jiguanensis* sp. nov., isolated from soil. *Antonie van Leeuwenhoek* 109, 1423–1431. doi: 10.1007/s10482-016-0742-1

Hug, J. J., Bader, C. D., Remskar, M., Cirnski, K., and Muller, R. (2018). Concepts and methods to access novel antibiotics from actinomycetes. *Antibiotics* 7:44. doi: 10.3390/antibiotics7020044

Inokoshi, J., Takagi, Y., Uchida, R., Masuma, R., Omure, S., and Tomoda, H. (2010). Production of a new type of amidepsine with a sugar moiety by static fermentation of *Humicola* sp. FO-2942. *J. Antibiot.* 63, 9–16. doi: 10.1038/ja.2009.110

Jiang, L., Pu, H., Xiang, J., Su, M., Yan, X., Yang, D., et al. (2018). Huanglongmycin A-C, cytotoxic polyketides biosynthesized by a putative type II polyketide synthase from *Streptomyces* sp. CB09001. *Front. Chem.* 6:254. doi: 10.3389/fchem.2018.00254

Jiang, Y., Li,., Q., Chen, X., and Jiang, C. (2016). "Isolation and cultivation methods of Actinobacteria" in *Actinobacteria- Basics and Biotechnological Applications*, ed. D. Dhanasekaran (Rijeka: InTech), 39–57.

Jiang, Z. K., Guo, L., Chen, C., Liu, S. W., Zhang, L., Dai, S. J., et al. (2015). Xiakemycin A, a novel pyranonaphthoquinone antibiotic, produced by the

Streptomyces sp. CC8-201 from the soil of a karst cave. *J. Antibiot.* 68, 771–774. doi: 10.1038/ja.2015.70

Jurado, V., , Kroppenstedt, R. M., Jimenez, C. S., Klenk, H.-P., Mounieé, D., Laiz, L., et al. (2009). *Hoyosella altamirensis* gen. nov., sp. nov., a new member of the order *Actinomycetales* isolated from a cave bioflim. *Int. J. Syst. Evol. Microbiol.* 59, 3105–3110. doi: 10.1099/ijs.0.008664-0

Jurado, V., Boiron, P., Kroppenstedt, R. M., Laurent, F., Couble, A., Laiz, L., et al. (2008). *Nocardia altamirensis* sp. nov., isolated from Altamira cave, Cantabria, Spain. *Int. J. Syst. Evol. Microbiol.* 58, 2210–2214. doi: 10.1099/ijs.0.65482-0

Jurado, V., Groth, I., Gonzalez, J. M., Laiz, L., and Jimenez, C. S. (2005). *Agromyces subbeticus* sp. nov., isolated from a cave in southern Spain. *Int. J. Syst. Evol. Microbiol.* 55, 1897–1901. doi: 10.1099/ijs.0.63637-0

Kay, S., Pathom-aree, W., and Cheeptham, N. (2013). Screening of volcanic cave actinomycetes for antimicrobial activity against *Paenibacillus larvae*, a honey bee pathogen. *Chiang Mai J. Sci.* 40. 26–33.

Kemung, H. M., Tan, L. T. H., Khan, T. M., Chan, K. G., Pusparajah, P., Goh, B. H., et al. (2018). *Streptomyces* as a prominent resource of future anti-MRSA drugs. *Front. Microbiol.* 9:2221. doi: 10.3389/fmicb.2018.02221

Kim, B. S., Lee, J. Y., and Hwang, B. K. (1998). Diversity of actinomycetes antagonistic to plant pathogenic fungi in cave and sea-mud soils of Korea. *J. Microbiol.* 36, 86–92.

Ko, D. H., and Lee, S. D. (2017). *Augustibacter spluncae* sp. nov., isolated from a lava cave stalactite. *Int. J. Syst. Evol. Microbiol.* 67, 3283–3288. doi: 10.1099/ijsem.0.002108

Ko, K. S., Kim, Y., Seong, C. N., and Lee, S. D. (2015). *Rhodococcus antrifimi* sp. nov., isolated from dried bat dung of a cave. *Int. J. Syst. Evol. Microbiol.* 65, 4043–4048. doi: 10.1099/ijsem.0.000534

Laiz, L., Gonzalez-Delvalle, M., Hermosin, B., Ortiz-Martinez, A., and Saiz-Jimenez, C. (2003). Isolation of cave bacteria and substrate utilization at different temperature. *Geomicrobiol. J.* 20, 479–489. doi: 10.1080/713851125

Laiz, L., Groth, I., Gonzalez, I., and Saiz-Jimenez, C. (1999). Microbiological study of the dripping waters in Altamira cave (Santillana del mar, Spain). *J. Microbiol. Methods* 36, 129–138. doi: 10.1016/S0167-7012(99)00018-4

Lee, S. D. (2006a). *Actinocorallia cavernae* sp. nov., isolated from a natural cave in Jeju, Korea. *Int. J. Syst. Evol. Microbiol.* 56, 1085–1088. doi: 10.1099/ijs.0.63895-0

Lee, S. D. (2006b). *Amycolatopsis jejuensis* sp. *nov. and Amycolatopsis halotolerans* sp. nov., novel actinomycetes isolated from a natural cave. *Int. J. Syst. Evol. Microbiol.* 56, 549–553. doi: 10.1099/ijs.0.63881-0

Lee, S. D. (2006c). *Nocardia jejuensis* sp. nov., novel actinomycetes isolated from a natural cave on Jeju island, Republic of Korea. *Int. J. Syst. Evol. Microbiol.* 56, 559–562. doi: 10.1099/ijs.0.63866-0

Lee, S. D. (2008). *Jiangella alkaliphila* sp. nov., an actinobacterium isolated from a cave. *Int. J. Syst. Evol. Microbiol.* 58, 1176–1179. doi: 10.1099/ijs.0.65479-0

Lee, S. D. (2013a). *Humibacter antri* sp. nov., an actinobactrium isolated from a natural cave, and emended description of the genus *Humibacter*. *Int. J. Syst. Evol. Microbiol.* 63, 4315–4319. doi: 10.1099/ijs.0.050708-0

Lee, S. D. (2013b). *Spelaeicoccus albus* gen. nov., sp. nov., an actinobacterium isolated from a natural cave. *Int. J. Syst. Evol. Microbiol.* 63, 3958–3963. doi: 10.1099/ijs.0.050732-0

Lee, S. D. (2015). *Antricoccus suffuscus* gen. nov., sp. nov., isolated from a natural cave. *Int. J. Syst. Evol. Microbiol.* 65, 4410–4416. doi: 10.1099/ijsem.0.000588

Lee, S. D. (2017). *Conexibacter stalactiti* sp. nov., isolated from stalactites in a lava cave and emended description of the genus *Conexibacter*. *Int. J. Syst. Evol. Microbiol.* 67, 3214–3218. doi: 10.1099/ijsem.0.002083

Lee, S. D., Kang, S. O., and Hah, Y. C. (2000a). *Catellatospora koreensis* sp. nov., a novel actinomycete isolated from a gold-mine cave. *Int. J. Syst. Evol. Microbiol.* 50, 1103–1111. doi: 10.1099/00207713-50-3-1103

Lee, S. D., Kim, E. S., Roe, J. H., Kim, J. H., Kang, S. O., and Hah, Y. C. (2000b). *Saccharothrix violacea* sp. nov., isolated from a gold mine cave, and *Saccharothrix albidocapillata* comb. nov. *Int. J. Syst. Evol. Microbiol.* 50, 1315–1323. doi: 10.1099/00207713-50-3-1315

Li, Q. Q., Han, M. X., Fang, B. Z., Jiao, J. Y., Liu, L., Yang, Z. W., et al. (2017). *Nocardia cavernae* sp. nov., a novel actinobacterium isolated from a karst cave sample. *Int. J. Syst. Evol. Microbiol.* 67, 2998–3003. doi: 10.1099/ijsem.0.002072

Li, W. J., Nimaichand, S., Jiang, Z., Liu, M. J., Khieu, T. N., Kim, C. J., et al. (2014). *Streptomyces canchipurensis* sp. nov., isolated from a limestone habitat. *Antonie van Leeuwenhoek* 106, 1119–1126. doi: 10.1007/s10482-014-0281-6

Li, X. J., Dai, S. J., Liu, S. W., Liu, J. M., Chen, L., Hu, L., et al. (2016). *Tenggerimyces flavus* sp. nov., isolated from soil in a karst cave, and emended description of the genus *Tenggerimyces*. *Int. J. Syst. Evol. Microbiol.* 66, 1499–1505. doi: 10.1099/ijsem.0.000908

Maciejewska, M., Adam, D., Martinet, L., Naome, A., Calusiriska, M., Delfosse, P., et al. (2016). A phenotypic and genotypic analysis of the antimicrobial potential of cultivable *Streptomyces* isolated from cave moonmilk deposits. *Front. Microbiol.* 7:1455. doi: 10.3389/fmicb.2016.01455

Maciejewska, M., Pessi, I. S., Arias, A. A., Noirfalise, P., Luis, G., Ongena, M., et al. (2015). *Streptomyces lunaelactis* sp. nov., a novel ferroverdin A-producing *Streptomyces* species isolated from a moonmilk speleothem. *Antonie van Leeuwenhoek* 107, 519–531. doi: 10.1007/s10482-014-0348-4

Margesin, R., Schumann, P., Spröer, C., and Gounot, A. M. (2004). *Arthrobacter psychrophenolicus* sp. nov., isolated from an alpine ice cave. *Int. J. Syst. Evol. Microbiol.* 54, 2067–2072. doi: 10.1099/ijs.0.63124-0

Mingma, R., Duangmal, K., Také, Inahashi, Y., Ōmura, S., Takahashi, Y., and Matsumoto, A. (2016). Proposal of *Sphaerimonospora cavernae* gen. nov., sp. nov. and transfer of *Microbispora mesophila* (Zhang et al., 1984). *Sphaerimonospora mesophila* comb. nov. and *Microbispora thailandensis* (Duangmal et al., 2012) to *Sphaerimonospora thailandensis* comb. nov. *Int. J. Syst. Evol. Microbiol.* 66, 1735–1744. doi: 10.1099/ijsem.0.000935

Moir, A., and Smith, D. A. (1990). The genetics of bacterial spore germination. *Annu. Rev. Microbiol.* 44, 531–553. doi: 10.1146/annurev.mi.44.100190.002531

Nakaew, N., Pathom-aree, W., and Lumyong, S. (2009a). First record of the isolation, identification and biological activity of a new strain of *Spirillospora albida* from Thai cave soils. *Actinomycetologica* 23, 1–7. doi: 10.3209/saj.SAJ230102

Nakaew, N., Pathom-aree, W., and Lumyong, S. (2009b). Generic diversity of rare actinomycetes from Thai cave soils and their possible use as new bioactive compounds. *Actinomycetologica* 23, 21–26. doi: 10.3209/saj.SAJ230201

Nakaew, N., Sungthong, R., Yokota, A., and Lumyong, S. (2012). *Nonomuraea monospora* sp. nov., an actinomycete isolated from cave soil in Thailand, and emended description of the genus *Nonomuraea*. *Int. J. Syst. Evol. Microbiol.* 62, 3007–3012. doi: 10.1099/ijs.0.035220-0

Nimaichand, S., Devi, A. M., Tamreihao, K., Ningthoujam, D. S., and Li, W.-J. (2015). Actinobacterial diversity in limestone deposit sites in Hundung, Manipur (India) and their antimicrobial activities. *Front. Microbiol* 6:413. doi: 10.3389/fmicb.2015.00413

Nimaichand, S., Sanasam, S., Zheng, L.-Q., Zhu, W.-Y., Yang, L.-L., Tang, S.-K., et al. (2013a). *Rhodococcus canchipurensis* sp. nov., an actinomycete isolated from a limestone deposit site. *Int. J. Syst. Evol. Microbiol.* 63, 114–118. doi: 10.1099/ijs.0.036087-0

Nimaichand, S., Tamrihao, K., Yang, L. L., Zhu, W. Y., Zhang, Y. G., Li, L., et al. (2013b). *Streptomyces hundungensis* sp. nov., a novel actinomycete with antifungal activity and plant growth promoting traits. *J. Antibiot.* 66, 205–209. doi: 10.1038/ja.2012.119

Nimaichand, S., Zhang, Y.-G., Cheng, J., Li, L., Zhang, D.-F., Zhou, E.-M., et al. (2013c). *Micromonospora kangleipakensis* sp. nov., isolated from a sample of limestone quarry. *Int. J. Syst. Evol. Microbiol.* 63, 4546–4551. doi: 10.1099/ijs.0.052746-0

Nimaichand, S., Zhu, W. Y., Yang, L. L., Ming, H., Nie, G. X., Tang, S. K., et al. (2012). *Streptomyces manipurensis* sp. nov., a novel actinomycete isolated from a limestone deposit site in Manipur, India. *Antonie van Leeuwenhoek* 102, 133–139. doi: 10.1007/s10482-012-9720-4

Niyomvong, N., Pathom-aree, W., Thamchaipenet, A., and Duangmal, K. (2012). Actinomycetes from tropical limestone caves. *Chiang Mai J. Sci.* 39, 373–388.

Northup, D. E., and Lavoie, K. H. (2001). Geomicrobiology of caves: a review. *Geomicrobiol. J.* 18, 199–222. doi: 10.1080/01490451552467750

Passari, A. K., Misha, V. K., Gupta, V. K., and Singh, B. P. (2018). "Chapter 1: methods used for the recovery of culturable endophytic actinobacteria: an overview," in *Actinobacteria: Diversity and Biotechnological Applications: New and Future Developments in Microbial Biotechnology and Bioengineering*, eds B. P. Singh, A. K. Passari, and V. K. Gupta (Amsterdam: Elsevier), 1–11.

Patel, K., Laille, R., Martin, M. T., Tilvi, S., Moriou, C., Gallard, J. F., et al. (2010). Unprecedented stylissazoles A-C from *Stylissa carteri*: another dimension for marine pyrrole-2-aminoimadazole metabolite diversity. *Angew. Chem. Int. Ed.* 49, 4775–4779. doi: 10.1002/anie.201000444

Pham, V. H., and Kim, J. (2012). Cultivation of unculturable soil bacteria. *Trends Biotechnol.* 30, 475–484. doi: 10.1016/j.tibtech.2012.05.007

Quadri, S. R., Tian, X. P., Zhang, J., Li, J., Nie, G. X., Tang, S. K., et al. (2015). *Nonomuraea indica* sp. nov., novel actinomycetes isolated from lime-stone open pit mine, India. *J. Antibiot.* 68, 491–495. doi: 10.1038/ja.2015.24

Ren, H., Wang, B., and Zhao, H. (2017). Breaking the silence: New strategies for discovering novel natural products. *Curr. Opin. Biotechnol.* 48, 21–27. doi: 10.1016/j.copbio.2017.02.008

Riquelme, C., Dapkevicius, M. L. E., Miller, A. Z., Charlop-Powers, Z., Brady, S., Mason, C., et al. (2017). Biotechnological potential of actinobacteria from Canadian and Azorean volcanic caves. *Appl. Microbiol. Biotechnol.* 101, 843–857. doi: 10.1007/s00253-016-7932-7

Schabereiter-Gurtner, C., Saiz-Jimenez„ C., Pinar, G., Lubitz, W., and Röllke, S. (2002). Altamira cave paleolithic paintings harbor partly unknown bacterial communities. *FEMS Microbiol. Lett.* 211, 7–11. doi: 10.1111/j.1574-6968.2002.tb11195.x

Scherlach, K., and Hertweck, C. (2009). Triggering cryptic natural product biosynthesis in microorganisms. *Org. Biomol. Chem.* 7, 1753–1760. doi: 10.1039/b821578b

Seo, J. P., Yun, Y. W., and Lee, S. D. (2007). *Nocardia speluncae* sp. nov., isolated from a cave. *Int. J. Syst. Evol. Microbiol.* 57, 2932–2935. doi: 10.1099/ijs.0.65085-0

Stankovic, N., Radulovic, V., Petkovic, M., Vuckovic, I., Jadranin, M., Vasiljevic, B., et al. (2012). *Streptomyces* sp. JS520 produces exceptionally high quantities of undecylprodigiosin with antibacterial, antioxidative, and UV-protective properties. *Appl. Microbiol. Biotechnol.* 96, 1217–1231. doi: 10.1007/s00253-012-4237-3

Takahashi, Y., and Nakashima, T. (2018). Actinomycetes, an inexhaustible source of naturally occurring antibiotics. *Antibiotics* 7:45. doi: 10.3390/antibiotics7020045

Také, A., Inahashi, Y., Ōmura, S., Takahashi, Y., and Matsumoto, A. (2018). *Streptomyces boninensis* sp. nov., isolated from soil from a limestone cave in the Ogasawara islands. *Int. J. Syst. Evol. Microbiol.* 68, 1795–1799. doi: 10.1099/ijsem.0.002753

Tawari, K., and Gupta, R. K. (2013). Diversity and isolation of rare actinomyctes: an overview. *Crit. Rev. Microbiol.* 39, 256–294. doi: 10.3109/1040841X.2012.709819

Tomczyk-Zak, K., and Zielenkiewicz, U. (2016). Microbial diversity in caves. *Geomicrobiol. J.* 33, 20–38. doi: 10.1080/01490451.2014.1003341

Tomoda, H., and Omura, S. (2001). "Screening for inhibitors of lipid metabolism," in *Enzyme Technologies for Pharmaceutical and Biotechnological Applications,* eds. H.A. Kirst, W.K. Yeh, M.J. Zmijewski, Jr. (New York, NY: Marcel Dekker Inc), 343–378.

Tuo, L., Liu, S. W., Liu, J. M., Zhang, Y. Q., Jiang, Z. K., Liu, X. F., et al. (2015). *Lysinibacter cavernae* gen. nov., sp. nov., a new member of the family *Microbacteriaceae* isolated from a karst cave. *Int. J. Syst. Evol. Microbiol.* 65, 3305–3312. doi: 10.1099/ijsem.0.000415

Velikonja, B. H., Tkave, R., and Pasic, L. (2014). Diversity of cultivable bacteria involved in the formation of macroscopic microscopic microbial colonies (cave silver) on the walls of a cave in Slovenia. *Int. J. Speleol.* 43, 45–46. doi: 10.5038/1827-806X.43.1.5

Wang, D. S., Xue, Q. H., Zhu, W. J., Zhao, J., Duan, J. L., and Shen, G. H. (2013). Microwave irradiation is a useful tool for improving isolation of actinomycetes from soil. *Microbiology* 82, 102–110. doi: 10.1134/S0026261712060161

Wang, Z. G., Wang, Y. X., Liu, J. H., Chen, Y. G., Zhang, X. X., Wen, M. L., et al. (2009). *Fodinibacter luteus* gen. *n*ov., sp. nov., an actinobactrium isolated from a salt mine. *Int. J. Syst. Evol. Microbiol.* 59, 2185–2190. doi: 10.1099/ijs.0.006882-0

Yücel, S., and Yamac, M. (2010). Selection of *Streptomyces* isolates from Turkish karstic caves against antibiotic resistant microorganisms. *Pak. J. Pharm. Sci.* 23, 1–6.

Zhang, L. Y., Ming, H., Meng, X. L., Fang, B. Z., Jiao, J. Y., Nimaichand, S., et al. (2018). *Ornithinimicrobium cavernae* sp. nov., an actinobactrium isolated from a karst cave. *Antonie van Leeuwenhoek.* 112, 179-186. doi: 10.1007/s10482-018-1141-6

Zhang, W. Q., Li, Y. Q., Liu, L., Nimaichand, S., Fang, B. Z., Wei, D. Q., et al. (2016). *Stackebrandtia cavernae* sp. nov., a novel actinobacterium isolated from a karst cave sample. *Int. J. Syst. Evol. Microbiol.* 66, 1206–1211. doi: 10.1099/ijsem.0.000859

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Bioactivities and Extract Dereplication of *Actinomycetales* Isolated From Marine Sponges

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In the beginning of the twenty-first century, humanity faces great challenges regarding diseases and health-related quality of life. A drastic rise in bacterial antibiotic resistance, in the number of cancer patients, in the obesity epidemics and in chronic diseases due to life expectation extension are some of these challenges. The discovery of novel therapeutics is fundamental and it may come from underexplored environments, like marine habitats, and microbial origin. *Actinobacteria* are well-known as treasure chests for the discovery of novel natural compounds. In this study, eighteen *Actinomycetales* isolated from marine sponges of three *Erylus* genera collected in Portuguese waters were tested for bioactivities with the main goal of isolating and characterizing the responsible bioactive metabolites. The screening comprehended antimicrobial, anti-fungal, anti-parasitic, anti-cancer and anti-obesity properties. Fermentations of the selected strains were prepared using ten different culturing media. Several bioactivities against the fungus *Aspergillus fumigatus*, the bacteria *Staphylococcus aureus* methicillin-resistant (MRSA) and the human liver cancer cell line HepG2 were obtained in small volume cultures. Screening in higher volumes showed consistent anti-fungal activity by strain *Dermacoccus* sp. #91-17 and *Micrococcus luteus* Berg02-26. *Gordonia* sp. Berg02-22.2 showed anti-parasitic (*Trypanosoma cruzi*) and anti-cancer activity against several cell lines (melanoma A2058, liver HepG2, colon HT29, breast MCF7 and pancreatic MiaPaca). For the anti-obesity assay, *Microbacterium foliorum* #91-29 and #91-40 induced lipid reduction on the larvae of zebrafish (*Danio rerio*). Dereplication of the extracts from several bacteria showed the existence of a variety of secondary metabolites, with some undiscovered molecules. This work showed that *Actinomycetales* are indeed good candidates for drug discovery.

Keywords: *Actinomycetales*, antimicrobials, anti-cancer, anti-parasitic, anti-obesogenic, marine sponges

INTRODUCTION

The technological advances made in the twenty and twenty-first centuries gave rise to the most prosperous society which has ever existed (International Monetary Fund Research Department [IMF], 2000). This well-being is associated with an overall drastic increase in the average life expectancy (Vaupel, 2010) and with several problems faced by the today's society. One of these problems is a result of the increase in caloric uptake and lack of exercise which results in an epidemic of overweight. Obese people present major challenges, as obesity entails a myriad of risk factors for chronic diseases like diabetes, heart diseases and even some cancers (Hruby and Hu, 2015). Furthermore, the extended life span associated with environmental factors such as tobacco smoking, industrialization and urbanization are causes behind the increase in cancer incidence rates (You and Henneberg, 2018). Antibiotic resistance is another emerging problem. Overabundance in the use of antibiotics as well as their intense misuse resulted in the phenomenon of antibiotic resistance, which is rapidly occurring worldwide, with many commonly used antibiotics having already been rendered useless (Ventola, 2015). Several are thus the challenges at the beginning of the twenty-first century.

In recent years, research into new compounds has been focused in the ocean and many marine organisms are proving to be good sources of interesting new leads (Imhoff et al., 2011) Drugs like xestospongin C and several manoalides were discovered in marine animals like sponges (Miyamoto et al., 2000; Stowe et al., 2011). Sponges have a diverse microbiological community, often showing the presence of archaea, fungi, microalgae and a great diversity of bacterial phyla (Taylor et al., 2007). While sponges are good candidates for drug discovery, a major setback is that sponges may only contain minute quantities of these compounds in their body, which may invalidate clinical trials (Mehbub et al., 2014). However, it seems that the symbiotic microbiological community is the true origin of several relevant compounds (Yoo Kyung et al., 2001). It is also known that *Actinobacteria* are copious producers of bioactive metabolites. Particularly prolific are species isolated from soil and affiliated to the *Actinomycetales*, most notably the genus *Streptomyces* (Miao and Davies, 2010). Less studied are marine *Actinobacteria* which have nonetheless already shown potential as sources for novel leads. Several bioactive molecules, ranging from antimalarians like salinipostins, cytotoxics such as marinomycins to antibacterials as abyssomicins, have all been isolated from *Actinobacteria* found in marine environments (Bister et al., 2004; Kwon et al., 2006; Schulze et al., 2015; Dhakal et al., 2017).

This study aimed to contribute to the need of finding new and more effective bioactive molecules against several of the earlier mentioned threats faced by human kind nowadays. For this, eighteen species from the *Actinomycetales* previously isolated from *Erylus* spp. sponges collected in Portuguese marine waters (Açores, Madeira, and continental shelf) were screened for antimicrobial, anti-cancer, anti-parasitic and anti-obesogenic activities.

MATERIALS AND METHODS

Biological Material

The bacteria under study belong to the order *Actinomycetales* within the phylum *Actinobacteria* and were isolated from marine sponges of the genus *Erylus, E. discophorus* (Berg01 and Berg02, from the continental shelf at Berlengas, Portugal) and *E. deficiens* (#91, from the continental shelf at Gorringe, Portugal) and *E. mamillaris* (SM, Açores, Portugal) (**Table 1**). Most of these strains showed the presence of interesting secondary metabolism genes and/or bioactivities in previous antimicrobial screenings (**Table 1**; Graca et al., 2013, 2015).

For the antimicrobial assays six different pathogens were tested, *Escherichia coli* ATCC25922 (EcoWT), *Klebsiella*

TABLE 1 | List of *Actinomycetales* used in this study evidencing their physiological affiliation and bioactive potential.

Strain ID	Affiliation	Secondary metabolism gene	Previous bioactivity
#91_17*	*Dermacoccus* sp. Ellin185; AF409027	NRPS	CA
#91_20	*Rhodococcus hoagii* CUB1156; AJ272469	N/D	NA
#91_29*	*Microbacterium foliorum* BJC15-C14; JX464206	N/D	CA
#91_31*	*Microbacterium hydrocarbonoxydans* 3084; EU714352	N/D	CA
#91_34*	*Microbacterium esteraromaticum* 2122; EU714337	N/D	CA
#91_35*	*Microbacterium phyllosphaerae (T)*; DSM 13468; P 369/06; AJ277840	PKS-I	CA; VA
#91_36.1*	*Rhodococcus equi* type strain: DSM20307; X80614	PKS-I; NRPS	CA; VA; EC
#91_37*	*Microbacterium foliorum* BJC15-C1; JX401513	N/D	CA; VA
#91_40*	*Microbacterium foliorum* BJC15-C14; JX464206	PKS-I	CA; VA
#91_44	*Rhodococcus* sp.	PKS-I; NRPS	NA
#91_54	*Rhodococcus qingshengii* KUDC1814; KC355321	NRPS	CA, VA
SM 115	*Agrococcus baldri* B-G-NA10	PKS-I	NA
SM 116	*Agrococcus baldri* B-G-NA10	PKS-I	NA
Berg01-119c	*Microbacterium sp.* ZJY-409	N/D	VA; VF
Berg02-22.2*	*Gordonia sp.* DEOB200; AY927227	PKS-I	BS
Berg02-26	*Micrococcus luteus;* KCL-1; DQ538135	N/D	NA
Berg02-78*	*Gordonia terrae* 3269aBRRJ; FJ200386	PKS-I; NRPS	BS
Berg02-79*	*Microbacterium sp.* M63-2; EF061897	N/D	BS

**Strains assayed for anti-obesity; PKS-I, Polyketide Synthase I; NRPS, Non-ribosomal Peptide Synthetases; N/D, Not determined; NA, No Activity; CA, Candida albicans; BS, Bacillus subtilis; EC, Escherichia coli; VA, Vibrio anguillarum; VF, Vibrio fisheri.*

pneumoniae ATCC 700603, Methicillin-resistant *Staphylococcus aureus* MB5393 (MRSA), *Staphylococcus aureus* ATCC29213 (MSSA), *Acinetobacter. baumannii* MB5973 *Aspergillus fumigatus* ATCC46645, and *Candida albicans* ATCC64124. For the anti-cancer assays the following cell lines were tested: human melanoma (A2058), human lung carcinoma (A549), human hepatocellular carcinoma (HepG2), human colon cancer (HT29), human breast cancer (MCF7), and human pancreatic cancer (MIA PaCa-2). Anti-parasitic activity was assessed using the parasite *Trypanosoma cruzi* Tulahuen C4 strain. As *T. cruzi* is an obligate intracellular parasite, it was cultivated inside host L6 rat skeletal muscle cells. Anti-obesogenic activity was tested using the zebrafish (*Danio rerio*) larvae.

Growth Media

The various *Actinomycetales* were cultivated and maintained in Marine Agar (MA) medium (Graca et al., 2013, 2015) at 25°C in darkness.

To conduct the screenings, liquid fermentations of the strains were prepared, using ten different culturing media. These different media provide a range of nutritional conditions (from oligotrophic to heterotrophic status) aiming to favor the production of bioactive metabolites. These include several ready prepared media such as Antibiotic Broth (AB) (1.0 g/L dextrose, 3.68 g/L K_2HPO_4, 1.5 g/L meat extract, 5.0 g/L peptone, 1.32 g/L KH_2PO_4, 3.5 g/L NaCl and 1.5 g/L yeast extract), Tryptic Soy Broth (TSB) (17 g/L tryptone, 3 g/L phytone, 5 g/L NaCl, 2.5 g/L K_2HPO_4 and 2.5 g/L glucose) and Marine Broth (MB) (5 g/L peptone, 1 g/L yeast extract, 40 g/L sea salts) CGY (Rojas et al., 2009), DEF-15 (Lam et al., 1995), IN-CRY (Obata et al., 1999), R358 (Jensen et al., 2007), and other media specified in **Table 2**. Moreover, as all the strains were previously isolated from a marine environment, Sea salts (Sigma-Aldrich) were added to all the media at a concentration of 30 g/L, except in the medium MB.

TABLE 2 | Composition of the media used for liquid culture extractions.

Reagents (g/L)	Medium		
	FPY-12	M016	R2A
Yeast Extract	–	1.0	$5.0^{*}\ 10^{-1}$
Casein hydrolysate	–	–	$5.0^{*}\ 10^{-1}$
Glucose	10	10	$5.0^{*}\ 10^{-1}$
Fructose	20	–	–
Maltose	10	10	–
Peptone	5	–	$5.0^{*}\ 10^{-1}$
Amicase	5	–	–
Starch	–	10	$5.0^{*}\ 10^{-1}$
Soytone	–	5.0	–
Tryptone	–	4.0	–
K_2HPO_4	–	$2.0^{*}\ 10^{-1}$	$3.0^{*}\ 10^{-1}$
NaCl	–	$2.0^{*}\ 10^{-2}$	–
KCl	–	$2.0^{*}\ 10^{-5}$	–
$MgSO_4.7H_2O$	–	$5.0^{*}\ 10^{-2}$	$4.0^{*}\ 10^{-2}$
KH_2PO_4	–	$1.0^{*}\ 10^{-1}$	–
$CaCl_2.H_2O$	–	$5.0^{*}\ 10^{-2}$	–
$C_3H_3NaO_3$	–	–	$3.0^{*}\ 10^{-1}$
$FeSO_4.7H_2O$	$5.0^{*}\ 10^{-4}$	–	–
$ZnCl_2$	–	$2.0^{*}\ 10^{-5}$	–
$ZnSO_4.7H_2O$	$5.0^{*}\ 10^{-4}$	–	–
$MnSO_4.H_2O$	$1.0^{*}\ 10^{-4}$	$1.0^{*}\ 10^{-4}$	–
$CuSO_4.5H_2O$	$5.0^{*}\ 10^{-5}$	–	–
$CoCl_2.6H_2O$	$5.0^{*}\ 10^{-5}$	$2.0^{*}\ 10^{-5}$	–
$SnCl_2.2H_2O$	–	$5.0^{*}10^{-6}$	–
H_3BO_3	–	$1.0^{*}\ 10^{-5}$	–
$Na_2MoO_4.2H_2O$	–	$1.2^{*}\ 10^{-5}$	–
$CuSO_4$	–	$1.5^{*}\ 10^{-5}$	–
$FeCl_3$	–	$5.8^{*}\ 10^{-3}$	–

Extraction Protocols

Small Scale-Culture Extraction

A first small-scale fermentation in 0.8 mL cultures was performed. Bacterial strains were grown in the 10 media in 96-well plates (Duetz system[1]) (Duetz et al., 2000; Minas et al., 2000; Duetz, 2007; Palomo et al., 2013; Pan et al., 2019) for 5 days at 25°C, 20% humidity and 300 revolutions per minute (rpm). After incubation, 0.8 mL acetone were added to each bacterial culture. The acetone/culture mixture was mixed for 1 h at 170 rpm and later evaporated up to a final volume of 0.6 mL in a Genevac® centrifugal evaporator. Due to a large presence of salts that might influence subsequent assays, the 0.6 mL were captured in a Waters™ Oasis® HLB extraction plates, with the sorbent Oasis® HLB. The Oasis® HLB plate was equilibrated using first methanol and after HPLC grade water. The extract was run though the Oasis® HLB plate in 0.2 mL steps, vacuum-eluted and the collected volume discarded. Subsequently, the secondary metabolites were captured with methanol. The methanol was, then removed in a Genevac® and the recovered metabolites were dissolved in 0.4 mL 20% DMSO. This allowed a twofold concentration increase of the extracts. Additionally, as controls, culture media were also extracted with the same protocol.

[1]www.enzyscreen.com

Medium-Scale Culture Extraction

Bacteria that showed bioactive extracts were re-fermented in higher volumes (EPA vials system – 40 mL). Ten milliliters of bacterial cultures were grown for 5 days at 25°C with 20% humidity and 220 rpm. After the 5 days period, 10 mL acetone were added (1:1) to each culture and mixed for 1 h. The acetone/culture mixture was then evaporated under nitrogen to 9 milliliters to remove all traces of acetone. To remove the salt contamination, the resin SEPABEADS® SP207ss was used. Two milliliters of a ready prepared suspension of SP207ss were added to the culture and mixed for 1 h. The vials were then centrifuged, and the supernatant discarded. The resin was washed with HPLC water. Ten mL of acetone were added to the resin, centrifuged and the acetone was collected, evaporated under nitrogen and dissolved in 2 mL of 20% DMSO. A fivefold increase of the extracts was achieved. As controls, media samples were also extracted with the same protocol.

Anti-obesogenic Assays Culture Extraction

To test possible anti-obesity compounds produced by the selected strains, each strain was grown in 100 mL Marine Broth at 25°C, 200 rpm for 5 days. For the extraction, cell pellets were lyophilized and mixed twice for 30 min with 50 mL methanol. The collected methanol was dried in a rotatory evaporator and the weight of the extract measured. The extracts were stored at −20°C and, when needed, dissolved in 100% DMSO at a concentration of 10 mg/mL.

Bioactivity Assays

Antimicrobial Assays

Previously described methods using pathogenic microorganisms from Fundación MEDINA's collection were performed to test for antibacterial and antifungal properties (Monteiro et al., 2012; Audoin et al., 2013). Briefly, single colonies of each microorganism were incubated overnight at 37°C and 220 rpm in their corresponding medium and then diluted in order to obtain assay inoculum of approximately 1.1×10^6 CFU/mL for methicillin-resistant *S. aureus* MB 5393, 5.0×10^5 CFU/mL for *A. baumannii* MB5973, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603 and *S. aureus* ATCC 29213 and 2.5×10^4 CFU/mL for *A. fumigatus* ATCC46645. For *C. albicans*, the OD at 660 nm of the liquid culture was adjusted to 0.25 and diluted 1:100 for assay inoculum.

For all the assays 90 μL/well of the corresponding diluted inoculum were mixed with 10 μL/well of extract. Positive and negative internal plate controls were included following the previously described methodologies. Absorbance or fluorescence were measured with an Envision plate reader. Genedata Screener software (Genedata, Inc., Basel, Switzerland) was used to analyze the data and to calculate the percentage of growth inhibition of the extracts and the RZ' factor to estimate the robustness of the assays (Zhang et al., 1999). In all experiments performed in this work, the RZ' factor obtained was between 0.87 and 0.92.

Anti-cancer Assays

The cytotoxic bioactivity of the extracts was tested by using the portfolio of HTS assays from MEDINA according to Cautain et al. (2015). Cytotoxic activity of the extracts (5 μL in 19 μL medium) was tested against human hepatocellular carcinoma (HepG2) for Duetz extracts and against human melanoma (A2058), human lung carcinoma (A549), human hepatocellular carcinoma (HepG2), human colon cancer (HT29), human breast cancer (MCF7), and human pancreatic cancer (MIA PaCa-2) for EPA vials extracts.

Anti-parasitic Assays

Anti-parasitic bioactivity of the extracts (5 μL of 1:1500 dilution) was tested against *T. cruzi* Tulahuen C4 strain according to Annang et al. (2015).

Anti-obesogenic Assays

The anti-obesity activity of the extracts taken from 11 strains arbitrarily chosen (**Table 1**) was tested using zebrafish larvae as described in Urbatzka et al. (2018). Briefly, hatched larvae were transferred to 48-well plates, 6–8 individual larvae per well, with 750 μL water and N-phenylthiourea. Larvae were then treated with 10 μg/mL extracts or 0.1% DMSO or 50 μM Resveratrol for 48 h. At 24 h, the solutions in the wells were renewed and 10 ng/ml Nile red added. For imaging, the larvae were anesthetized with 0.03% tricaine, fluorescence intensity acquired in a fluorescence microscope (Leica DM6000B) and the images analyzed with the software ImageJ. Statistical significance was evaluated with an ANOVA and Dunnett's test.

Dereplication

Dereplication of the extracts was performed by Liquid Chromatography/High-Resolution Mass Spectroscopy (LC/HRMS) which was performed in an Agilent 1200 Rapid Resolution HPLC interfaced to a Bruker maXis mass spectrometer. The column used for separation was a Zorbax SB-C8 column (2.1 × 30 mm, 3.5 mm particle size), with two solvents used for the mobile phase. Both solvents were composed of water and acetonitrile in a 90:10 ratio for solvent A and in a 10:90 ratio for B. Both solvents contained 13 mM ammonium formiate and 0.01% trifluoracetic acid. The mass spectrometer was operated in positive ESI mode. The retention time and exact mass of the components were compared against Fundación MEDINA's high resolution mass spectrometry database, and when a match was obtained it was reported as a named compound. For the components with no matches in the MEDINA database, the predicted molecular formula and exact mass were searched for in the Chapman and Hall Dictionary of Natural Products database. If a plausible match was found, considering the exact mass/molecular formula, the producing microorganism and the target assay, the molecule was reported as a suggested component of the fraction (Perez-Victoria et al., 2016).

RESULTS

Small-scale extracts (0.8 mL cultures, performed in Duets plates) are useful for a rapid evaluation of the bioactive profile of the strains. However, due to the small volume of the extracts, only a limited number of assays could be performed. For this reason, only the antimicrobial and anti-parasitic assays were run with the full number of targets, while in the anti-cancer assay, only the HepG2 cell line was assayed. This cell line is used as a model system for studies of liver toxicity (Mersch-Sundermann et al., 2004) which is an important characteristic of the drugability of any molecule.

From the 18 different strains tested in the 10 media, only 6 strains demonstrated bioactivity in one or more of the assays performed (**Table 3**). Antifungal activity against *A. fumigatus* ATCC46645 was obtained in several media with strains #91_17, Berg02-26, Berg02-22.2, and Berg02-78. Strain Berg02-79 showed activity against the methicillin-resistant *S. aureus* (MRSA) and strains #91_40, Berg02-22.2 Berg02-78 and Berg02-79 were effective against HepG2 cells. Berg02-78 extracts proved to be bioactive against *T. cruzi* Tulahuen C4. As extracts from strains #91_40, Berg02-22.2, Berg02-78, and Berg02-79 in medium

IN-CRY proved to be hepatotoxic, no further testing was done with these extracts.

With the medium scale extracts (10 mL culture – EPA vials) and as the volume was no longer a limitation, all microbiological, parasitic and cancer cell lines targets and assays were tested. Antifungal activity against *A. fumigatus* ATCC46645 and *C. albicans* ATCC64124 was obtained with strains #91_17 (in media CGY and M016) and Berg02-26 (in medium IN-CRY) (**Table 4**). Regarding the anticancer assays, the extracts from AB medium of strain Berg02-22.2 showed activity against human melanoma cell line (A2058), human hepatocellular carcinoma cell line (HepG2), human colon cancer cell line (HT29), human breast cancer cell line (MCF7), and human pancreatic cancer cell line (MIA PaCa-2) (**Table 4**). No activity was observed against the human lung carcinoma cell line (A549). Furthermore, strain Berg02-22.2 also showed anti-parasitic activity against *T. cruzi* Tulahuen C4 (**Table 4**). With the medium scale extracts, the bioactivities previously detected in the small-scale extracts of strains Berg02-78 and Berg02-79 were not confirmed (**Tables 3**, **4**).

The results of the anti-obesity activity obtained are shown in **Figures 1**, **2**. Larvae incubated with only DMSO, the solvent control of the experiment, showed an intense red fluorescent staining which was considered as the 100% level of lipid content (**Figure 1B**). As DMSO has low toxicity and does not affect lipid accumulation on zebrafish larvae, as shown by Jones et al. (2008), it is the commonly used solvent in the fish embryo tests (Kais et al., 2013). When larvae were treated with final concentration of 50 μM resveratrol, the positive control of the experiment, a complete absence of red fluorescent staining was visible (**Figures 1D**, **2**) which is indicative of reduction of lipid accumulation.

TABLE 3 | Summary of the bioactivities obtained with the Duetz extracts.

Strain ID	Affiliation	Bioactivity		
		Target	Medium	% inhibition or death
#91_17	*Dermacoccus* sp.	AF	CGY	66
			IN-CRY	86
			M016	60
#91_40	*Microbacterium foliorum*	HepG2	IN-CRY	66
Berg02-22.2	*Gordonia* sp.	AF	AB	64
		HepG2	IN-CRY	62
Berg02-26	*Micrococcus luteus*	AF	IN-CRY	69
			R358	57
Berg02-78	*Gordonia terrae*	AF	AB	51
		TC	IN-CRY	50
Berg02-79	*Microbacterium* sp.	MRSA	R358	100
		HepG2	IN-CRY	56

AF, A. fumigatus ATCC46645; CA, C. albicans ATCC64124; MRSA, Methicillin-resistant S. aureus MB 5393; TC, T. cruzi Tulahuen C4.

TABLE 4 | Summary of the bioactivities obtained with the EPA extracts.

Strain ID	Affiliation	Bioactivity		
		Target	Medium	% inhibition or death
#91_17	*Dermacoccus* sp.	AF	CGY	67
			M016	93
		CA	CGY	82
			M016	100
Berg02-22.2	*Gordonia* sp.	TC	AB	91
		A2058	AB	71
		HepG2	AB	88
		HT29	AB	78
		MCF7	AB	82
		MIAPaCa-2	AB	72
Berg02-26	*Micrococcus luteus*	AF	IN-CRY	58
		CA	IN-CRY	75

AF, A. fumigatus ATCC46645; CA, C. albicans ATCC64124; MRSA, Methicillin-resistant S. aureus MB 5393; TC, T. cruzi Tulahuen C4.

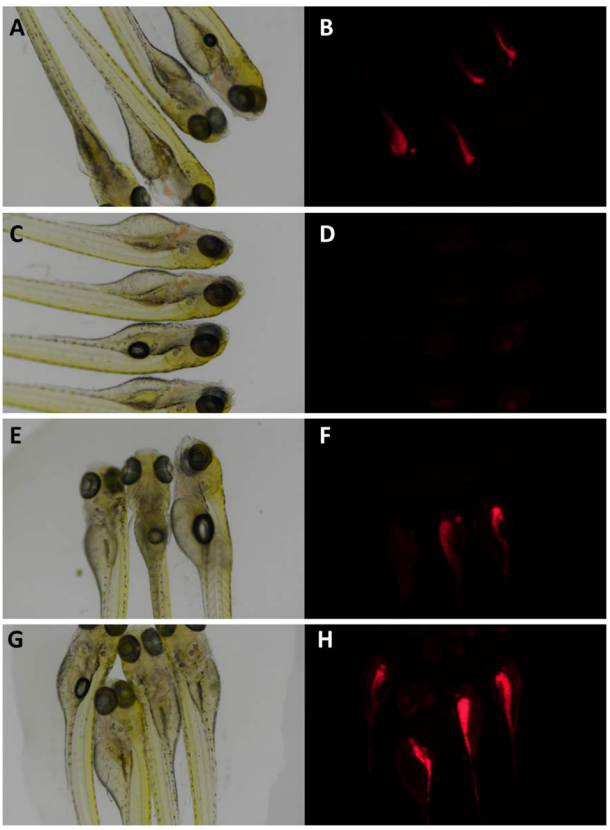

FIGURE 1 | Zebrafish larvae under brightfield **(A,C,E,G)** and fluorescence microscopy **(B,D,F,H)**. In **(A,B)** larvae were exposed only to DMSO, showing a normal lipid buildup, situated mostly among the lower abdomen; in **(C,D)** larvae were exposed to resveratrol, showing a decrease of neutral lipid staining; in **(E,F)** larvae were exposed to extract from strain #91–40; in **(G,H)** larvae were exposed to extract from strain #91-17. These larvae showed a visible decrease and increase in fluorescence when compared to the DMSO treated larvae, respectively. This implies that the extract altered the accumulation of lipids in treated larvae.

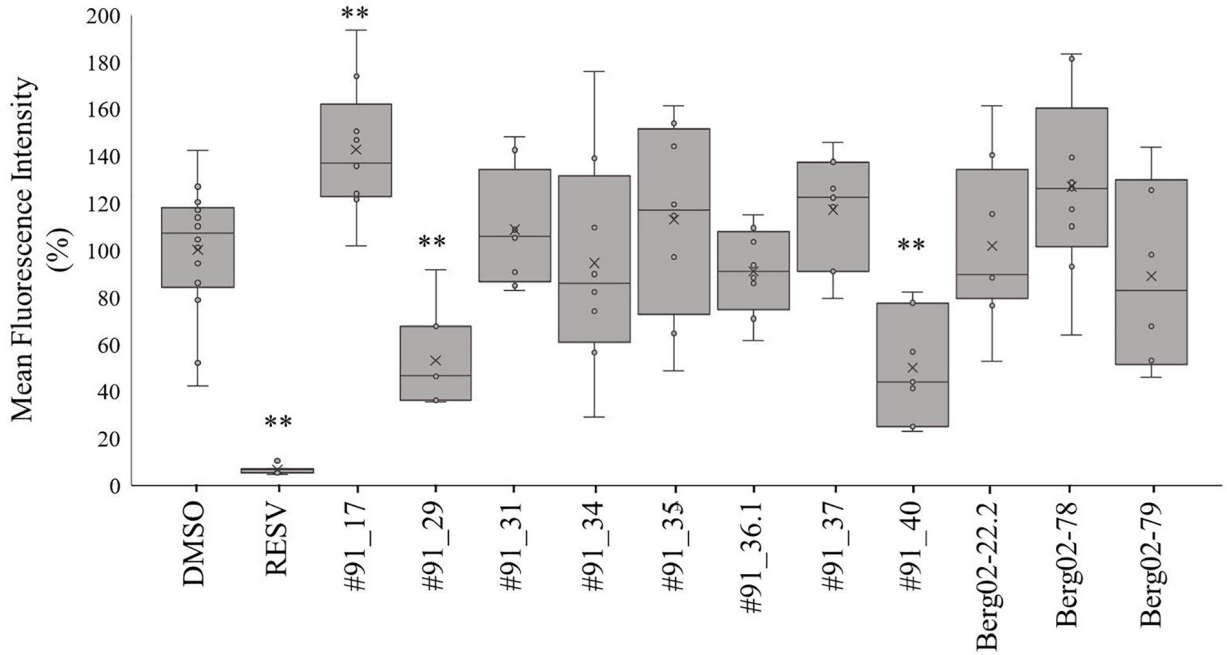

FIGURE 2 | Percentage of fluorescent staining of neutral lipids in zebrafish larvae. Values are presented as mean fluorescence intensity relative to the DMSO group in a box-whisker plot. Statistical differences are represented as asterisks, $^{**}p < 0.01$.

The zebrafish larvae treated with the majority of extracts did not show different fluorescence levels compared to the DMSO control. However, extracts from two strains, #91_29 and #91_40, reduced significantly the level of fluorescence by 47% and 50% (**Figures 1F**, **2**), respectively. The extract from strain #91_17 induced a significant increase (43%) in fluorescence levels (**Figures 1H**, **2**). Statistical analysis showed that these results are statistically different from the control [$F_{[19,\ 133]} = 8.97$; $p < 0.01$].

HPLC-HRMS results showed that the extract from strain #91_17 (antifungal activity and increase in neutral lipids) has a high complexity of already identified molecules (**Table 5**) which are several diketopiperazines and the plant hormone indole acetic acid (IAA). The four diketopiperazines identified in both the extracts from media AB and M016 were cyclo(prolyltyrosyl), cyclo([iso]leucylprolyl), cyclo(phenylalanylprolyl), and cyclo(prolylvalyl). Moreover, four components with the following formulae, $C_{41}H_{60}N_2O_9$, $C_{43}H_{64}N_2O_9$, $C_{22}H_{38}N_4O_5$, and $C_{22}H_{21}N_3O_3$, were also identified in the extracts. These are not described in the Chapman and Hall Dictionary of Natural Products database.

In the extract from the AB medium of strain Berg02-22.2 (antifungal in Duetz and antiparasitic and anticancer activity in EPA vials), both Nocardichelin A ($C_{40}H_{65}N_5O_8$) and B ($C_{38}H_{61}N_5O_8$) were found, as well as a component with $C_{42}H_{69}N_5O_8$ as molecular formula and UV spectrum and ionization pattern similar to both nocardachelins, suggesting that this component is a new nocardachelin not described previously (**Figure 3**). The peptide Gly-Pro-Phe-Pro-Ile and the diketopiperazines cyclo([iso]leucylprolyl), cyclo(phenylalanylprolyl) were also detected. Furthermore, components with the formulae $C_{52}H_{74}N_{10}O_{11}$, $C_{35}H_{28}N_6O_8$, $C_{28}H_{41}N_5O_{10}$ were found to be present.

The extract from the IN-CRY medium of strain Berg02-26 (antifungal activity) showed the presence of two diketopiperazines and two other compounds. Compound $C_{22}H_{21}N_3O_3$ seems to be related to the diketopiperazine cyclo(prolyltryrosyl), as the UV spectrum is similar. Component $C_{13}H_{14}N_2O_3$ matches caerulomycin G in the DNP. However, the UV spectrum is also similar to cyclo(prolyltryrosyl).

TABLE 5 | Dereplication of the selected active extracts.

Strain ID	Culture medium	Putatively detected components
#91_17	CGY	Cyclo(prolyltyrosyl), cyclo([iso]leucylprolyl), cyclo(phenylalanylprolyl), cyclo(prolylvalyl), 1*H*-indole-3-acetic acid, $C_{41}H_{60}N_2O_9$, and $C_{43}H_{64}N_2O_9$.
#91_17	M016	Cyclo(prolyltyrosyl), cyclo([iso]leucylprolyl), cyclo(phenylalanylprolyl), cyclo(prolylvalyl), 1*H*-indole-3-acetic acid, $C_{22}H_{38}N_4O_5$ and $C_{22}H_{21}N_3O_3$
Berg02-22.2	AB	Cyclo([iso]leucylprolyl), Cyclo(phenylalanylprolyl), $C_{52}H_{74}N_{10}O_{11}$, $C_{35}H_{28}N_6O_8$, $C_{28}H_{41}N_5O_{10}$, Gly-Pro-Phe-Pro-Ile peptide, nocardichelin A, nocardichelin B and an undescribed nocardichelin ($C_{42}H_{69}N_5O_8$).
Berg02-26	IN-CRY	Cyclo([iso]leucylprolyl), cyclo(prolyltyrosyl) $C_{22}H_{21}N_3O_3$ and $C_{13}H_{14}N_2O_3$ (coincidence with caerulomycin G.)

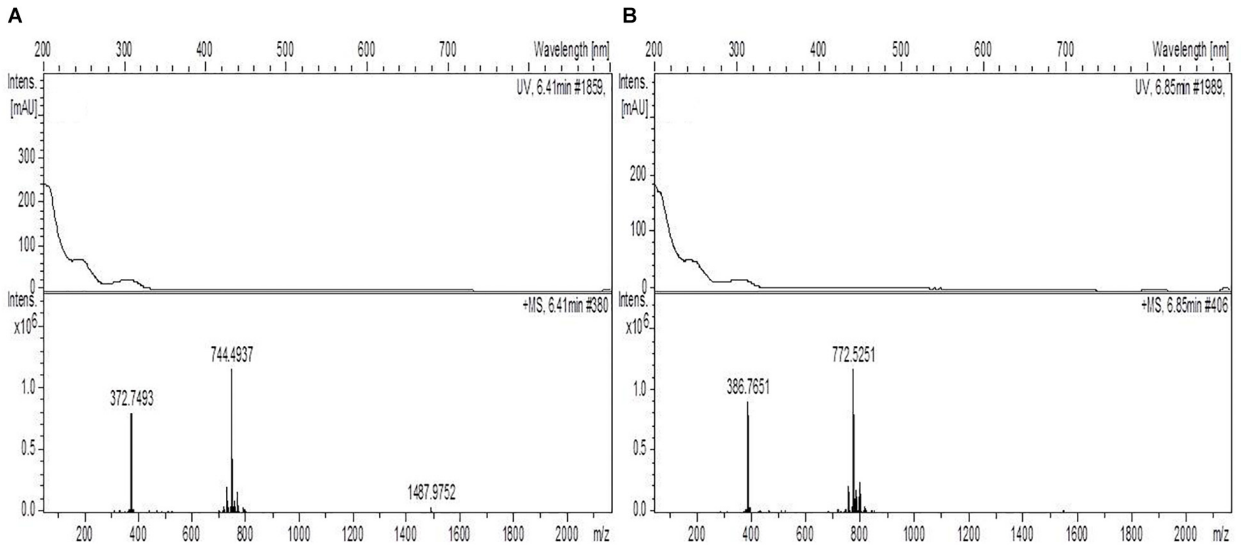

FIGURE 3 | UV and mass spectra of **(A)** nocardichelin A and **(B)** component $C_{42}H_{69}N_5O_8$ detected in the extract from the AB medium of strain Berg02-22.2. The comparison of the spectra shows the similarities between the two components which suggest that the component $C_{42}H_{69}N_5O_8$ is a new nocardichelin.

DISCUSSION

The selected strains under study all belong to the *Actinomycetales*, an order that is known for the production of a great number of useful secondary metabolites (Manivasagan et al., 2014; van Keulen and Dyson, 2014; Barka et al., 2016). Although these strains do not belong to the most prolific genera, *Streptomyces* (Ser et al., 2015; Tan et al., 2016; Lee et al., 2018) or *Salinispora* (Jensen et al., 2015), they hold great potential for novel pharmaceutically relevant molecules. The strains here studied were chosen based on previous molecular and screening evidences of bioactive potential (Graca et al., 2013, 2015). As evidenced in **Table 1**, many of the strains hold key genes to produce secondary metabolites, like polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) and demonstrated antimicrobial bioactivity.

For an overall view of the results obtained, bioactivities and extracts dereplication results are summarized in **Table 6**.

Our small-scale results not only confirmed the bioactive potential seen previously but proved that several of the selected strains possessed a variable repertoire of bioactivities (**Table 3**). In the small-scale extraction results, *Dermacoccus* sp. strain #91_17 showed activity against *A. fumigatus* but not against *C. albicans*, as had previously been shown (Graca et al., 2015). However, with medium-scale extraction, *C. albicans* bioactivity was restored (**Table 4**). Bioactive compounds have been discovered in *Dermacoccus* genus. *Dermacoccus nishinomiyaensis* has displayed the capability to produce monensins A and B, which are produced in a classic polyketide pathway (AlMatar et al., 2017). Monensin has been tested as a chelating agent for the treatment of lead poisoning, with promising results in mice (Ivanova et al., 2016). Phenazine-type pigments, dermacozines, have been isolated from *Dermacoccus abyssi*. Dermacozines F and G have been shown to induce moderate cytotoxic against leukemia cell line K562 (Manivasagan et al., 2014). Hence, *Dermacoccus* has shown to be a genus with a good potential biotechnological value. In this study, *Dermacoccus* sp. strain #91_17 did not display any anti-cancer bioactivity but good anti-fungal activity against *C. albicans* and *A. fumigatus*. Dereplication of the medium-scale extracts pointed to the presence of several bioactive diketopiperazines, the plant hormone IAA and some unidentified molecules (**Table 5**). The diketopiperazine cyclo(prolyltyrosyl) in the form of (3S, 8aS) can have antibacterial and cytotoxic effects (Bycroft et al., 1988). Cyclo(phenylalanylprolyl) can show different configurations, with one configuration (3R, 8aS) showing some phytotoxic activity and another (3S, 8aS) showing broad antibacterial activity and gastrointestinal cell maturation enhancing activity (Blunt and Munro, 2008). Cyclo(prolylvalyl) is an diketopiperazine antibiotic, with a wide spectrum of activity, with the form (3R, 8aR) active against *Vibrio anguillarum* and the (3S, 8aS) showing a broad antibacterial spectrum (Bycroft et al., 1988). In fact, *Dermacoccus* sp. strain #91_17 demonstrated activity against *V. anguillarum* in a previous study (Graca et al., 2015).

Gordonia sp. Berg02-22.2 and *Gordonia terrae* Berg02-78 possess at least one PKS-I gene and showed activity against *Bacillus subtilis* in the previous studies (Graca et al., 2013). Neither *Gordonia* did show any activity against any of the Gram-positive strains tested in our study (although *B. subtilis* was not tested) but, instead, displayed activity against *A. fumigatus* (**Table 3**). However, antifungal activity of EPA extracts from Berg02-22.2 and Berg02-78 was not observed. Different culture conditions such as the ones in Duetz and EPA can influence the production of bioactive metabolites (Wei et al., 2010). Yet, special relevance can be attributed to strain Berg02-22.2, as these extracts demonstrated anti parasitic and several different anti-cancer activities. Several bioactive capacities of *Gordonia* spp. were referred by Sowani et al. (2018), namely a great antimicrobial

TABLE 6 | Summarized results from the work.

Strain	Afiliation	Lipid assay activity	Bioctive Duetz extracts		Bioctive EPA extracts		Detected components in bioactive EPA extracts
			Extract	Bioactivity	Extract	Bioactivity	
#91_17	*Dermacoccus* sp.	−43%	CGY IN-CRY	AF	CGY	AF; CA	Cyclo(prolyltyrosyl), cyclo([iso]leucylprolyl), cyclo(phenylalanylprolyl), cyclo(prolylvalyl), 1H-Indole-3-acetic acid, C41H60N2O9 and C43H64N2O9
			M016		M016	AF; CA	Cyclo(prolyltyrosyl), cyclo([iso]leucylprolyl), cyclo(phenylalanylprolyl), cyclo(prolylvalyl), 1H-Indole-3-acetic acid, C22H38N4O5 and C22H21N3O3
Berg02-22.2	*Gordonia* sp.	NA	AB IN-CRY	AF HepG2	AB	TC A2058 HepG2 HT29 MCF7 MIAPaCa-2	Cyclo([iso]leucylprolyl), cyclo(phenylalanylprolyl), C52H74N10O11, C35H28N6O8, C28H41N5O10, the peptide Gly-Pro-Phe-Pro-Ile, nocardichelin A, nocardichelin B and C42H69N5O8
Berg02-78	*Gordonia terrae*	NA	AB IN-CRY	AF TC	N/T		N/T
#91_29	*Microbacterium foliorum*	47%	NA		NA		N/T
#91_40	*Microbacterium foliorum*	50%	IN-CRY	HepG2	N/T		N/T
Berg02-79	*Microbacterium* sp.	NA	R358 IN-CRY	MRSA HepG2	N/T		N/T
Berg02-26	*Micrococcus luteus*	NA	IN-CRY R358	AF	IN-CRY	AF CA	Cyclo([iso]leucylprolyl), cyclo(prolyltyrosyl), C22H21N3O3 and C13H14N2O3

AF, A. fumigatus ATCC46645; CA, C. albicans ATCC64124; MRSA, Methicillin-resistant S. aureus MB 5393; TC, T. cruzi Tulahuen C4, N/T, Not tested.

capacity of a strain of *G. terrae* isolated from a sponge (Elfalah et al., 2013). Furthermore, Sowani et al. (2018) also reported the various compounds already known to be produced by *Gordonia* genus. These include circumcin A, kurasoin B, soraphinol C; bendigole A, canthxanthin, c-carotene, glycolipids, peptidolipids, and exopolysaccharides. Our work also evidenced the anti-parasitic bioactivity of *Gordonia* sp. Berg02-22.2 against *T. cruzi*. *G. terrae* was isolated from the gut of a *Triatomore* sp. (Gumiel et al., 2015), which is the insect vectors of *T. cruzi* (Lent and Wygodzinsky, 1979). This actinobacterium along with other bacteria present in the gut microbiota of this insect are believed to play a role in the epidemiology of Chagas disease by competing with *T. cruzi* (Gumiel et al., 2015). Moreover, Davila et al. (2011) showed that immunized rats with *G. bronchialis* and challenged with *T. cruzi* had a reduction of parasitemia in offspring. The rat's immunological system was activated with increased levels of interferons and reduced levels of interleukins. *Gordonia* is, thus, a genus with an extensive biotechnological value. The dereplication of the extract of *Gordonia* sp. strain Berg02-22.2 points to the presence of both nocardichelin A and B, as well as a novel nocardichelin. Both nocardichelin A and B were first isolated from a strain from the genus *Nocardia* and were shown to strongly inhibit human cell lines from gastric adenocarcinoma, breast carcinoma, and hepatocellular carcinoma (Schneider et al., 2007). The peptide Gly-Pro-Phe-Pro-Ile was also detected. This peptide is the result of a pancreatic digestion of β-casein and has been described as a competitive inhibitor for cathepsin B which is upregulated in some cancers (Lee and Lee, 2000). The diketopiperazine cyclo(phenylalanylprolyl) was also detected. Furthermore, components with the formulae $C_{52}H_{74}N_{10}O_{11}$, $C_{35}H_{28}N_6O_8$, $C_{28}H_{41}N_5O_{10}$ were detected in the extract but are of an unknown nature.

Species of the genus *Micrococcus* produce pigmented colonies which are sources of carotenoid pigments. Rostami et al. (2016) showed that carotenoid pigments produced by *Micrococcus roseus* proved to have some antioxidant, antitumor and potent antibacterial activities, in particular against Gram-positive bacteria. *M. luteus* has very few genes associated with secondary metabolism and possesses one of the smallest genomes from the phylum *Actinobacteria*, comprised of a single circular chromosome with 2.5 Mb in size (Young et al., 2010). Bacteria with genome sizes below 3 Mb usually have fewer or none secondary metabolism genes, while above 5 Mb, there appears to exist a linear correlation between genome size and the presence of these genes (Donadio et al., 2007). This may imply a reduced capacity for the production of bioactive molecules for microorganisms like *M. luteus*. Nevertheless, in both our small-scale and medium-scale extractions, *M. luteus* Berg02-26 proved to produce anti-fungal bioactive molecules and no hepatotoxicity. The dereplication of the extract pointed to the presence of one bioactive diketopiperazine, cyclo(prolyltyrosyl). As discussed above, this diketopiperazine in the form of (3S, 8aS) can have antibacterial and cytoxic effects. Several unidentified molecules were also detected, with compound $C_{13}H_{14}N_2O_3$ showing a coincidence with caerulomycin G in the DNP. Caerulomycin G is a bypridine alkaloids with a range of activities against

E. coli, *Aerobacter aerogenes* and *C. albicans* and cytotoxic activity against the cell lines HL-60, A549 (Kim, 2013).

The small-scale extracts from *Microbacterium* sp. strain Berg02-79 displayed activity against methicillin-resistant *Staphylococcus aureus* and in the HepG2 cell line. Previous studies had shown activity against *Bacillus subtilis* (Graca et al., 2013). Curiously, the methicillin-sensitive strain *Staphylococcus aureus* was not affected by the extract. However, in the medium-scale assay no bioactivity was observed. Once again, the culture conditions may justify this different behavior. *Microbacterium foliorum*, strain #91_40, showed only hepatotoxic activity in the small-scale screening. Additionally, in the zebrafish fat metabolism assay, the extract from this strain displayed possible anti-lipid accumulation activity. Similarly, *Microbacterium foliorum* strain #91_29 also displayed this activity. The zebrafish fat metabolism assay was previously used to identify bioactive compounds from marine fungi and plant polyphenols and has the advantage to mirror the complexity of a whole small animal model and to indicate extracts/compounds with physiologically relevant activities (Noinart et al., 2017; Urbatzka et al., 2018). Natural products isolated from different organisms from marine environments were described to have anti-obesity activities (Castro et al., 2016). *Microbacterium* strains have already shown to possess a varied and interesting repertoire of bioactive secondary metabolites. Microbacterins A and B isolated from a deep-sea strain of *Microbacterium sediminis*, are peptaibols that showed significant inhibitory effects against human tumor cell lines like HCT-8, Bel-7402, BGC-823, A549, and A2780 (Liu et al., 2015). Peptaibols are a class of non-ribosomal linear or cyclic peptides. Additionally, several glycoglycerolipids were isolated from *Microbacterium* sp., and the glycoglycerol GGL.2 showed promising antitumor activities (Wicke et al., 2000).

As the Duetz extraction requires only a very small volume of culture, a great variety of growth conditions could be tested. From the 10 media used for culturing the *Actinomycetales*, only extracts from 5 media were bioactive but differently for each strain. These five media (AB, CGY, IN-CRY, M016, and R358) all have very different composition. With strain #91-17, media CGY, IN-CRY, and M016 were the ones that induced more bioactivities. For strain #91-40 it was medium IN-CRY. Berg02-22.2 and Berg02-78 had better secondary metabolite production in media AB and IN-CRY while Berg02-26 and Berg02-79 the production was favored by media IN-CRY and R358. Remarkably, in the maintenance medium (Marine Broth) no bacterium showed bioactivity. These results give further support to the fact that medium composition is crucial for the activation of the metabolic pathways to produce secondary metabolites. Furthermore, the results obtained illustrated quite well the extended biosynthetic potential of single strains through the "One strain/many compounds" approach (Wei et al., 2010).

The dereplication of our extracts revealed the complexity of molecules possibly produced by the bacterial strains. This molecular diversity can justify the various bioactivities (antifungal, anti-cancer, anti-parasitic, and anti-obesity) obtained. Many of the detected molecules likely possess already known bioactivity. This is the case of molecules like nocardichelins, diketopiperazines, and bipyridine alkaloids. Additionally, eight non-identified compounds were also detected. The variety of components encountered proves that the *Actinomycetales* tested are prolific biosynthesizers, reinforcing the bioactive characteristics of this group.

ETHICS STATEMENT

An approval by an ethics committee was not necessary for the presented work, since chosen procedures are not considered animal experimentation according to the EC Directive 86/609/EEC for animal experiments.

AUTHOR CONTRIBUTIONS

The design of the experiments were by JS, RU, FV, and OL. The performing of the scientific work by JS, IV, MC, CD, BC, FA, GP-M, IG, JT, JM, and RU. Manusript writing by JS, IV, MC, CD, BC, FA, GP-M, IG, JT, JM, RU, FV, and OL.

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REFERENCES

AlMatar, M., Eldeeb, M., Makky, E. A., Koksal, F., Var, I., and Kayar, B. (2017). Are there any other compounds isolated from *Dermacoccus* spp at All? *Curr. Microbiol.* 74, 132–144. doi: 10.1007/s00284-016-1152-3

Annang, F., Perez-Moreno, G., Garcia-Hernandez, R., Cordon-Obras, C., Martin, J., Tormo, J. R., et al. (2015). High-throughput screening platform for natural product-based drug discovery against 3 neglected tropical diseases: human African trypanosomiasis, leishmaniasis, and Chagas disease. *J. Biomol. Screen* 20, 82–91. doi: 10.1177/1087057114555846

Audoin, C., Bonhomme, D., Ivanisevic, J., de la Cruz, M., Cautain, B., Monteiro, M. C., et al. (2013). Balibalosides, an original family of glucosylated sesterterpenes produced by the mediterranean sponge Oscarella balibaloi. *Mar. Drugs* 11, 1477–1489. doi: 10.3390/md11051477

Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Meier-Kolthoff, J. P., et al. (2016). Taxonomy, physiology, and natural products of actinobacteria. *Microbiol. Mol. Biol. Rev.* 80, 1–43. doi: 10.1128/MMBR.00019-15

Bister, B., Bischoff, D., Strobele, M., Riedlinger, J., Reicke, A., Wolter, F., et al. (2004). Abyssomicin C-A polycyclic antibiotic from a marine *Verrucosispora* strain as an inhibitor of the p-aminobenzoic acid/tetrahydrofolate biosynthesis pathway. *Angew. Chem. Int. Ed. Engl.* 43, 2574–2576. doi: 10.1002/anie.200353160

Blunt, J. W., and Munro, M. H. G. (2008). *Dictionary of Marine Natural Products, with CD-ROM*. Boca Raton: Chapman & Hall/CRC.

Bycroft, B. W., Higton, A. A., and Roberts, A. D. (1988). *Dictionary of Antibiotics and Related Substances*. New York, NY: Chapman and Hall.

Castro, M., Preto, M., Vasconcelos, V., and Urbatzka, R. (2016). Obesity: the metabolic disease, advances on drug discovery and natural product research. *Curr. Top. Med. Chem.* 16, 2577–2604. doi: 10.2174/1568026616666160415155644

Cautain, B., de Pedro, N., Schulz, C., Pascual, J., Sousa Tda, S., Martin, J., et al. (2015). Identification of the lipodepsipeptide MDN-0066, a novel inhibitor of VHL/HIF pathway produced by a new *Pseudomonas* species. *PLoS One* 10:e0125221. doi: 10.1371/journal.pone.0125221

Davila, H., Didoli, G., Bottasso, O., and Stanford, J. (2011). Maternal immunization with actinomycetales immunomodulators reduces parasitemias in offspring challenged with Trypanosoma cruzi. *Immunotherapy* 3, 577–583. doi: 10.2217/imt.11.14

Dhakal, D., Pokhrel, A. R., Shrestha, B., and Sohng, J. K. (2017). Marine rare actinobacteria: isolation, characterization, and strategies for harnessing bioactive compounds. *Front. Microbiol.* 8:1106. doi: 10.3389/fmicb.2017.01106

Donadio, S., Monciardini, P., and Sosio, M. (2007). Polyketide synthases and nonribosomal peptide synthetases: the emerging view from bacterial genomics. *Nat. Prod. Rep.* 24, 1073–1109. doi: 10.1039/b514050c

Duetz, W. A. (2007). Microtiter plates as mini-bioreactors: miniaturization of fermentation methods. *Trends Microbiol.* 15, 469–475. doi: 10.1016/j.tim.2007.09.004

Duetz, W. A., Ruedi, L., Hermann, R., O'Connor, K., Buchs, J., and Witholt, B. (2000). Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. *Appl. Environ. Microbiol.* 66, 2641–2646. doi: 10.1128/AEM.66.6.2641-2646.2000

Elfalah, H., Usup, G., and Ahmad, A. (2013). Anti-microbial properties of secondary metabolites of marine gordonia tearrae extract. *J. Agric. Sci.* 5, 94–101. doi: 10.5539/jas.v5n6p94

Graca, A. P., Bondoso, J., Gaspar, H., Xavier, J. R., Monteiro, M. C., de la Cruz, M., et al. (2013). Antimicrobial activity of heterotrophic bacterial communities from the marine sponge *Erylus* discophorus (Astrophorida, Geodiidae). *PLoS One* 8:e78992. doi: 10.1371/journal.pone.0078992

Graca, A. P., Viana, F., Bondoso, J., Correia, M. I., Gomes, L., Humanes, M., et al. (2015). The antimicrobial activity of heterotrophic bacteria isolated from the marine sponge *Erylus* deficiens (Astrophorida, Geodiidae). *Front. Microbiol.* 6:389. doi: 10.3389/fmicb.2015.00389

Gumiel, M., da Mota, F. F., Rizzo Vde, S., Sarquis, O., de Castro, D. P., Lima, M. M., et al. (2015). Characterization of the microbiota in the guts of *Triatoma brasiliensis* and *Triatoma pseudomaculata* infected by *Trypanosoma cruzi* in natural conditions using culture independent methods. *Parasit. Vectors* 8, 245. doi: 10.1186/s13071-015-0836-z

Hruby, A., and Hu, F. B. (2015). The epidemiology of obesity: a big picture. *Pharmacoeconomics* 33, 673–689. doi: 10.1007/s40273-014-0243-x

Imhoff, J. F., Labes, A., and Wiese, J. (2011). Bio-mining the microbial treasures of the ocean: new natural products. *Biotechnol. Adv.* 29, 468–482. doi: 10.1016/j.biotechadv.2011.03.001

International Monetary Fund Research Department [IMF]. (2000). *World Economic Outlook, May 2000 : Asset Prices and the Business Cycle*. Washington, D.C: International Monetary Fund.

Ivanova, J., Gluhcheva, Y., Dimova, D., Pavlova, E., and Arpadjan, S. (2016). Comparative assessment of the effects of salinomycin and monensin on the biodistribution of lead and some essential metal ions in mice, subjected to subacute lead intoxication. *J. Trace Elem. Med. Biol.* 33, 31–36. doi: 10.1016/j.jtemb.2015.08.003

Jensen, P. R., Moore, B. S., and Fenical, W. (2015). The marine actinomycete genus *Salinispora*: a model organism for secondary metabolite discovery. *Nat. Prod. Rep.* 32, 738–751. doi: 10.1039/c4np00167b

Jensen, P. R., Williams, P. G., Oh, D. C., Zeigler, L., and Fenical, W. (2007). Species-specific secondary metabolite production in marine actinomycetes of the genus Salinispora. *Appl. Environ. Microbiol.* 73, 1146–1152. doi: 10.1128/AEM.01891-06

Jones, K. S., Alimov, A. P., Rilo, H. L., Jandacek, R. J., Woollett, L. A., and Penberthy, W. T. (2008). A high throughput live transparent animal bioassay to identify non-toxic small molecules or genes that regulate vertebrate fat metabolism for obesity drug development. *Nutr. Metab.* 5, 23. doi: 10.1186/1743-7075-5-23

Kais, B., Schneider, K. E., Keiter, S., Henn, K., Ackermann, C., and Braunbeck, T. (2013). DMSO modifies the permeability of the zebrafish (Danio rerio) chorion-implications for the fish embryo test (FET). *Aquat. Toxicol.* 14, 229–238. doi: 10.1016/j.aquatox.2013.05.022

Kim, S.-K. (2013). *Marine Microbiology : Bioactive Compounds and Biotechnological Applications*. Hoboken, NJ: Wiley. doi: 10.1002/9783527665259

Kwon, H. C., Kauffman, C. A., Jensen, P. R., and Fenical, W. (2006). Marinomycins A-D, antitumor-antibiotics of a new structure class from a marine actinomycete of the recently discovered genus "marinispora". *J. Am. Chem. Soc.* 128, 1622–1632. doi: 10.1021/ja0558948

Lam, K. S., Veitch, J. A., Golik, J., Rose, W. C., Doyle, T. W., and Forenza, S. (1995). Production and isolation of two novel esperamicins in a chemically defined medium. *J. Antibiot.* 48, 1497–1501. doi: 10.7164/antibiotics.48.1497

Lee, H. S., and Lee, K. J. (2000). Cathepsin B inhibitory peptides derived from beta-casein. *Peptides* 21, 807–809. doi: 10.1016/S0196-9781(00)00212-6

Lee, L. H., Chan, K. G., Stach, J., Wellington, E. M. H., and Goh, B. H. (2018). Editorial: the search for biological active agent(s) from actinobacteria. *Front. Microbiol.* 9:824. doi: 10.3389/fmicb.2018.00824

Lent, H., and Wygodzinsky, P. W. (1979). *Revision of the Triatominae (Hemiptera, Reduviidae), and their Significance as Vectors of Chagas' Disease*. New York, NY: American Museum of Natural History.

Liu, D., Lin, H., Proksch, P., Tang, X., Shao, Z., and Lin, W. (2015). Microbacterins A and B, new peptaibols from the deep sea actinomycete *Microbacterium sediminis* sp. *nov. YLB*-01(T). *Org. Lett.* 17, 1220–1223. doi: 10.1021/acs.orglett.5b00172

Manivasagan, P., Venkatesan, J., Sivakumar, K., and Kim, S. K. (2014). Pharmaceutically active secondary metabolites of marine actinobacteria. *Microbiol. Res.* 169, 262–278. doi: 10.1016/j.micres.2013.07.014

Mehbub, M. F., Lei, J., Franco, C., and Zhang, W. (2014). Marine sponge derived natural products between 2001 and 2010: trends and opportunities for discovery of bioactives. *Mar. Drugs* 12, 4539–4577. doi: 10.3390/md12084539

Mersch-Sundermann, V., Knasmuller, S., Wu, X. J., Darroudi, F., and Kassie, F. (2004). Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. *Toxicology* 198, 329–340. doi: 10.1016/j.tox.2004.02.009

Miao, V., and Davies, J. (2010). Actinobacteria: the good, the bad, and the ugly. *Antonie Van Leeuwenhoek* 98, 143–150. doi: 10.1007/s10482-010-9440-6

Minas, W., Bailey, J. E., and Duetz, W. (2000). Streptomycetes in micro-cultures: growth, production of secondary metabolites, and storage and retrieval in the 96-well format. *Antonie Van Leeuwenhoek* 78, 297–305. doi: 10.1023/A:1010254013352

Miyamoto, S., Izumi, M., Hori, M., Kobayashi, M., Ozaki, H., and Karaki, H. (2000). Xestospongin C, a selective and membrane-permeable inhibitor of IP(3) receptor, attenuates the positive inotropic effect of alpha-adrenergic stimulation in guinea-pig papillary muscle. *Br. J. Pharmacol.* 130, 650–654. doi: 10.1038/sj.bjp.0703358

Monteiro, M. C., de la Cruz, M., Cantizani, J., Moreno, C., Tormo, J. R., Mellado, E., et al. (2012). A new approach to drug discovery: high-throughput screening of microbial natural extracts against *Aspergillus fumigatus* using resazurin. *J. Biomol. Screen* 17, 542–549. doi: 10.1177/1087057111433459

Noinart, J., Buttachon, S., Dethoup, T., Gales, L., Pereira, J. A., Urbatzka, R., et al. (2017). A new ergosterol analog, a new bis-anthraquinone and

anti-obesity activity of anthraquinones from the marine sponge-associated fungus *Talaromyces* stipitatus KUFA 0207. *Mar. Drugs* 15, E139. doi: 10.3390/md15050139

Obata, H., Muryoi, N., Kawahara, H., Yamade, K., and Nishikawa, J. (1999). Identification of a novel ice-nucleating bacterium of Antarctic origin and its ice nucleation properties. *Cryobiology* 38, 131–139. doi: 10.1006/cryo.1999.2156

Palomo, S., Gonzalez, I., de la Cruz, M., Martin, J., Tormo, J. R., Anderson, M., et al. (2013). Sponge-derived Kocuria and *Micrococcus* spp. as sources of the new thiazolyl peptide antibiotic kocurin. *Mar. Drugs* 11, 1071–1086. doi: 10.3390/md11041071

Pan, R., Bai, X., Chen, J., Zhang, H., and Wang, H. (2019). Exploring structural diversity of microbe secondary metabolites using OSMAC strategy: a literature review. *Front. Microbiol.* 10:294. doi: 10.3389/fmicb.2019.00294

Perez-Victoria, I., Martin, J., and Reyes, F. (2016). Combined LC/UV/MS and NMR strategies for the dereplication of marine natural products. *Planta Med.* 82, 857–871. doi: 10.1055/s-0042-101763

Rojas, J. L., Martin, J., Tormo, J. R., Vicente, F., Brunati, M., Ciciliato, I., et al. (2009). Bacterial diversity from benthic mats of Antarctic lakes as a source of new bioactive metabolites. *Mar. Genom.* 2, 33–41. doi: 10.1016/j.margen.2009.03.005

Rostami, H., Hamedi, H., and Yolmeh, M. (2016). Some biological activities of pigments extracted from Micrococcus roseus (PTCC 1411) and *Rhodotorula glutinis* (PTCC 5257). *Int. J. Immunopathol. Pharmacol.* 29, 684–695. doi: 10.1177/0394632016673846

Schneider, K., Rose, I., Vikineswary, S., Jones, A. L., Goodfellow, M., Nicholson, G., et al. (2007). Nocardichelins A and B, siderophores from nocardia strain acta 3026. *J. Nat. Prod.* 70, 932–935. doi: 10.1021/np060612i

Schulze, C. J., Navarro, G., Ebert, D., DeRisi, J., and Linington, R. G. (2015). Salinipostins A-K, long-chain bicyclic phosphotriesters as a potent and selective antimalarial chemotype. *J. Org. Chem.* 80, 1312–1320. doi: 10.1021/jo5024409

Ser, H. L., Palanisamy, U. D., Yin, W. F., Abd Malek, S. N., Chan, K. G., Goh, B. H., et al. (2015). Presence of antioxidative agent, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- in newly isolated *Streptomyces* mangrovisoli sp. nov. *Front. Microbiol.* 6:854. doi: 10.3389/fmicb.2015.00854

Sowani, H., Kulkarni, M., and Zinjarde, S. (2018). An insight into the ecology, diversity and adaptations of *Gordonia* species. *Crit. Rev. Microbiol.* 44, 393–413. doi: 10.1080/1040841X.2017.1418286

Stowe, S. D., Richards, J. J., Tucker, A. T., Thompson, R., Melander, C., and Cavanagh, J. (2011). Anti-biofilm compounds derived from marine sponges. *Mar. Drugs* 9, 2010–2035. doi: 10.3390/md9102010

Tan, L. T., Chan, K. G., Lee, L. H., and Goh, B. H. (2016). *Streptomyces* bacteria as potential probiotics in aquaculture. *Front. Microbiol.* 7:79. doi: 10.3389/fmicb.2016.00079

Taylor, M. W., Radax, R., Steger, D., and Wagner, M. (2007). Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiol. Mol. Biol. Rev.* 71, 295–347. doi: 10.1128/MMBR.00040-06

Urbatzka, R., Freitas, S., Palmeira, A., Almeida, T., Moreira, J., Azevedo, C., et al. (2018). Lipid reducing activity and toxicity profiles of a library of polyphenol derivatives. *Eur. J. Med. Chem.* 151, 272–284. doi: 10.1016/j.ejmech.2018.03.036

van Keulen, G., and Dyson, P. J. (2014). Production of specialized metabolites by *Streptomyces* coelicolor A3(2). *Adv. Appl. Microbiol.* 89, 217–266. doi: 10.1016/B978-0-12-800259-9.00006-8

Vaupel, J. W. (2010). Biodemography of human ageing. *Nature* 464, 536–542. doi: 10.1038/nature08984

Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *P T* 40, 277–283.

Wei, H., Lin, Z., Li, D., Gu, Q., and Zhu, T. (2010). [OSMAC (one strain many compounds) approach in the research of microbial metabolites–a review]. *Wei Sheng Wu Xue Bao* 50, 701–709.

Wicke, C., Huners, M., Wray, V., Nimtz, M., Bilitewski, U., and Lang, S. (2000). Production and structure elucidation of glycoglycerolipids from a marine sponge-associated microbacterium species. *J. Nat. Prod.* 63, 621–626. doi: 10.1021/np990313b

Yoo Kyung, L., Jung-Hyun, L., and Hong Kum, L. (2001). Microbial symbiosis in marine sponges. *J. Microbiol.* 39, 254–264.

You, W., and Henneberg, M. (2018). Cancer incidence increasing globally: the role of relaxed natural selection. *Evol. Appl.* 11, 140–152. doi: 10.1111/eva.12523

Young, M., Artsatbanov, V., Beller, H. R., Chandra, G., Chater, K. F., Dover, L. G., et al. (2010). Genome sequence of the fleming strain of *Micrococcus luteus*, a simple free-living actinobacterium. *J. Bacteriol.* 192, 841–860. doi: 10.1128/JB.01254-09

Zhang, J. H., Chung, T. D., and Oldenburg, K. R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen* 4, 67–73. doi: 10.1177/108705719900400206

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LmbU, a Cluster-Situated Regulator for Lincomycin, Consists of a DNA-Binding Domain, an Auto-Inhibitory Domain, and Forms Homodimer

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Few studies were reported about the regulatory mechanism of lincomycin biosynthesis since it was found in 1962. Although we have proved that a cluster-situated regulator (CSR) LmbU (GenBank Accession No. ABX00623.1) positively modulates lincomycin biosynthesis in *Streptomyces lincolnensis* NRRL 2936, the molecular mechanism of LmbU regulation is still unclear. In this study, we demonstrated that LmbU binds to the target *lmbAp* by a central DNA-binding domain (DBD), which interacts with the binding sites through the helix-turn-helix (HTH) motif. N-terminal of LmbU includes an auto-inhibitory domain (AID), inhibiting the DNA-binding activity of LmbU. Without the AID, LmbU variant can bind to its own promoter. Interestingly, compared to other LmbU homologs, the homologs within the biosynthetic gene clusters (BGCs) of known antibiotics generally contain N-terminal AIDs, which offer them the abilities to play complex regulatory functions. In addition, cysteine 12 (C12) has been proved to be mainly responsible for LmbU homodimer formation *in vitro*. In conclusion, LmbU homologs naturally exist in hundreds of actinomycetes, and belong to a new regulatory family, LmbU family. The present study reveals the DBD, AID and dimerization of LmbU, and sheds new light on the regulatory mechanism of LmbU and its homologs.

Keywords: LmbU, CSR, functional domain, HTH, homodimer, regulatory mechanism

INTRODUCTION

Streptomycetes are well known as prolific producers of bioactive secondary metabolites including more than half of antibiotics as well as antitumor agents, antifungal compounds and vitamins, which have remarkable pharmacological, and industrial importance. Biosynthetic genes for antibiotics and other secondary metabolites are typically clustered together on the chromosomes (Cundliffe, 2006; Liu et al., 2013) or the plasmids (O'Rourke et al., 2009), designated as BGC, and are subject to multi-level and complex regulation cascades. Among them, CSRs provide direct contributions to the biosynthesis of antibiotics by responding to pleiotropic regulators (Ohnishi et al., 2005), global regulators (Uguru et al., 2005; Higo et al., 2011; Iqbal et al., 2012), and

different kinds of regulatory small molecules (Wang et al., 2009; Zhang et al., 2013), subsequently regulating expression of the other biosynthetic genes within their cognate clusters. However, not all the BGCs share a same regulatory scheme. Some of them, such as jadomycin BGC, harbor multiple CSRs (Zou et al., 2014), some of them, such as sansanmycin BGC, contain a single CSR (Li et al., 2013), while the others, such as lincomycin BGC, lack any distinct CSRs (Hou et al., 2017). Generally, CSRs belong to a variety of regulatory protein families, which are divided by sequence or structural similarities, including SARP (*Streptomyces* antibiotic regulatory protein) family, LAL (large ATP-binding regulators of the LuxR) family, TetR family, and so on.

The most common and best studied CSRs are those of the SARP family in *Streptomyces*, including ActII-ORF4 within actinorhodine BGC as well as RedD within undecylprodigiosin BGC in *Streptomyces coelicolor* (Takano et al., 1992; Arias et al., 1999), DnrI within daunorubicin BGC in *Streptomyces peucetius* (Sheldon et al., 2002), and CcaR within cephamycin-clavulanic acid BGC in *Streptomyces clavuligerus* (Santamarta et al., 2011). These members are classified by having an N-terminal HTH DBD subject to OmpR-type and a transcriptional activation domain (Wietzorrek and Bibb, 1997), which generally positively regulate the biosynthesis of secondary metabolites (Sheldon et al., 2002; Tanaka et al., 2007). The target promoters of these members usually contain direct repeats, for instance, direct heptameric repeats (5'-TCGAGXX-3') with 4 bp spacers are conserved upstream the -10 regions of the promoters targeted by ActII-ORF4 and DnrI (Tanaka et al., 2007). The LAL family members usually function as activators in *Streptomyces* too, and comprise an N-terminal ATP-binding domain with a C-terminal LuxR-type DBD, including PikD within pikromycin BGC in *Streptomyces venezuelae* (Wilson et al., 2001), RapH within rapamycin BGC in *Streptomyces hygroscopicus* (Kuscer et al., 2007), and AveR within avermectin BGC in *Streptomyces avermitilis* (Guo et al., 2010). Compared to the SARP and LAL families, TetR family members are widely distributed in various bacteria, including ActR within actinorhodin BGC in *S. coelicolor* (Tahlan et al., 2008), TetR in *Escherichia coli* (Kisker et al., 1995), RolR in *Corynebacterium glutamicum* (Li et al., 2012), LplR in *Rhodococcus erythropolis* (Si et al., 2012), and VtpR in *Vibrio tubiashii* (Hasegawa and Häse, 2009). The TetR family members usually function as transcriptional repressors and consist of an N-terminal DBD and a C-terminal functional domain, which can bind to one or more ligands, subsequently losing the DNA-binding activity and turning on transcription of the target genes (Yu et al., 2010; Cuthbertson and Nodwell, 2013).

Previously, we have reported that LmbU functions as an activator belonging to a novel regulatory family, LmbU family (Hou et al., 2017; van der Heul et al., 2018), and promotes lincomycin biosynthesis by directly regulating transcription of the biosynthetic genes (Hou et al., 2017). The conserved binding site of LmbU is a palindromic sequence 5'-CGCCGGCG-3', which was found in the promoter regions of the *lmbA* and *lmbW* genes. While, the regulatory mechanism of LmbU to other genes lack of the conserved motif is still unknown. In addition, because LmbU and its homologs have no significant sequence and structural similarities to other known CSRs, the binding pattern and functional domains of LmbU are also unclear. In the present study, we characterized the DBD (HTH motif) of LmbU and demonstrated that N-terminal of LmbU contains an AID, which was found in LmbU homologs within the BGCs of antibiotics, but not in that outside BGC of antibiotics. LmbU inhibits transcription of its own gene *in vivo*, and LmbU variant without AID can bind to *lmbUp* promoter. In addition, we revealed that LmbU can form homodimer by a disulfide bond *in vitro*.

MATERIALS AND METHODS

Homology Modeling, Sequence Alignment, and Phylogenetic Tree Analysis

Secondary structure analysis of LmbU was performed by using an online software PredictProtein[1]. Homology modeling of LmbU was performed by using an online software SwissModel[2]. The templates used for LmbU modeling were chosen by ranking, including *Tt*Spo0J derived from *Thermus thermophiles* (GenBank Accession No. WP_011173975.1), *Hp*Spo0J derived from *Helicobacter pylori* (GenBank Accession No. ACJ08256.1), AtaR derived from *E. coli* (GenBank accession no. 6AJN_F), ParG derived from a multidrug resistance plasmid TP228 from *E. coli* (GenBank Accession No. ACV89876.1), AmrZ derived from *Pseudomonas aeruginosa* (GenBank Accession No. APJ53923.1), ω repressor derived from a plasmid Psm19035 from *Streptococcus pyogenes* (GenBank accession no. AAR27202.1), and Arc repressor derived from *Salmonella* bacteriophage P22 (GenBank Accession No. AAM81381.1). Sequence alignment of LmbU with its homologs and the targets of LmbU were carried out by using DNAMAN (Hou et al., 2017). Phylogenetic tree analysis was inferred by using MEGA v7.0.14 with the maximum likelihood method, the LmbU homologs were chosen by ranking (Bown et al., 2017; Hou et al., 2018).

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in **Table 1**. *E. coli* JM83, BL21 (DE3) and ET12567/pUZ8002 strains were used for plasmids construction, protein overexpression and *E. coli-Streptomyces* conjugation, respectively. *Streptomyces lincolnensis* wild-type strain NRRL 2936 and *lmbU* disruption strain JLUa2 were used for *xylTE* reporter assays *in vivo* (Mao et al., 2015; Hou et al., 2018). The pET-28a (+) plasmid was used for overexpression of LmbU and its variants, and the pIB139 plasmid was used for *xylTE* reporter analysis. *E. coli* strains were grown in liquid or on solid Luria-Bertani media at 37°C. *S. lincolnensis* NRRL 2936 and mutants were grown in liquid YEME medium or on solid SMA and ISP4 media at 28°C as described previously (Hou et al., 2017). The media were added

[1]https://open.predictprotein.org/

[2]https://www.swissmodel.expasy.org/interactive

with 50 μg/ml kanamycin, 50 μg/ml apramycin, and 30 μg/ml chloramphenicol as appropriate.

Construction, Overexpression, and Purification of LmbU and Its Variants in *E. coli*

To construct LmbU truncated variants, DNA fragments covering different regions of *lmbU* gene were amplified by PCR using primer pairs U02-F28a/R28, U03-F28a/R28, U04-F28a/R28, U05-F28a/R28, U06-F28a/R28, U07-F28a/R28, and U13-F28a/R28 listed in **Supplementary Table S1**. The amplified DNA fragments were inserted into the *Nde*I/*Eco*RI restriction sites of the pET-28a (+) vector, resulting in various expression plasmids pLU-02, pLU-03, pLU-04, pLU-05, pLU-06, pLU-07, and pLU-13, which were used for expression of $LmbU_{1-161}$, $LmbU_{86-225}$, $LmbU_{1-142}$, $LmbU_{1-131}$, $LmbU_{58-225}$, $LmbU_{58-161}$, and $LmbU_{113-225}$.

To construct LmbU point-mutant variants, DNA fragments covering different upstream or downstream within *lmbU* genes were, respectively amplified by PCR using the primer pairs listed in **Supplementary Table S1**. Among them, primer pairs U-P1/U-RR-P2 with U-RR-P3/U-P4 were used for combined mutation of R101 and R102, primer pairs U-P1/U-R101-P2 with U-R101-P3/U-P4 were used for mutation of R101, primer pairs U-P1/U-R102-P2 with U-R102-P3/U-P4 were used for mutation

TABLE 1 | Strains and plasmids used in this study.

Strain or plasmid	Genotype and/or description	Source or reference
Strains		
S. lincolnensis		
NRRL 2936	Wild-type, lincomycin producer	NRRL, United States
JLUa2	NRRL 2936 Δ*lmbU*	Hou et al., 2017
LNA	NRRL 2936 attBΦC31::pATE152	Hou et al., 2018
LUA	JLUa2 attBΦC31::pATE152	Hou et al., 2018
JAU01	JLUa2 attBΦC31::pAU01	This study
JAU02	JLUa2 attBΦC31::pAU02	This study
JAU03	JLUa2 attBΦC31::pAU03	This study
JAU06	JLUa2 attBΦC31::pAU06	This study
JAU07	JLUa2 attBΦC31::pAU07	This study
LNU	NRRL 2936 attBΦC31::pUTE152	This study
LUU	JLUa2 attBΦC31::pUTE152	This study
E. coli		
JM83	F', ara, Δ(*lac-pro* AB), *rpsL*, (Str^r), Φ80, *lacZ*ΔM15	Hou et al., 2017
BL21 (DE3)	F^- *ompT hsdS gal dcm*	Novagen
ET12567/pUZ8002	*dam-13*::Tn9 *dcm-6 hsdM*; containing the non-transmissible RP4 derivative plasmid pUZ8002	Huang and Grove, 2013
Plasmids		
pET-28a (+)	*E. coli* expression vector	Novagen
pLU-02	$LmbU_{1-161}$ cloned in *Nde*I/*Eco*RI sites of pET-28a (+)	This study
pLU-03	$LmbU_{86-225}$ cloned in *Nde*I/*Eco*RI sites of pET-28a (+)	This study
pLU-04	$LmbU_{1-142}$ cloned in *Nde*I/*Eco*RI sites of pET-28a (+)	This study
pLU-05	$LmbU_{1-131}$ cloned in *Nde*I/*Eco*RI sites of pET-28a (+)	This study
pLU-06	$LmbU_{58-225}$ cloned in *Nde*I/*Eco*RI sites of pET-28a (+)	This study
pLU-07	$LmbU_{58-161}$ cloned in *Nde*I/*Eco*RI sites of pET-28a (+)	This study
pLU-13	$LmbU_{113-225}$ cloned in *Nde*I/*Eco*RI sites of pET-28a (+)	This study
pLU-14	$LmbU_{C12G}$ cloned in *Nde*I/*Eco*RI sites of pET-28a (+)	This study
pLU-15	$LmbU_{C63G}$ cloned in *Nde*I/*Eco*RI sites of pET-28a (+)	This study
pLU-16	$LmbU_{R101A}$ cloned in *Nde*I/*Eco*RI sites of pET-28a (+)	This study
pLU-17	$LmbU_{R102A}$ cloned in *Nde*I/*Eco*RI sites of pET-28a (+)	This study
pSET152	Integrative vector based on ΦC31 integrase	Bierman et al., 1992
pATE152	pSET152 carrying *xylTE* reporter gene controlled by *lmbAp* promoter	Hou et al., 2018
pEU139	pIB139 with *lmbU* inserted downstream of *ermE*p*	Hou et al., 2017
pAU01	pATE152 inserted with LmbU expression cassette	This study
pAU02	pATE152 inserted with $LmbU_{1-161}$ expression cassette	This study
pAU03	pATE152 inserted with $LmbU_{86-225}$ expression cassette	This study
pAU06	pATE152 inserted with $LmbU_{58-225}$ expression cassette	This study
pAU07	pATE152 inserted with $LmbU_{58-161}$ expression cassette	This study
pUTE152	pSET152 carrying *xylTE* reporter gene controlled by *lmbUp* promoter	This study

of R102, primer pairs U-P1/U-C63-P2 with U-C63-P3/U-P4 were used for mutation of C63. The mutations were introduced by primers P2 and P3, R was replaced with A, and C was replaced with G. The corresponding DNA fragments of upstream and downstream of *lmbU* were inserted into the *Nde*I/*Eco*RI restriction sites of the pET-28a (+) vector by using Super Efficiency Fast Seamless Cloning kits (DoGene, China), resulting in various expression plasmids pLU-08, pLU-16, pLU-17, and pLU-15, which were used for expression of $LmbU_{RR}$, $LmbU_{R101A}$, $LmbU_{R102A}$, and $LmbU_{C63G}$. In addition, to construct LmbU point-mutant variant $LmbU_{C63G}$, a DNA fragment was amplified by PCR using primer pairs U-C12-P1/U-R28a, and inserted into the *Nde*I/*Eco*RI restriction sites of the pET-28a (+) vector, resulting in expression plasmids pLU-14.

The obtained plasmids were transformed into *E. coli* BL21 (DE3) for protein expression as described previously (Hou et al., 2017). Briefly, The strains were cultivated at 37°C until OD_{600} reached about 0.6, isopropyl–D-1-thiogalactopyranoside (IPTG) was added and the cultures were then incubated at 16°C overnight. The proteins were released by sonication on ice and were purified using nickel-iminodiacetic acid–agarose chromatography (WeiShiBoHui, China). After dialysis using binding buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.2 mM dithiothreitol, 20 g/ml bovine serum albumin, 1.2% glycerol) and concentration using 10 or 3-kDa-cutoff centrifugal filter units (Millipore, Billerica, MA, United States), the proteins were analyzed and quantified using 12% SDS-PAGE and Bradford assay, respectively (Bradford, 1976).

Electrophoretic Mobility Shift Assay (EMSA)

Electrophoretic mobility shift assay were carried out as described previously (Hou et al., 2017). Briefly, biotin-labeled probe *lmbAp* (5 ng) was incubated with His_6-LmbU or variants (different concentrations) in the binding reaction mixture contained 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.2 mM dithiothreitol, 20 g/mL bovine serum albumin, 1.2% glycerol, and 50 g/mL poly (dI-C). After incubation at 28°C for 20 min, the samples were separated on 6% non-denaturing polyacrylamide gels in 0.5 × TBE buffer (54 g/L Tris, 1.86 g/L EDTA and 27.5 g/L boric acid, pH 8.0) in ice-water bath at 100 V, and transferred to the positively charged nylon membrane. The biotin-labeled probes were detected by streptavidin- horseradish-peroxidase (HRP) conjugate and BeyoECL Plus (Beyotime Biotechnology, China). Each experiment was at least repeated two times, and the representative images are shown.

xylTE Reporter Assays

To analyze the function of LmbU and its variants *in vivo*, we performed *xylTE* reporter assays. DNA fragments covering LmbU expression cassettes (*ermE*p* promoter plus *lmbU* gene or *lmbU* variants) were amplified by PCR using primer pairs E*p-lmbU-F/R with pLU-1, pLU-03, and pLU-06 as templates, and using primer pairs E*p-lmbU-F/lmbU4-R with pLU-2 and pLU-7 as templates. The amplified fragments were inserted into the *Nhe*I restriction sites of the pATE152 plasmid by using T4 DNA ligase (TAKARA, Japan), resulting in pAU01, pAU02, pAU03, pAU06, and pAU07 plasmids. The obtained plasmids were then introduced into the *lmbU* disruption strain JLUa2 and integrated into the *attB* site of the chromosome to generate reporter strains JAU01, JAU02, JAU03, JAU06, and JAU07. The reporter plasmid pUTE152 was constructed as pATE152 described previously (Hou et al., 2018). The region upstream (relative to the translation start codon) of the *lmbU* gene (-329 – 1 bp) was amplified using primer pairs pUxyl-1/pUxyl-2, and the *xylTE* gene was amplified by PCR using primer pair pAxyl-3/pAxyl-4. Two fragments were inserted into the *Pvu*II site of the plasmid pSET152 using Super Efficiency Fast Seamless Cloning kits (Do Gene, China). The obtained plasmid was introduced into wild-type strain NRRL 2936 and *lmbU* disruption strain JLUa2, and integrated into the *attB* site of the chromosome to generate reporter strains LNU and LUU.

The analysis of catechol dioxygenase activity was performed as described previously (Hou et al., 2018). *Streptomyces* strains were grown in YEME medium at 28°C for 1 day, cells were washed in 20 mM potassium phosphate buffer, and suspended in 1 ml sample buffer (100 mM potassium phosphate, pH 7.5, 20 mM EDTA, 10% acetone). Total proteins were harvested by sonication, and quantified using the Bradford method (Bradford, 1976). 20 μl total proteins were added to 180 μl assay buffer (100 mM potassium phosphate, pH 7.5, 1 mM catechol), and were detected at 375 nm at 1, 2, 3, 4, 5, and 6 min, respectively. The activity was calculated as the rate of change per minute per milligram of protein and converted to milliunits per milligram. Data represent means ± standard deviations of results from three independent experiments. Statistical significance is indicated vs. the results of wild-type LmbU by using *T* test (Kim, 2015), ns, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$.

Dimerization Analysis of LmbU

The purified LmbU protein and variants were dealt with different loading buffers, which contained or did not contain SDS or DTT. The total loading buffer consists of 50 mM Tris–HCl (pH 6.8), 2% SDS (m/v), 0.1% bromophenol blue (m/v), 10% glycerin (m/v), and 100 mM DTT. The samples were analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (**Supplementary Table S2**) and stained with Coomassie brilliant blue R-250. The electrophoresis buffer consists of 3 g/L Tris, 19 g/L glycine and 1 g/L SDS. The molecular weights of LmbU dimer (56 kDa) and LmbU monomer (28 kDa) were standardized by the protein marker (TAKARA, Japan).

RESULTS

Bioinformatics Analysis of the Structure of LmbU

In our previous study, we have characterized LmbU as a DNA-binding protein involved in lincomycin biosynthesis, and identified the target genes and the binding site of LmbU (Hou et al., 2017). To further investigate the regulatory mechanism of LmbU, we performed bioinformatics analysis of the structure of LmbU. Secondary structure analysis showed that LmbU protein

contains 9 α-helices and 2 β-strands. In addition, 14 protein binding regions, one RNA-binding region and 3 DNA-binding regions were predicted in LmbU (**Supplementary Figure S1**). Structure modeling demonstrated that two potential DBDs, a putative HTH motif including amino acid (aa) 80–102, and a putative ribbon-helix-helix (RHH) motif including aa 167–206, were predicted in LmbU (**Figure 1**). However, all the templates used for LmbU modeling are not derived from *Streptomyces*, indicating the regulatory pattern of LmbU may be complex and novel compared to other CSRs.

In addition, LmbU homologs naturally exist in hundreds of actinomycetes (**Supplementary Figure S2**), indicating LmbU homologs might play important roles in metabolism of natural products. Sequence alignment of LmbU with eight selected homologs revealed that the HTH motifs are highly conserved, 11 out of 23 amino acids, especially 10 out of 13 amino acids in the latter helix, are totally identical. In contrast, only 7 out of 40 amino acids in the RHH motif are totally identical (**Supplementary Figure S3**). These data indicated that the

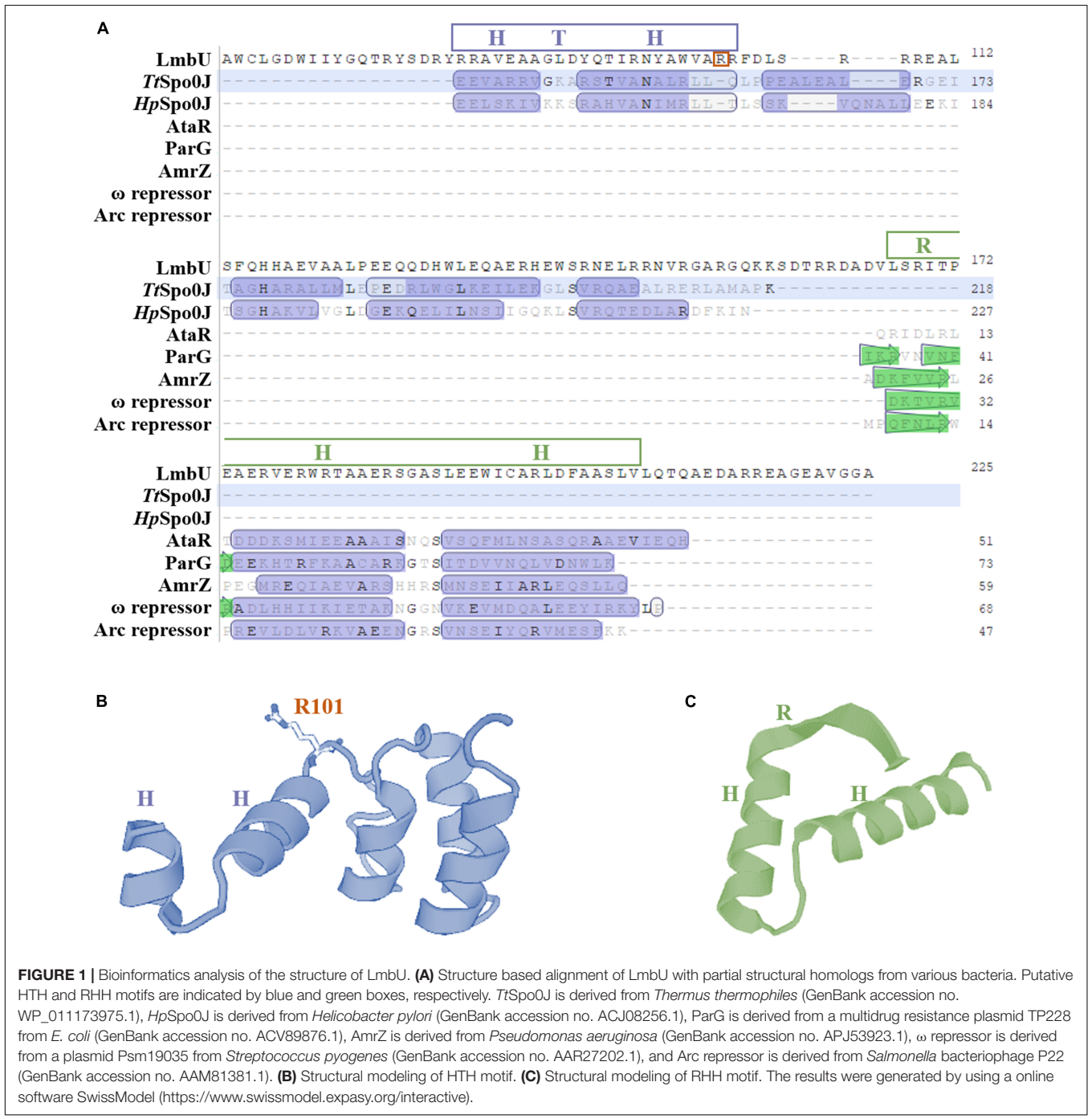

FIGURE 1 | Bioinformatics analysis of the structure of LmbU. **(A)** Structure based alignment of LmbU with partial structural homologs from various bacteria. Putative HTH and RHH motifs are indicated by blue and green boxes, respectively. *Tt*Spo0J is derived from *Thermus thermophiles* (GenBank accession no. WP_011173975.1), *Hp*Spo0J is derived from *Helicobacter pylori* (GenBank accession no. ACJ08256.1), ParG is derived from a multidrug resistance plasmid TP228 from *E. coli* (GenBank accession no. ACV89876.1), AmrZ is derived from *Pseudomonas aeruginosa* (GenBank accession no. APJ53923.1), ω repressor is derived from a plasmid Psm19035 from *Streptococcus pyogenes* (GenBank accession no. AAR27202.1), and Arc repressor is derived from *Salmonella* bacteriophage P22 (GenBank accession no. AAM81381.1). **(B)** Structural modeling of HTH motif. **(C)** Structural modeling of RHH motif. The results were generated by using a online software SwissModel (https://www.swissmodel.expasy.org/interactive).

HTH motif might be more important for DNA-binding than the RHH motif.

Identification of the DNA-Binding Function of HTH and RHH Motifs

To identify whether the HTH or/and RHH motifs were relative to the DNA-binding of LmbU, two deletion variants were constructed and expressed, one deleted the HTH motif (designed as $LmbU_{DH}$) and the other deleted the RHH motif (designed as $LmbU_{DR}$) (**Supplementary Figure S4A**). Unfortunately, His_6-$LmbU_{DH}$ failed to express in *E. coli* BL21 (DE3). EMSA analysis revealed that His_6-$LmbU_{DR}$ had the DNA-binding activity (**Supplementary Figure S4B**), suggesting the RHH motif is not critical for DNA-binding of LmbU. To further verify the DNA-binding activities of RHH and HTH motifs, we expressed and purified the His_6-$LmbU_{1-161}$ (aa 1–161) and His_6-$LmbU_{86-225}$ (aa 86_225) variants, which contained the intact HTH motif, and the intact RHH motif, respectively (**Figure 2A**). EMSA analysis demonstrated that His_6-$LmbU_{1-161}$ could bind to the *lmbAp* probe, while His_6-$LmbU_{86-225}$ could not (**Figure 2B**), which also indicated that the RHH motif is not a critical DBD, and the DBD may exist in $LmbU_{1-161}$. Subsequently, *xylTE* reporter assay was carried out to identify the function of the LmbU variants *in vivo*. The reporter plasmid pATE152, where *xylTE* gene was controlled by *lmbAp* promoter, was introduced into wild-type strain NRRL 2936 and *lmbU* disruption strain JLUa2, resulting in reporter strains LNA and LUA, respectively. The data showed that LmbU activates *lmbAp* promoter (**Supplementary Figure S5**), which is available and coincident with that of *neo*^{r} reporter assay (Hou et al., 2017). In addition, the enzyme activities of total proteins extracted from the cells cultured for 1 day were observed higher than that from the cells cultured for 2 days (**Supplementary Figure S5**), thus, in the following study, we just detected the enzyme activities at day 1. Reporter plasmids pAUTE1 and pAUTE2 were constructed and introduced into JLUa2 strain, respectively, where the *xylTE* gene was controlled by the *lmbAp* promoter and the *lmbU* mutant genes were controlled by *ermE*p*. The data showed that $LmbU_{1-161}$ rather than $LmbU_{86-225}$ could activate the *lmbAp* promoter (**Figure 2C**), which is consistent with the results of EMSA, showing that $LmbU_{1-161}$ variant contains a pivotal DBD.

Verification of the DBD and AID of LmbU

To narrow down the region of potential DBD of LmbU, we further truncated $LmbU_{1-161}$ to the $LmbU_{1-141}$ and $LmbU_{1-131}$ variants. However, both His_6-$LmbU_{1-141}$ and His_6-$LmbU_{1-131}$ were failed to express in *E. coli* BL21 (DE3). Therefore, semi-quantitative reverse transcription and polymerase chain reaction (sqRT-PCR) and Western blotting were performed to check the RNA levels and the protein levels of LmbU variants, respectively. The data demonstrated that RNA levels of $lmbU_{1-141}$ and $lmbU_{1-131}$ had no differences with that of *lmbU*, but protein levels of them were severely reduced compared to that of LmbU (**Supplementary Figure S6**). Then, we extended $LmbU_{86-225}$ to $LmbU_{58-225}$ (**Figure 3A**) and performed EMSA. The data revealed that His_6-$LmbU_{58-225}$ could recover the DNA-binding activity, and the affinity to the target seemed enhanced compared to LmbU. Further EMSA analysis showed that the complex bands were observed when 0.2 μM His_6-$LmbU_{58-225}$ was added (**Figure 3B**), but that was observed when 3.2 μM His_6-LmbU was added, indicating N-terminal of LmbU contains an AID against DNA-binding.

Given that both His_6-$LmbU_{1-161}$ and His_6-$LmbU_{58-225}$ have DNA-binding activities, we speculated that the DBD of LmbU was located in the overlapping region of the two variants. Thus, we constructed and expressed the His_6-$LmbU_{58-161}$ variant (**Figure 3A**) and performed EMSA. As expected, His_6-$LmbU_{58-161}$ was found to bind to the *lmbAp* probe as well. In addition, the complex bands were observed when 0.1 μM His_6-$LmbU_{58-161}$ was added, and 0.2 μM protein could completely impede the migration of the *lmbAp* probe (**Figure 3B**), indicating that $LmbU_{58-161}$ has a better affinity to the target compared to LmbU and $LmbU_{58-225}$. Thus, we demonstrate that $LmbU_{58-161}$ has a DNA-binding activity, and the HTH motif is located in this region, suggesting the HTH motif is possibly a crucial DBD of LmbU. Furthermore, *xylTE* reporter assays showed that $LmbU_{58-25}$ could activate *lmbAp* promoter, but not like the result of EMSA, the activity of $LmbU_{58-225}$ for *lmbAp* promoter was not enhanced compared to that of LmbU (**Figure 3C**). While, $LmbU_{58-161}$ could not activate *lmbAp* promoter (**Figure 3C**), indicating that C-terminal of LmbU performed a certain function to regulate the activity of *lmbAp* promoter *in vivo*.

It has been reported that polar and positively charged amino acids are usually important for DNA-binding of regulators, such as arginine (Davis et al., 2013; Bhukya et al., 2014). To further verify whether the HTH motif is responsible for DNA-binding, two arginines in the motif, R101 and R102, were, respectively substituted with either an alanine or a similarly charged lysine, resulting in $LmbU_{R101A}$, $LmbU_{R102A}$, $LmbU_{R101K}$, and $LmbU_{R102K}$. EMSA analysis revealed that His_6-$LmbU_{R102A}$ and His_6-$LmbU_{R102K}$ could bind to the *lmbAp* probe (**Figure 4A**) while His_6-$LmbU_{R101A}$ and His_6-$LmbU_{R101K}$ could not (**Figure 4B**), indicating that the HTH motif is a critical DBD and R101 plays a key role in DNA-binding. These data also demonstrated that the HTH motif, not the RHH motif is the DBD of LmbU.

Insight Into Regulation of LmbU to *lmbUp*

In our previous study, we found that LmbU regulates the *lmbC*, *lmbK* and *lmbU* genes, but does not bind to their promoters (Hou et al., 2017). Considering the DNA-binding activities of $LmbU_{58-225}$ and $LmbU_{58-161}$ were enhanced compared to that of LmbU, we performed EMSA using $LmbU_{58-225}$ and $LmbU_{58-161}$ with *lmbCp*, *lmbKp* and *lmbUp* probes, the $P_{V-W}3$ probe was used as a positive control. The data showed that both His_6-$LmbU_{58-225}$ and His_6-$LmbU_{58-161}$ could not bind to the *lmbCp* and *lmbKp* probes, but seemed to bind to the *lmbUp* probe (**Supplementary Figure S7**). Subsequently, further EMSA with competition analysis were carried out using His_6-$LmbU_{58-225}$ and $LmbU_{58-161}$ with the *lmbUp* probe. The results showed that both of the two variants can bind to *lmbUp* directly and

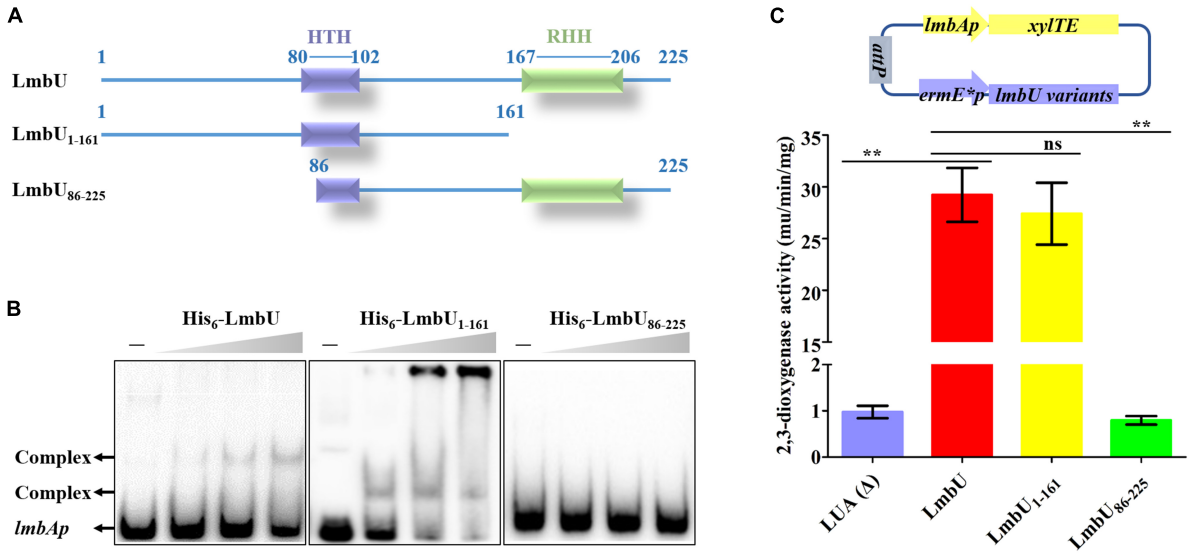

FIGURE 2 | Functional analysis of LmbU variants $LmbU_{1-161}$ and $LmbU_{86-225}$. **(A)** Sketch map of LmbU protein and LmbU variants showing the location of the HTH (aa 80–102) and RHH motif (aa 167–206). **(B)** EMSA analysis of LmbU and variants with *lmbAp* probe. Biotin-labeled *lmbAp* (263 bp, 5 ng) was incubated with increasing concentrations (0, 3.2, 6.4, and 12.8 μM) of His_6-LmbU, His_6-$LmbU_{1-161}$, and His_6-$LmbU_{86-225}$, respectively. The DNA-protein complexes and the free probes are indicated by arrows. **(C)** *XylTE* reporter assay of LmbU and variants to *lmbAp in vivo*. The reporter plasmids were constructed with *xylTE* reporter gene controlled by *lmbAp* and *lmbU* or *lmbU* variants controlled by *ermE*p*. The reporter plasmids were integrated into the *attB* site of the chromosome of JLUa2 to generate reporter strains. In addition, LUA was used as a negative control, which was derived from JLUa2 harboring *xylTE* reporter gene controlled by *lmbAp*. Data represent means ± standard deviations of results from three independent experiments. Statistical significance is indicated vs. the results of wild-type LmbU by using *T* test (Kim, 2015), ns, not significant, $^{**}P < 0.01$.

specifically with a concentration-dependent manner (**Figure 5A** and **Supplementary Figure S8**). In addition, *xylTE* reporter assay showed that LmbU represses the activity of the *lmbUp* promoter *in vivo* (**Figure 5B**), indicating that LmbU might regulate the activity of *lmbUp* promoter by binding to *lmbUp* in a different pattern compared to *lmbAp* and *lmbWp*.

Identification of Dimerization of LmbU

Generally, regulatory proteins perform their functions by forming homodimers (Bhukya et al., 2014; Hayashi et al., 2014). To investigate the polymeric form of LmbU, we performed SDS-PAGE by using purified LmbU and variants from *E. coli* BL21 (DE3), which were dealt with different loading buffers (containing DTT/SDS or not). The data showed that LmbU could form a homodimer, which was affected by DTT, but not by SDS (**Figure 6A**), indicating that the homodimer is likely to be formed by disulfide bond among cysteines. Sequence analysis showed that LmbU contains three cysteines, C12, C63, and C196, the first two of which are included in $LmbU_{1-161}$ and the last one is included in $LmbU_{113-225}$. To figure this out, we firstly carried out SDS-PAGE using $LmbU_{1-161}$ and $LmbU_{113-225}$, respectively, and found that the former could form homodimer, but the latter could not (**Figure 6B**), suggesting the crucial cysteines for dimerization were located in aa 1–161. Then, the two cysteines C12 and C63 were mutated to glycines, resulting in the $LmbU_{C12G}$, $LmbU_{C63G}$ and $LmbU_{C12G/C63G}$ variants. SDS-PAGE analysis revealed that $LmbU_{C12G}$ and $LmbU_{C12G/C63G}$ could not form homodimer, but $LmbU_{C63G}$ could form homodimer partly (**Figure 6C**), indicating C12 plays a key role in forming LmbU homodimer, and C63 plays a supporting role.

DISCUSSION

In our previous study, we have demonstrated that a global regulator BldA (Hou et al., 2018) and a CSR LmbU (Hou et al., 2017) are involved in lincomycin biosynthesis. Recently, a TetR-type regulator SLCG_2919 has been proved to negatively regulate lincomycin biosynthesis (Xu et al., 2018). However, the regulatory mechanism of lincomycin biosynthesis is still unclear at present.

Importantly, we found that hundreds of LmbU homologs exist in or outside the BGCs of different natural products derived from a variety of actinomycetes (**Supplementary Figure S2**), indicating LmbU homologs might play important roles in metabolism and do not only act as CSRs of natural products. Although there are so much LmbU homologs, few studies have been reported. SACE_5599, a homolog of LmbU outside the BGCs of natural products, can regulate not only erythromycin production, but also morphological differentiation in *Saccharopolyspora erythraea* (Kirm et al., 2013), which has been shown to bind to the promoter regions of *lmbAp*, and *lmbWp* within *S. lincolnensis* as well in our previous study (Hou et al., 2017). HmtD, a homolog of LmbU in the BGC of himastatin, positively regulates the biosynthesis of himastatin in *Streptomyces hygroscopicus*, however, the relevant mechanism is still unknown (Xie et al., 2019). In addition, structural prediction of LmbU demonstrated that LmbU protein does not include

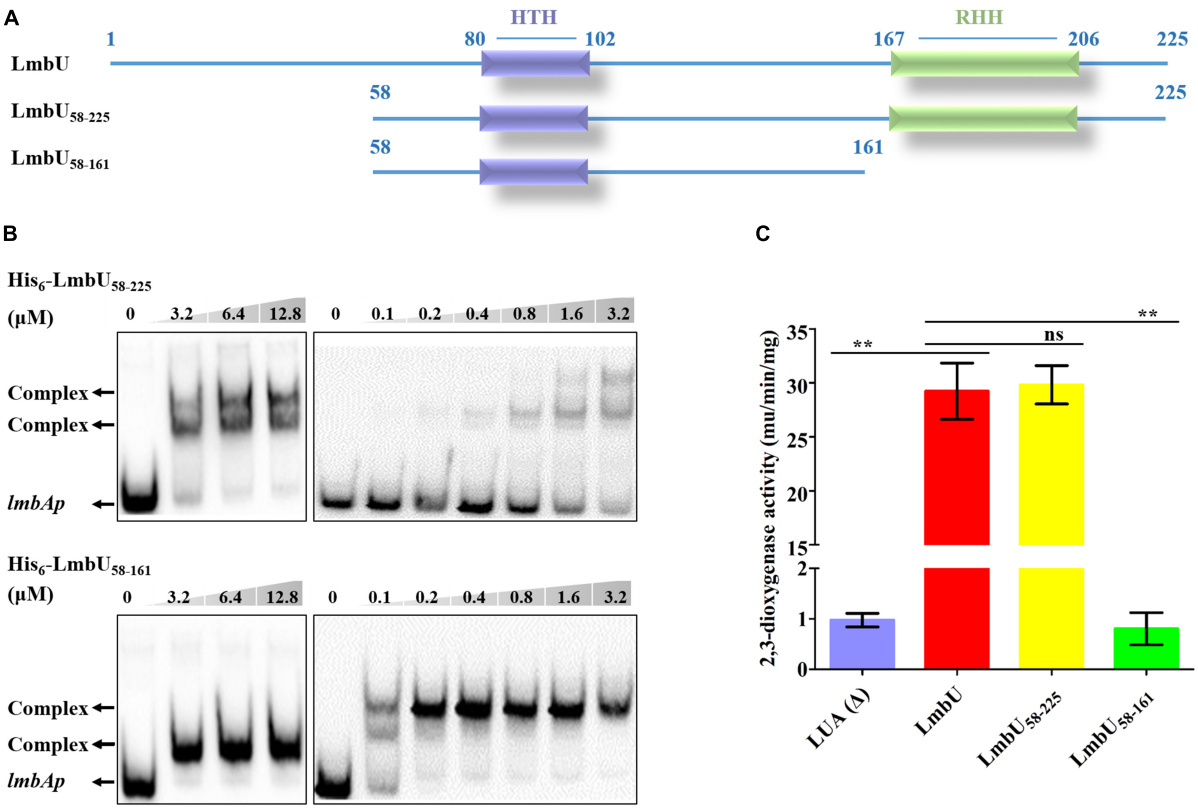

FIGURE 3 | Functional analysis of LmbU variants $LmbU_{58-225}$ and $LmbU_{58-161}$. **(A)** Sketch map of LmbU protein and LmbU variants. $LmbU_{58-225}$ contains intact HTH and RHH motifs, $LmbU_{58-225}$ only contains intact HTH motif. **(B)** EMSA analysis of LmbU and variants with *lmbAp* probe. Biotin-labeled *lmbAp* (263 bp, 5 ng) was incubated with increasing concentrations of His_6-$LmbU_{58-225}$ and His_6-$LmbU_{58-161}$, respectively. The DNA-protein complexes and the free probes are indicated by arrows. **(C)** *XylTE* reporter assay of LmbU and variants to *lmbAp in vivo*. Data represent means ± standard deviations of results from three independent experiments. Statistical significance is indicated vs. the results of wild-type LmbU by using *T* test (Kim, 2015), ns, not significant; $^{**}P < 0.01$.

a known domain similar to that of other CSRs, indicating the regulatory pattern of LmbU and its homologs was novel and complex compared to other CSRs. In the present study, we illuminate the functional domains of LmbU, including DBD and AID, and insight into the regulatory pattern of LmbU.

We demonstrated that LmbU consists of three functional domains, including a N-terminal AID (aa 1–57), a central DBD (aa 80–102), and a C-terminal unknown domain (aa 162–225) (**Figure 7**). To our knowledge, HTH motif is the best known and widely used DBD, although LmbU has been shown to bind to the targets by HTH motif as well, the sequence, and structure of HTH motif within LmbU is unlike the most of the regulators in *Streptomyces* (Natsume et al., 2004; Guo et al., 2010; Hayashi et al., 2013), indicating LmbU and its homologs function in a novel regulatory mechanism.

Furthermore, we found that the N-terminal AID represses the DNA-binding of LmbU, and without this domain, LmbU variants can bind to its own promoter region and inhibit transcription of itself, forming negative feedback regulation (**Figure 5**). Moreover, we found that LmbU homologs within the BGCs of the known antibiotics all contain the N-terminal AID (**Supplementary Figure S3**), such as HrmB (GenBank Accession No. AEH41782.1) for hormaomycin (Hofer et al., 2011), HmtD (GenBank accession no. CBZ42138.1) for himastatin (Ma et al., 2011), and AcmO (GenBank accession no. ADG27350.1) for actinomycin (Keller et al., 2010). Thus, we speculate that whether LmbU and its homologs within the BGCs of antibiotics function in a more complicated manner, by forming different conformations or different variants. These kinds of cases are not common in the known CSRs, but were found in global regulators. For instance, in *Bacillus subtilis*, the C-terminal of GlnR acts as an auto-inhibitory domain (AID) repressing dimer formation and DNA-binding, when interacting with DNA, GlnR changes its conformational and oligomeric state, resulting in a stable complex (Fisher and Wray, 2008; Wray and Fisher, 2008; Schumacher et al., 2015). In *S. coelicolor*, BldD undergoes degradation of the C-terminal domain, resulting in two forms, which may play roles at vegetative stage or at the late stage of life cycle, respectively (Lee et al., 2007). Interestingly, two PmbA (TldE)-TldD family proteins, LmbIH and LmbQ, are found in the lincomycin BGC. As reported, in *E. coli*, TldD and TldE participate in the cleavage of the modified MccB17 precursor peptide to mature antibiotic by forming heterodimer (Allali et al., 2002; Rodriguez-Sainz et al., 1990; Ghilarov et al., 2017). These studies promote us to speculate that LmbU may undergo accurate post-translational modification by LmbIH and LmbQ in the late growth stage,

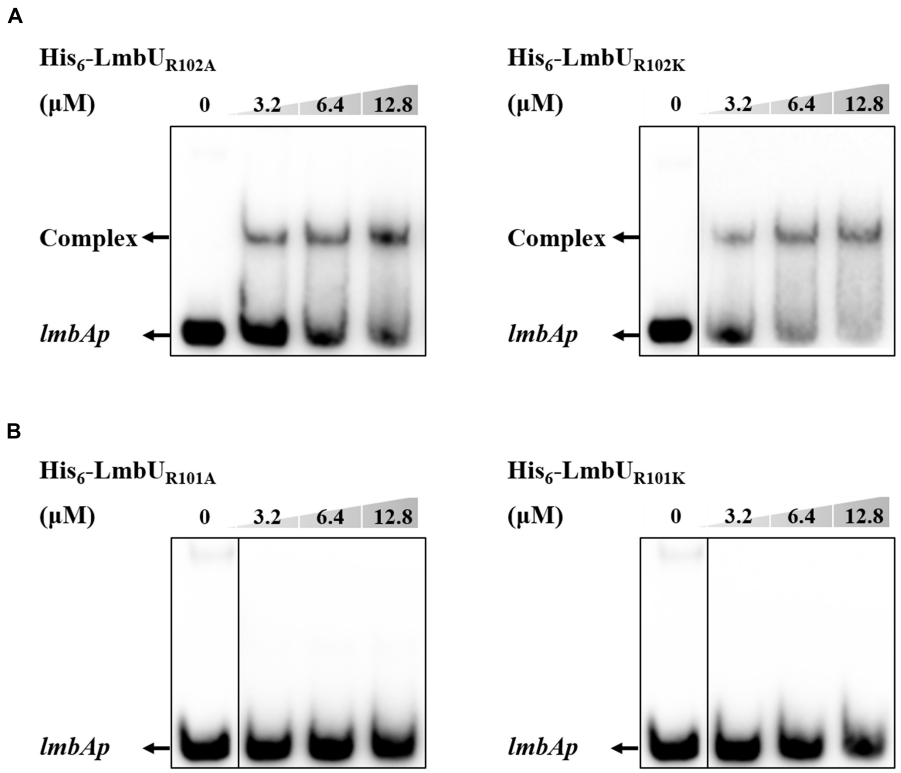

FIGURE 4 | Effect of R102 and R101 on the DNA-binding activity of LmbU. **(A)** EMSA analysis of LmbU variants His$_6$-LmbU$_{R102A}$ and His$_6$-LmbU$_{R102K}$ with *lmbAp* probe. **(B)** EMSA analysis of LmbU variants His$_6$-LmbU$_{R101A}$ and His$_6$-LmbU$_{R101K}$ with *lmbAp* probe.

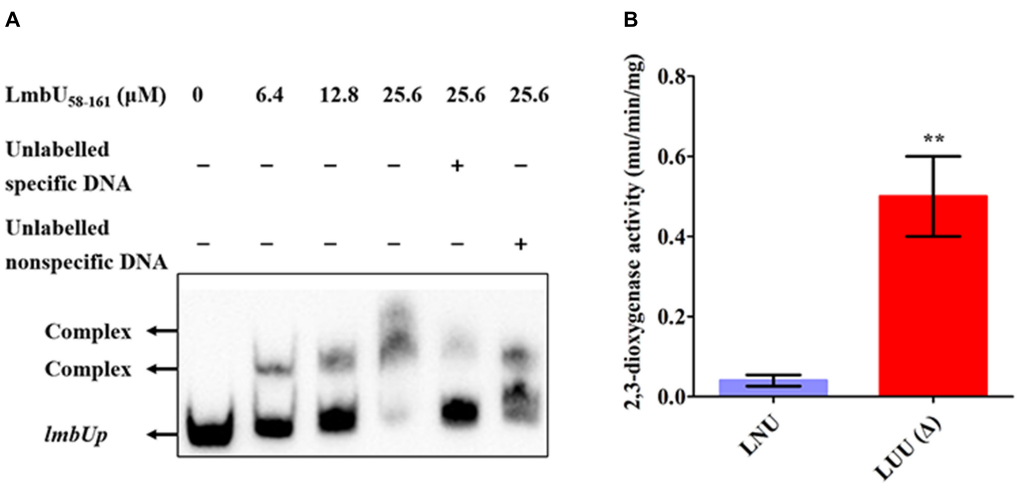

FIGURE 5 | Regulation of LmbU to its own promoter *lmbUp*. **(A)** EMSAs of LmbU 58–161 with *lmbUp* probe. Biotin-labeled *lmbUp* (415 bp, 5 ng) probes were incubated with increasing His6-LmbU 58–161 (0, 6.4, 12.8, and 25.6 μM). EMSAs with 200-fold excess of unlabeled specific DNA or non-specific DNA were added as controls, to confirm specificity of the band shifts. The DNA-protein complexes and the free probes are indicated by arrows. **(B)** *XylTE* reporter analysis of the effect of LmbU to *lmbUp in vivo*. LNU, wild-type strain NRRL 2936 harboring the reporter plasmid pUTE152; LUU, *lmbU* disruption strain JLUa2 harboring the reporter plasmid pUTE152. Data represent means ± standard deviations of results from three independent experiments. Statistical significance is indicated vs. the results of wild-type LmbU by using *T* test (Kim, 2015), $^{**}P < 0.01$.

resulting in functional variant, which binds to its own promoter, and inhibits the biosynthesis of lincomycin. However, further studies are needed to confirm these hypotheses.

In addition, we demonstrated that compared to the functional variant LmbU$_{58-225}$, LmbU$_{58-161}$ can bind to the target DNA *in vitro* with a better affinity, but can not activate transcription

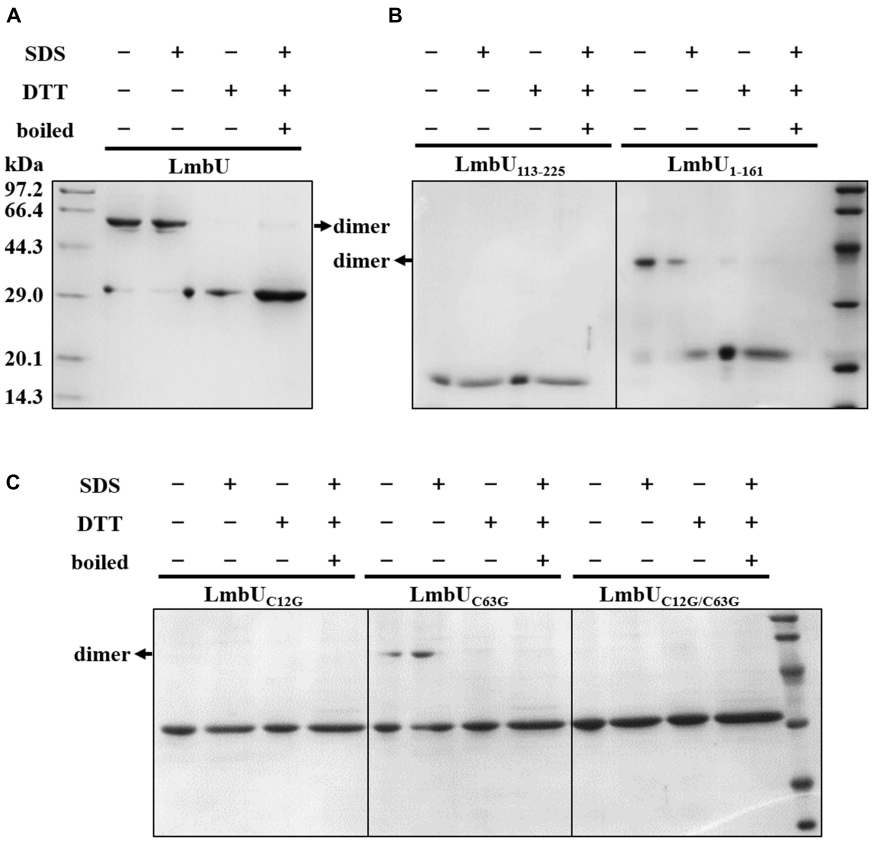

FIGURE 6 | Dimerization analysis of LmbU. **(A)** The LmbU protein was dealt with SDS, DTT, or was boiled for 5 min. −, not dealt with; + dealt with. **(B)** Dimerization analysis of LmbU variants LmbU113-225 and LmbU1-161. **(C)** Effect of C12 and C63 on LmbU dimer.

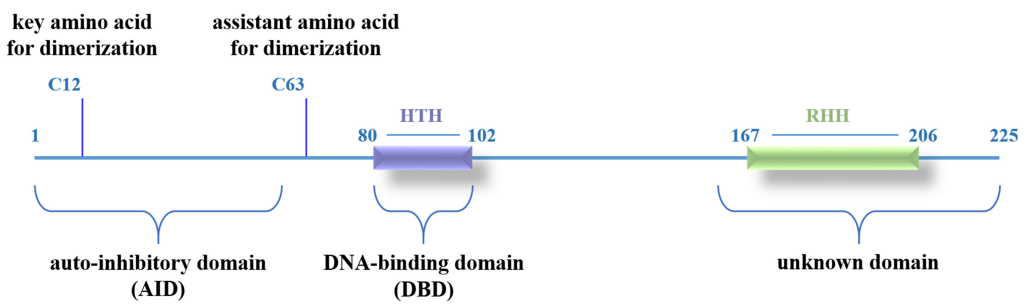

FIGURE 7 | A hypothetical model for the functional domains of LmbU.

of the reporter gene *in vivo*. Considering $LmbU_{1-161}$ without aa 162–225 has a similar DNA-binding affinity to LmbU, thus we thought there is no inhibitory domain within the C-terminal, the better affinity of $LmbU_{58-161}$ might be due to the exposure of the DBD domain. In addition, these data showed that the C-terminal amino acids play important roles as an unknown domain, either in structural stability or in interaction with ligands. However, we also found that $LmbU_{1-161}$ has activity to *lmbAp* promoter both *in vitro* and *in vivo*, indicating the C-terminal domain is not necessary, which appeared to be different from the hypothesis mentioned above. In consideration of the unclear regulatory mechanism of LmbU to the target *lmbCp* and *lmbKp* with no identified binding sites, the function of the C-terminal domain needs to be further illuminated.

Generally, transcriptional regulators bind to the target DNA by forming homodimers. For instance, in *Streptomyces griseus*, a global regulator AdpA either binds to two sites with different lengths in the target DNA (type I or type I'), or binds to a single site in the target DNA with one subunit of the homodimer (type II) (Yamazaki et al., 2004); in *S. coelicolor* A3 (2), a γ-butyrolactone receptor CprB interacts with the target DNA

through two individual CprB homodimers (Bhukya et al., 2014). Here, we showed that LmbU can form homodimer mainly via C12. And the DNA-binding mode of LmbU need to be further investigated.

In summary, we have demonstrated the functional domains of LmbU, which is a representative of the LmbU family transcriptional regulators. LmbU consists of an N-terminal AID, a central DBD and a C-terminal unknown domain. In addition, LmbU forms homodimer mainly via the C12 *in vitro*. By applying this knowledge, we speculate that the unusual properties of LmbU will be exploited for future applications in the realization of high-yield of lincomycin, and in the functional research of LmbU family proteins.

AUTHOR CONTRIBUTIONS

BH, HZ, and HW designed the experiments. BH, XZ, and YK carried out the experiments. BH, JY, HW, and HZ analyzed the data. BH and HW wrote the manuscript. RW discussed the experimental design and contributed to the manuscript. All authors assisted with critical reading of 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.2019.00989/full#supplementary-material

REFERENCES

Allali, N., Afif, H., Couturier, M., and Van Melderen, L. (2002). The highly conserved TldD and TldE proteins of *Escherichia coli* are involved in microcin B17 processing and in CcdA degradation. *J. Bacteriol.* 184, 3224–3231. doi: 10.1128/jb.184.12.3224-3231.2002

Arias, P., Fernandez-Moreno, M. A., and Malpartida, F. (1999). Characterization of the pathway-specific positive transcriptional regulator for actinorhodin biosynthesis in *Streptomyces coelicolor* A3(2) as a DNA-binding protein. *J. Bacteriol.* 181, 6958–6968.

Bhukya, H., Bhujbalrao, R., Bitra, A., and Anand, R. (2014). Structural and functional basis of transcriptional regulation by TetR family protein CprB from *S. coelicolor* A3(2). *Nucleic Acids Res.* 42, 10122–10133. doi: 10.1093/nar/gku587

Bierman, M., Logan, R., O'Brien, K., Seno, E. T., Rao, R. N., and Schoner, B. E. (1992). Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* 116, 43–49. doi: 10.1016/0378-1119(92)90627-2

Bown, L., Li, Y., Berrue, F., Verhoeven, J. T. P., Dufour, S. C., and Bignell, D. R. D. (2017). Biosynthesis and evolution of coronafacoyl phytotoxin production in the common scab pathogen *Streptomyces scabies*. *Appl. Environ. Microbiol.* 83, e1169–e1117.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1006/abio.1976.9999

Cundliffe, E. (2006). Antibiotic production by actinomycetes: the janus faces of regulation. *J. Ind. Microbiol. Biotechnol.* 33, 500–506. doi: 10.1007/s10295-006-0083-6

Cuthbertson, L., and Nodwell, J. R. (2013). The TetR family of regulators. *Microbiol. Mol. Biol. Rev.* 77, 440–475. doi: 10.1128/MMBR.00018-13

Davis, J. R., Brown, B. L., Page, R., and Sello, J. K. (2013). Study of PcaV from *Streptomyces coelicolor* yields new insights into ligand-responsive MarR family transcription factors. *Nucleic Acids Res.* 41, 3888–3900. doi: 10.1093/nar/gkt009

Fisher, S. H., and Wray, L. V. (2008). *Bacillus subtilis* glutamine synthetase regulates its own synthesis by acting as a chaperone to stabilize GlnR–DNA complexes. *Proc. Natl. Acad. Sci. U.S.A.* 105, 1014–1019. doi: 10.1073/pnas.0709949105

Ghilarov, D., Serebryakova, M., Stevenson, C. E. M., Hearnshaw, S. J., Volkov, D. S., Maxwell, A., et al. (2017). The origins of specificity in the microcin-processing protease TldD/E. *Structure* 25, 1549–1561. doi: 10.1016/j.str.2017.08.006

Guo, J., Zhao, J. L., Li, L. L., Chen, Z., Wen, Y., and Li, J. L. (2010). The pathway-specific regulator AveR from *Streptomyces avermitilis* positively regulates avermectin production while it negatively affects oligomycin biosynthesis. *Mol. Genet. Genomics* 283, 123–133. doi: 10.1007/s00438-009-0502-2

Hasegawa, H., and Häse, C. C. (2009). TetR-type transcriptional regulator VtpR functions as a global regulator in *Vibrio tubiashii*. *Appl. Environ. Microb.* 75, 7602–7609. doi: 10.1128/AEM.01016-09

Hayashi, T., Tanaka, Y., Sakai, N., Okada, U., Yao, M., Watanabe, N., et al. (2013). SCO4008, a putative TetR rranscriptional repressor from *Streptomyces coelicolor* A3(2), regulates rranscription of sco4007 by multidrug recognition. *J. Mol. Biol.* 425, 3289–3300. doi: 10.1016/j.jmb.2013.06.013

Hayashi, T., Tanaka, Y., Sakai, N., Okada, U., Yao, M., Watanabe, N., et al. (2014). Structural and genomic DNA analysis of the putative TetR transcriptional repressor SCO7518 from *Streptomyces coelicolor* A3(2). *FEBS Lett.* 588, 4311–4318. doi: 10.1016/j.febslet.2014.09.037

Higo, A., Horinouchi, S., and Ohnishi, Y. (2011). Strict regulation of morphological differentiation and secondary metabolism by a positive feedback loop between two global regulators AdpA and BldA in *Streptomyces griseus*. *Mol. Microbiol.* 81, 1607–1622. doi: 10.1111/j.1365-2958.2011.07795.x

Hofer, I., Crusemann, M., Radzom, M., Geers, B., Flachshaar, D., Cai, X. F., et al. (2011). Insights into the biosynthesis of hormaomycin, an exceptionally complex bacterial signaling metabolite. *Chem. Biol.* 18, 381–391. doi: 10.1016/j.chembiol.2010.12.018

Hou, B., Lin, Y., Wu, H., Guo, M., Petkovic, H., Tao, L., et al. (2017). The novel transcriptional regulator LmbU promotes lincomycin biosynthesis through regulating expression of its target genes in *Streptomyces lincolnensis*. *J. Bacteriol.* 200, e447–e417. doi: 10.1128/JB.00447-17

Hou, B., Tao, L., Zhu, X., Wu, W., Guo, M., Ye, J., et al. (2018). Global regulator BldA regulates morphological differentiation and lincomycin production in *Streptomyces lincolnensis*. *Appl. Microbiol. Biotechnol.* 102, 4101–4115. doi: 10.1007/s00253-018-8900-1

Huang, H., and Grove, A. (2013). The transcriptional regulator TamR from *Streptomyces coelicolor* controls a key step in central metabolism during oxidative stress. *Mol. Microbiol.* 87, 1151–1166. doi: 10.1111/mmi.12156

Iqbal, M., Mast, Y., Amin, R., Hodgson, D. A., Consortium, S., Wohlleben, W., et al. (2012). Extracting regulator activity profiles by integration of de novo motifs and expression data: characterizing key regulators of nutrient depletion responses in *Streptomyces coelicolor*. *Nucleic Acids Res.* 40, 5227–5239. doi: 10.1093/nar/gks205

Keller, U., Lang, M., Crnovcic, I., Pfennig, F., and Schauwecker, F. (2010). The actinomycin biosynthetic gene cluster of *Streptomyces chrysomallus*: a genetic hall of mirrors for synthesis of a molecule with mirror symmetry. *J. Bacteriol.* 192, 2583–2595. doi: 10.1128/JB.01526-09

Kim, T. K. (2015). T test as a parametric statistic. *Korean J. Anesthesiol.* 68, 540–546. doi: 10.4097/kjae.2015.68.6.540

Kirm, B., Maqdevska, V., Tome, M., Horvat, M., Karnièar, K., Petek, M., et al. (2013). SACE_5599, a putative regulatory protein, is involved in morphological differentiation and erythromycin production in *Saccharopolyspora erythraea*. *Microb. Cell Fact.* 12:126. doi: 10.1186/1475-2859-12-126

Kisker, C., Hinrichs, W., Tovar, K., Hillen, W., and Saenger, W. (1995). The complex formed between Tet repressor and tetracycline-Mg2+ reveals mechanism of antibiotic resistance. *J. Mol. Biol.* 247, 260–280. doi: 10.1006/jmbi.1994.0138

Kuscer, E., Coates, N., Challis, I., Gregory, M., Wilkinson, B., Sheridan, R., et al. (2007). Roles of rapH and rapG in positive regulation of rapamycin biosynthesis in *Streptomyces hygroscopicus*. *J. Bacteriol.* 189, 4756–4763. doi: 10.1128/jb.00129-07

Lee, C. J., Won, H. S., Kim, J. M., Lee, B. J., and Kang, S. O. (2007). Molecular domain organization of BldD, an essential transcriptional regulator for developmental process of *Streptomyces coelicolor* A3(2). *Proteins* 68, 344–352. doi: 10.1002/prot.21338

Li, Q. L., Wang, L. F., Xie, Y. Y., Wang, S. M., Chen, R. X., and Hong, B. (2013). SsaA, a member of a novel class of transcriptional regulators, controls sansanmycin production in *Streptomyces* sp. strain SS through a feedback mechanism. *J. Bacteriol.* 195, 2232–2243. doi: 10.1128/JB.00054-13

Li, T., Zhao, K. X., Huang, V., Li, D. F., Jiang, C. Y., Zhou, N., et al. (2012). The TetR-type transcriptional repressor RolR from *Corynebacterium glutamicum* regulates resorcinol catabolism by binding to a unique operator, rolO. *Appl. Environ. Microb.* 78, 6009–6016. doi: 10.1128/AEM.01304-12

Liu, G., Chater, K. F., Chandra, G., Niu, G., and Tan, H. (2013). Molecular regulation of antibiotic biosynthesis in *Streptomyces*. *Microbiol. Mol. Biol. Rev.* 77, 112–143. doi: 10.1128/MMBR.00054-12

Ma, J., Wang, Z., Huang, H., Luo, M., Zuo, D., Wang, B., et al. (2011). Biosynthesis of himastatin: assembly line and characterization of three cytochrome P450 enzymes involved in the post-tailoring oxidative steps. *Angew. Chem. Int. Ed. Engl.* 50, 7797–7802. doi: 10.1002/anie.201102305

Mao, X. M., Luo, S., Zhou, R. C., Wang, F., Yu, P., Sun, N., et al. (2015). Transcriptional regulation of the daptomycin gene cluster in *Streptomyces roseosporus* by an autoregulator. AtrA. *J. Biol. Chem.* 290, 7992–8001. doi: 10.1074/jbc.M114.608273

Natsume, R., Ohnishi, Y., Senda, T., and Horinouchi, S. (2004). Crystal structure of a γ-butyrolactone autoregulator receptor protein in *Streptomyces coelicolor* A3(2). *J. Mol. Biol.* 336, 409–419. doi: 10.1016/j.jmb.2003.12.040

Ohnishi, Y., Yamazaki, H., Kato, J. Y., Tomono, A., and Horinouchi, S. (2005). AdpA, a central transcriptional regulator in the A-factor regulatory cascade that leads to morphological development and secondary metabolism in *Streptomyces griseus*. *Biosci. Biotechnol. Biochem.* 69, 431–439. doi: 10.1271/bbb.69.431

O'Rourke, S., Wietzorrek, A., Fowler, K., Corre, C., Challis, G. L., and Chater, K. F. (2009). Extracellular signalling, translational control, two repressors and an activator all contribute to the regulation of methylenomycin production in *Streptomyces coelicolor*. *Mol. Microbiol.* 71, 763–778. doi: 10.1111/j.1365-2958.2008.06560.x

Rodriguez-Sainz, M. C., Hernandez-Chico, C., and Moreno, F. (1990). Molecular characterization of pmbA, an *Escherichia coli* chromosomal gene required for the production of the antibiotic peptide MccB17. *Mol. Microbiol.* 4, 1921–1932. doi: 10.1111/j.1365-2958.1990.tb02041.x

Santamarta, I., Lopez-Garcia, M. T., Kurt, A., Nardiz, N., Alvarez-Alvarez, R., Perez-Redondo, R., et al. (2011). Characterization of DNA-binding sequences for CcaR in the cephamycin-clavulanic acid supercluster of *Streptomyces clavuligerus*. *Mol. Microbiol.* 81, 968–981. doi: 10.1111/j.1365-2958.2011.07743.x

Schumacher, M. A., Chinnam, N. B., Cuthbert, B., Tonthat, N. K., and Whitfill, T. (2015). Structures of regulatory machinery reveal novel molecular mechanisms controlling *B. subtilis* nitrogen homeostasis. *Genes Dev.* 29, 451–464. doi: 10.1101/gad.254714.114

Sheldon, P. J., Busarow, S. B., and Hutchinson, C. R. (2002). Mapping the DNA-binding domain and target sequences of the *Streptomyces peucetius* daunorubicin biosynthesis regulatory protein. DnrI. *Mol. Microbiol.* 44, 449–460. doi: 10.1046/j.1365-2958.2002.02886.x

Si, D., Urano, N., Shimizu, S., and Kataoka, M. (2012). LplR, a repressor belonging to the TetR family, regulates expression of the L-pantoyl lactone dehydrogenase gene in *Rhodococcus erythropolis*. *Appl. Environ. Microbiol.* 78, 7923–7930. doi: 10.1128/AEM.01583-12

Tahlan, K., Yu, Z., Xu, Y., Davidson, A. R., and Nodwell, J. R. (2008). Ligand recognition by ActR, a TetR-like regulator of actinorhodin export. *J. Mol. Biol.* 383, 753–761. doi: 10.1016/j.jmb.2008.08.081

Takano, E., Gramajo, H. C., Strauch, E., Andres, N., White, J., and Bibb, M. J. (1992). Transcriptional regulation of the redD transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* 6, 2797–2804. doi: 10.1111/j.1365-2958.1992.tb01459.x

Tanaka, A., Takano, Y., Ohnishi, Y., and Horinouchi, S. (2007). AfsR recruits RNA polymerase to the afsS promoter: a model for transcriptional activation by SARPs. *J. Mol. Biol.* 369, 322–333. doi: 10.1016/j.jmb.2007.02.096

Uguru, G. C., Stephens, K. E., Stead, J. A., Towle, J. E., Baumberg, S., and McDowall, K. J. (2005). Transcriptional activation of the pathway-specific regulator of the actinorhodin biosynthetic genes in *Streptomyces coelicolor*. *Mol. Microbiol.* 58, 131–150. doi: 10.1111/j.1365-2958.2005.04817.x

van der Heul, H. U., Bilyk, B. L., McDowall, K. J., Seipke, R. F., and van Wezel, G. P. (2018). Regulation of antibiotic production in actinobacteria: new perspectives from the post-genomic era. *Nat. Prod. Rep.* 35, 575–604. doi: 10.1039/c8np00012c

Wang, L., Tian, X., Wang, J., Yang, H., Fan, K., Xu, G., et al. (2009). Autoregulation of antibiotic biosynthesis by binding of the end product to an atypical response regulator. *Proc. Natl. Acad. Sci. U.S.A.* 106, 8617–8622. doi: 10.1073/pnas.0900592106

Wietzorrek, A., and Bibb, M. (1997). A novel family of proteins that regulates antibiotic production in *streptomycetes* appears to contain an OmpR-like DNA-binding fold. *Mol. Microbiol.* 25, 1181–1184. doi: 10.1046/j.1365-2958.1997.5421903.x

Wilson, D. J., Xue, Y., Reynolds, K. A., and Sherman, D. H. (2001). Characterization and analysis of the PikD regulatory factor in the pikromycin biosynthetic pathway of *Streptomyces venezuelae*. *J. Bacteriol.* 183, 3468–3475. doi: 10.1128/jb.183.11.3468-3475.2001

Wray, L. V., and Fisher, S. H. (2008). *Bacillus subtilis* GlnR contains an autoinhibitory C-terminal domain required for the interaction with glutamine synthetase. *Mol. Microbiol.* 68, 277–285. doi: 10.1111/j.1365-2958.2008.06162.x

Xie, Y., Li, Q., Qin, X., Ju, J., and Ma, J. (2019). Enhancement of himastatin bioproduction via inactivation of atypical repressors in *Streptomyces hygroscopicus*. *Metab. Eng. Commun.* 8:e00084. doi: 10.1016/j.mec.2018.e00084

Xu, Y., Ke, M., Li, J., Tang, Y., Wang, N., Tan, G., et al. (2018). Negative Involvement of the TetR-type regulator SLCG_2919 in the regulation of lincomycin biosynthesis in *Streptomyces lincolnensis*. *Appl. Environ. Microbiol.* 85:e02091-18. doi: 10.1128/AEM.02091-18

Yamazaki, H., Tomono, A., Ohnishi, Y., and Horinouchi, S. (2004). DNA-binding specificity of AdpA, a transcriptional activator in the A-factor regulatory cascade in *Streptomyces griseus*. *Mol. Microbiol.* 53, 555–572. doi: 10.1111/j.1365-2958.2004.04153.x

Yu, Z., Reichheld, S. E., Savchenko, A., Parkinson, J., and Davidson, A. R. (2010). A comprehensive analysis of structural and sequence conservation in the TetR family transcriptional

regulators. *J. Mol. Biol.* 400, 847–864. doi: 10.1016/j.jmb.2010.05.062

Zhang, Y., Pan, G., Zou, Z., Fan, K., Yang, K., and Tan, H. (2013). JadR*-mediated feed-forward regulation of cofactor supply in jadomycin biosynthesis. *Mol. Microbiol.* 90, 884–897. doi: 10.1111/mmi.12406

Zou, Z. Z., Du, D. Y., Zhang, Y. Y., Zhang, J. H., Niu, G. Q., and Tan, H. R. (2014). A γ-butyrolactone-sensing activator/repressor, JadR3, controls a regulatory mini-network for jadomycin biosynthesis. *Mol. Microbiol.* 94, 490–505. doi: 10.1111/mmi.12752

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New Metabolites From the Co-culture of Marine-Derived Actinomycete *Streptomyces rochei* MB037 and Fungus *Rhinocladiella similis* 35

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Co-culture of different microbes simulating the natural state of microbial community may produce potentially new compounds because of nutrition or space competition. To mine its metabolic potential in depth, co-culture of *Streptomyces rochei* MB037 with a gorgonian-derived fungus *Rhinocladiella similis* 35 was carried out to stimulate the production of new metabolites in this study, using pure cultivation as control. Five metabolites were isolated successfully from co-culture broth, including two new fatty acids with rare nitrile group, borrelidins J and K (**1** and **2**), one chromone derivative as a new natural product, 7-methoxy-2,3-dimethylchromone-4-one (**3**), together with two known 18-membered macrolides, borrelidin (**4**) and borrelidin F (**5**). The structures of **1**–**3** were elucidated by using a combination of NMR and MS spectroscopy, ester hydrolysis, and optical rotation methods. Interestingly, **1** and **2** were obtained only in co-culture. Though **3** was gained from either co-culture or single culture, its production was increased significantly by co-culture. Compound **1** exhibited significant antibacterial activity against methicillin-resistant *Staphylococcus aureus* with a MIC value of 0.195 μg/mL.

Keywords: co-culture, actinomycete, fungus, borrelidin, antibacterial activity

INTRODUCTION

In nature, microbes generally exist in a community. One microbe may produce biological products to inhibit other microbes for limited nutrition or space competition or against pathogenic microbes. Thus, co-culture of microorganisms which involves the cultivation of two or more microorganisms in the same confined environment may produce potentially new compounds by stimulating the silent genes or gene clusters of one partner or increase the yields of previously described metabolites. For example, Sung et al. (2017) researched increased production of three antibiotics and enhanced biological activity against the Gram positive human pathogens via co-cultures of a

marine-derived *Streptomyces* sp. with human pathogens (Sung et al., 2017), and Zuck et al. (2011) gained a cytotoxic *N*,*N*′-((1*Z*,3*Z*)-1,4-*bis*(4-methoxyphenyl)buta-1,3-diene-2,3-diyl)diformamide by co-culture of the fungus *Aspergillus fumigatus* with the actinomycete *Streptomyces peucetius*.

Macrolide borrelidin has been reported to show broad-spectrum activities (Anderson et al., 1989; Ishiyama et al., 2011; Mirando et al., 2015). In our previous study, borrelidin was isolated and elucidated as a major product from a sponge-derived actinomycete *Streptomyces rochei* MB037 (Li et al., 2018). To mine its more metabolic potential, co-culture approach was applied on *S. rochei* MB037. A gorgonian-derived fungal strain, *Rhinocladiella similis* 35, was selected as a partner against actinomycete *S. rochei* MB037. A literature survey revealed that a few new and bioactive compounds were separated from the fungus *Rhinocladiella* sp. (Wagenaar et al., 2000; Zhang et al., 2014). The co-culture of *S. rochei* MB037 and *R. similis* 35 stimulated the production of new metabolites successfully. Herein, we report the isolation, structural elucidation, and evaluation of biological activities of the metabolites **1**–**5** (**Figure 1**) produced by co-culture of *S. rochei* MB037 and *R. similis* 35. A plausible biosynthesis pathway for the metabolites was also proposed and discussed.

MATERIALS AND METHODS

Instrumentation

Optical rotations were determined on a JASCO P-2000 polarimeter. 1D and 2D NMR spectra were recorded on an Avance III 600 MHz NMR spectrometer. High-resolution electrospray ionization mass spectroscopy (HR-ESI-MS) was recorded with an ACQUITYTM UPLC and Q-TOF mass spectrometer. High-performance liquid chromatography (HPLC) analysis was performed with an Agilent 1200 detector (G1322A), using a Durashell 150 Å C_{18} column (4.6 mm × 250 mm, 5 μm, Agela) and HPLC preparative-scale purification was performed with an Eclipse XDB C_{18} column (4.6 mm × 150 mm, 5 μm, Agela). The FT-IR spectra were recorded using a Nicolet 6700 spectrometer.

Microbial Strains

The actinomycete *S. rochei* MB037 was derived from sponge *Dysidea arenaria* collected from Yongxin Island (112° 20′ E; 16° 50′ N) in the South China Sea (Karuppiah et al., 2015). It was identified as *S. rochei* on the basis of the 100% 16S rDNA sequence identity with the type strain of this species, under the GenBank Accession No. AA2017041 (Li et al., 2018). The fungal strain *R. similis* 35 was isolated from a staghorn gorgonian collected from Luhuitou fringing reefs (109° 25′ E; 18° 15′ N) in the South China Sea in July 2014. The strain was identified as *R. similis* according to its morphologic traits and molecular identification. Its 616 base pair ITS sequence had 100% sequence identity to that of *R. similis* (KY680425) isolate CMRP1259. The sequence data have been submitted to GenBank, under the Accession No. MH481284. Both of the strains were stored at −80°C after their arrival to the Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, China.

Fermentation, Extraction, and Isolation

The actinomycete *S. rochei* MB037 and fungal *R. similis* 35 were cultivated in 25 L of ISP2 medium [malt extract 10 g, anhydrous dextrose 4 g, yeast extract 4 g in 1 L of artificial seawater (NaCl 132.6 g, $MgCl_2{\cdot}6H_2O$ 55.86 g, $CaCl_2$ 5.705 g, KCl 3.625 g, $NaHCO_3$ 1.01 g, NaBr 0.415 g), pH value 7.0] at 28°C with shaking at 180 rpm, respectively. On day 3, 200 mL of actinomycete culture was inoculated into each 200 mL of the fungal cultures (1:1 v/v) to initiate the co-culture experiment. The changes of secondary metabolite production between co-culture and single culture were analyzed by reverse-phased HPLC.

After incubation for 11 additional days, the co-culture broth was extracted with 50 L ethyl acetate using rotary evaporator to yield 12 g of reddish brown oil material. The organic extract (12 g) was subjected to Sephadex LH-20 eluting by MeOH to obtain four fractions (Fr. 1–4). Fr. 2–4 was subjected to reversed phase chromatograph eluting with MeOH-H_2O (Cheetah Fs-9200t C_{18}, linear gradient 10, 30, 50, 70, 90, and 100% aqueous CH_3OH for 30 min, respectively, at a flow rate of 20 mL/min) to give three fractions (Fr. 2–7, 3–18, and 4–8). Fr. 2–7 was further purified twice by preparative HPLC (Eclipse XDB C_{18} column, 4.6 mm × 150 mm, 5 μm, Agela) with 40 to 44% aqueous CH_3CN to yield **5** (7 mg). Fr. 3–18 was further purified using Sephadex LH-20 with CH_3OH and then followed by preparative HPLC with 50% aqueous CH_3CN to give **2** (5 mg), **4** (20 mg) and **5** (4 mg), respectively. Fr. 4–8 was further purified by preparative HPLC with 38% aqueous CH_3CN to provide **3** (10 mg).

Borrelidin J (1)

A light yellow oil; $[\alpha]^{25}_D$ – 25.5 (*c* 4.0, MeOH); ^1H and ^{13}C NMR spectroscopic data, **Tables 1**, **2**; (−)-HR-ESI-MS m/z 506.3117 [M-H]− (calcd for $C_{28}H_{44}NO_7$, 506.3114).

Borrelidin K (2)

A light yellow oil; $[\alpha]^{25}_D$ – 14.0 (*c* 2.5, MeOH); ^1H and ^{13}C NMR spectroscopic data, **Tables 1**, **2**; (−)-HR-ESI-MS m/z 520.3264 $[M-H]^-$ (calcd for $C_{29}H_{46}NO_7$, 520.3261).

7-Methoxy-2,3-Dimethylchromone-4-One (3)

A brown yellow solid; ^1H and ^{13}C NMR spectroscopic data, **Table 3**; (+)-HR-ESI-MS m/z 205.0885 $[M+H]^+$(calcd for $C_{12}H_{13}O_3$, 205.0888).

Borrelidin (4)

A white amorphous powder; $[\alpha]^{25}_D$ – 16.0 (*c* 2.5, MeOH); ^1H and ^{13}C NMR spectroscopic data, **Tables 1**, **2**; (−)-HR-ESI-MS m/z 520.3264 $[M-H]^-$ (calcd for $C_{29}H_{46}NO_7$, 520.3261).

Borrelidin F (5)

A white amorphous powder; $[\alpha]^{25}_D$ + 14.0 (*c* 2.5, MeOH); ^1H and ^{13}C NMR spectroscopic data, **Tables 1**, **2**; (−)-HR-ESI-MS m/z 520.3264 $[M-H]^-$ (calcd for $C_{29}H_{46}NO_7$, 520.3261).

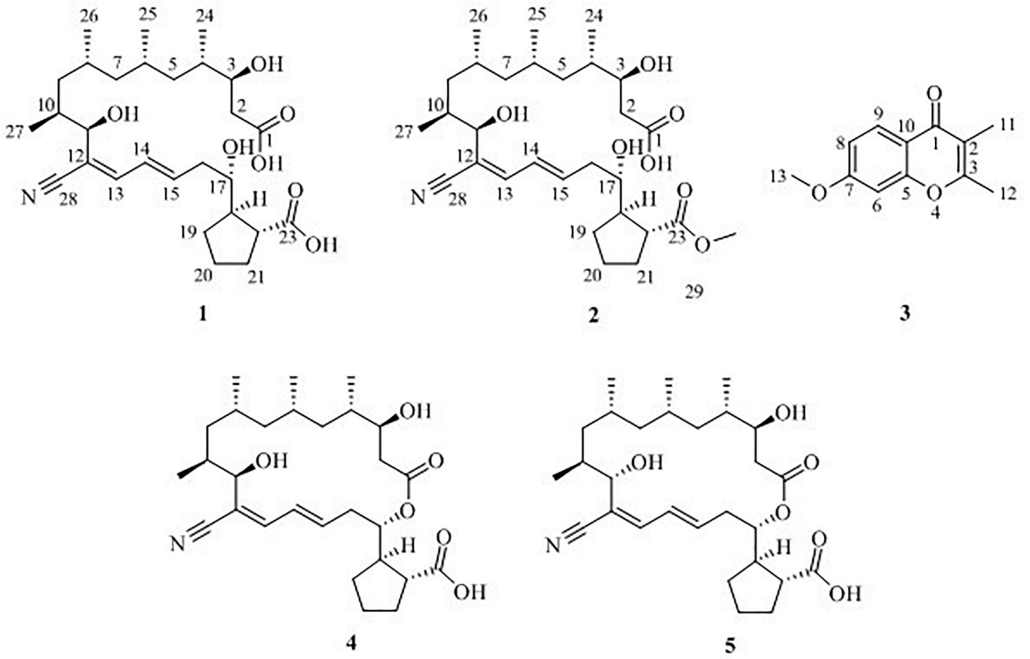

FIGURE 1 | Chemical structures of compounds **1**–**5**.

Antibacterial Bioassay

Antibacterial activity was evaluated by the conventional broth dilution assay (Appendino et al., 2008). Five bacterial strains: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Bacillus mycoides* were used, and ciprofloxacin was used as a positive control.

RESULTS

Co-culture of *S. rochei* MB037 and *R. similis* 35

The co-culture of sponge-derived actinomycete *S. rochei* MB037 with multiple marine microorganisms (bacteria and fungi) was tested. Based on a series of screening of co-cultivation with single strain cultivation as control, significant changes in metabolites in the fermentation broth were observed in the co-culture of a gorgonian-derived fungal strain, *R. similis* 35 with *S. rochei* MB037. The actinomycete stain *S. rochei* MB037 and fungal strain *R. similis* 35 were cultured for 3 days, respectively, and then co-cultivated for 11 days. As a control, the single cultivation was carried out for 14 days. Then the EtOAc extracts of fermentation broth were compared by HPLC (**Figure 2**). In single strain cultivation, two peaks (**4** and **5**) appeared for *S. rochei* MB037 (**Figure 2A**); a weak peak (**3**) was detected in *R. similis* 35 (**Figure 2B**). Compared with the control, two extra peaks (**1** and **2**) and one obvious increased peak (**3**) were detected in the co-cultural EtOAc extracts (**Figure 2C**). HR-ESI-MS analysis confirmed that peaks **1** and **2** were not detected in single cultural broth of actinomycete *S. rochei* MB037, while peak **3** with the molecular weight 204 ($[M + H]^+$ *m/z* at 205.0885) was detected in the single cultural broth of fungal strain *R. similis* 35 (**Figure 3**). This result indicated that the fungus *R. similis* 35 successfully induced the actinomycete *S. rochei* MB037 to produce new metabolites.

Five compounds (**1–5**) (**Figure 1**) were isolated from the co-culture broth of *S. rochei* MB037 and *R. similis* 35 using column chromatography and preparative HPLC. Besides the known macrolide borrelidin (**4**) and borrelidin F (**5**), *S. rochei* MB037 was induced to produce two new fatty acids, borrelidins J (**1**) and K (**2**). Correspondingly, *R. similis* 35 was also stimulated to enhance the production of a chromone derivative 7-methoxy-2,3-dimethylchromone-4-one (**3**), which has never been reported as a natural product.

Structure Elucidation

Borrelidin J (**1**) was isolated as a yellow oil. Its molecular formula $C_{28}H_{45}NO_7$ was confirmed by negative ion HR-ESI-MS ($[M - H]^-$ calcd. 506.3117, $C_{28}H_{44}NO_7^-$) (**Supplementary Figure S1**) with seven degrees of unsaturation. The IR spectra showed absorption at 3375, 2210, and 1637 cm^{-1}, suggesting the presence of hydroxyl, nitrile group, and double-bond protons, respectively. The NMR of **1** (**Supplementary Figures S2–S5**) closely resembled that of the known compound borrelidin (**4**) expect for the differences at C-1 and C-17 proton signals. The C-1 proton signal at δ_C 172.1 in **4** was shifted to δ_C 175.4 in **1**. The H-17 proton signal at δ_H 5.29 (1H, d, J = 10.0 Hz) in **4** was shifted to δ_H 3.83 (1H, m) in **1**, and this change was consistent with the difference between **4** and **1** of ^{13}C signals, the C-17 at δ_C 75.8 for **4** was shifted to δ_C 74.0 for **1** on the ^{13}C NMR (**Table 2** and **Supplementary Figure S4**). Also, on the HMBC (**Supplementary Figure S6**), C-1 was related to H-17 in

TABLE 1 | ^{1}H NMR (600 MHz) data for **1**, **2**, **4**, and **5** in pyridine-d_5.

Position	1		2		4		5	
	δ_H	Mult (*J* in Hz)	δ_H	Mult (*J* in Hz)	δ_H	Mult (*J* in Hz)	δ_H	Mult (*J* in Hz)
2	2.93	m	2.94	m	2.73	m	2.70	m
	2.82	d (13.5)	2.82	m	-	-	2.56	m
3	4.54	m	4.55	m	4.35	m	4.31	d (9.0)
4	1.91	m	2.22	m	1.97	m	2.04	m
5	1.75	m	1.75	m	1.29	m	1.38	m
	1.15	m	1.16	m	0.92	m	0.90	m
6	1.80	m	1.76	m	2.10	m	2.08	m
7	1.27	m	1.28	m	1.02	m	1.11	m
	0.95	m	0.96	m	0.96	m	1.02	m
8	1.75	m	1.78	m	1.72	m	1.57	m
9	1.37	m	1.37	m	1.38	m	1.25	m
	1.23	m	1.23	m	0.94	m	1.05	m
10	2.21	m	1.92	m	2.25	m	2.24	m
11	4.71	d (8.0)	4.71	d (8.0)	4.53	d (8.0)	3.99	d (8.5)
13	6.95	d (11.0)	6.96	d (11.0)	6.82	d (11.0)	6.78	d (11.0)
14	6.85	m	6.84	m	6.65	m	6.69	m
15	6.48	m	6.42	m	6.28	m	6.07	m
16	2.63	m	2.53	m	2.54	m	2.56	m
	2.55	m	2.45	m	2.44	m	2.37	m
17	3.83	m	3.72	m	5.29	m	5.40	m
18	2.75	m	2.62	m	2.84	m	2.82	m
19	1.84	m	1.42	m	1.87	m	1.80	m
	1.48	m	1.25	m	1.25	m	1.29	m
20	1.62	m	1.62	m	1.59	m	1.68	m
21	2.16	m	1.92	m	2.06	m	2.02	m
	2.05	m	1.87	m	1.97	m	1.98	m
22	3.23	m	3.05	dd (8.0, 8.0)	3.04	ddd (8.0, 8.0, 8.0)	2.88	ddd (8.0, 8.0, 8.0)
24	1.11	d (6.5)	1.11	d (6.5)	0.80	d (6.5)	0.78	d (6.5)
25	0.92	d (6.5)	0.93	d (6.5)	0.94	d (6.5)	0.92	d (6.5)
26	0.93	d (6.5)	0.94	d (6.5)	0.87	d (6.5)	0.89	d (6.5)
27	1.27	d (6.5)	1.28	d (6.5)	1.26	d (6.5)	1.22	d (6.5)
29	–	–	3.64	s	–	–	–	–

4, however, there was no correlation between them in **1**. Besides, compound **4** has one less unsaturation and one more H_2O on the formula and molecular weight than **1**. On the basis of these data (**Supplementary Figures S1–S9**), we postulated that **1** was the ester hydrolysis product of **4**.

Borrelidin K (**2**) was purified as a yellow oil and its molecular formula was determined to be $C_{29}H_{47}NO_7$ based on the analysis of negative ion HR-ESI-MS ($[M - H]^-$ calcd. 520.3264, $C_{29}H_{46}NO_7^-$) (**Supplementary Figure S10**). The NMR data (**Supplementary Figures S11–S14**) of **2** were analogous to those of **1** (**Tables 1**, **2**), except for a carbon signal at δ_C 51.3 (**Table 2** and **Supplementary Figure S13**), presumed to be an oxygen carbon signal. HSQC (**Supplementary Figure S15**) correlation of H-29 (δ_H 3.64, 3H) to C-29 (δ_C 51.3) and HMBC correlation of H-29 (δ_H 3.64, 3H) to C-23 (δ_C 177.3) further confirmed the existence of methoxy group of C-29 (**Figure 4** and **Supplementary Figure S16**). Combined with the relevant information of COSY (**Figure 4** and **Supplementary Figure S17**) and NOSEY spectra (**Supplementary Figure S18**), the structure of 2 was determined. Therefore, it was confirmed that the methyl group replaced the H atom of the C-23 carboxylic acid in compound **1** to form a methyl ester.

7-Methoxy-2,3-dimethylchromone-4-one (**3**) was isolated as a brown yellow powder. Its molecular formula was deduced as $C_{12}H_{12}O_3$ on the basis of (+)-HR-ESI-MS analysis ($[M + H]^+$ calcd. 205.0885, $C_{12}H_{13}O_3^+$) (**Supplementary Figure S19**). The ^{1}H NMR (**Table 3** and **Supplementary Figure S20**) of **5** in DMSO-d_6 showed three aromatic hydrogens including H-6 (δ_H 7.06, d, J = 2.4 Hz), H-8 (δ_H 7.01, dd, J = 8.9, 2.4 Hz) and H-9 (δ_H 7.91, d, J = 8.9 Hz), and three methyl protons including H-11 (δ_H 1.93, s), H-12 (δ_H 2.40, s), and H-13 (δ_H 3.88, s). ^{13}C NMR data showed (**Table 3** and **Supplementary Figures S21**, **S22**) six quaternary carbons, including one carbonyl carbons C-1 (δ_C 176.4), two double-bond carbons C-2 (δ_C 162.2), and C-3 (δ_C 116.2), and three aromatic carbons C-7 (δ_C 163.8), C-10 (δ_C 157.6), and C-5 (δ_C 116.3). Moreover, the ^{13}C NMR data revealed the presence of one methoxy group C-13 (δ_C 56.5) and three aromatic tertiary carbons including C-9 (δ_C 127.0),

TABLE 2 | ^{13}C NMR (150 MHz) data for **1**, **2**, **4**, and **5** in pyridine-d_5.

Position	1		2		4		5	
	δ_C	Type	δ_C	Type	δ_C	Type	δ_C	Type
1	175.4	C	175.3	C	172.1	C	173.0	C
2	41.2	CH_2	41.1	CH_2	38.6	CH_2	38.1	CH_2
3	70.7	CH	70.7	CH	70.8	CH	70.8	CH
4	36.5	CH	36.5	CH	36.0	CH	35.7	CH
5	41.5	CH_2	41.5	CH_2	43.6	CH_2	43.2	CH_2
6	27.3	CH	27.3	CH	27.3	CH	26.9	CH
7	45.9	CH_2	45.9	CH_2	48.0	CH_2	48.4	CH_2
8	27.2	CH	27.2	CH	26.5	CH	26.4	CH
9	39.7	CH_2	39.8	CH_2	36.2	CH_2	37.7	CH_2
10	35.8	CH	35.8	CH	35.4	CH	35.6	CH
11	71.5	CH	71.5	CH	72.0	CH	78.9	CH
12	117.2	C	120.3	C	118.0	C	117.0	C
13	144.1	CH	144.0	CH	143.4	CH	143.8	CH
14	126.4	CH	126.5	CH	127.5	CH	129.0	CH
15	143.3	CH	143.0	CH	138.7	CH	139.9	CH
16	40.5	CH_2	40.5	CH_2	37.7	CH_2	37.9	CH_2
17	74.0	CH	73.7	CH	75.8	CH	75.3	CH
18	49.7	CH	50.3	CH	49.4	CH	48.9	CH
19	30.3	CH_2	29.9	CH_2	29.6	CH_2	29.5	CH_2
20	26.1	CH_2	25.8	CH_2	25.4	CH_2	25.5	CH_2
21	31.7	CH_2	31.7	CH_2	31.6	CH_2	31.5	CH_2
22	47.2	CH	46.8	CH	46.2	CH	48.3	CH
23	179.4	C	177.3	C	179.0	C	178.7	C
24	14.7	CH_3	14.7	CH_3	18.1	CH_3	17.9	CH_3
25	20.0	CH_3	20.0	CH_3	18.3	CH_3	18.4	CH_3
26	20.9	CH_3	20.9	CH_3	20.4	CH_3	20.2	CH_3
27	15.4	CH_3	15.3	CH_3	15.1	CH_3	15.8	CH_3
28	120.4	C	117.3	C	119.9	C	117.4	C
29	–	–	51.3	CH_3	–	–	–	–

TABLE 3 | ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for **3** in DMSO-d_6.

C/H	3			
	δC	δH	Mult (*J* in Hz)	HMBC
1	176.4	–	–	9
2	162.2	–	–	–
3	116.1	–	–	–
5	116.3	–	–	–
6	100.6	7.06	d (2.4)	8, 9
7	163.8	–	–	–
8	114.7	7.01	dd (8.9, 2.4)	6, 9
9	127.0	7.91	d (8.9)	6, 8
10	157.6		–	6, 8, 9
11	10.2	1.93	s	12
12	18.7	2.40	s	–
13	56.5	3.88	s	–

C-8 (δ_C 114.7), and C-6 (δ_C 100.6). Correlations of H-9/C-1, C-6, C-7, C-8, and C-10, H-8/C-5, C-6, and C-10, H-13/C-7, and H-11/C-1 were observed in HMBC spectrum (**Figure 5** and **Supplementary Figure S23**). Supported by other NMR data showed in **Table 3**, and relevant HSQC (**Supplementary Figure S24**) and COSY (**Supplementary Figure S25**) spectra data, the structure of **3** was elucidated as a new natural product, 7-methoxy-2,3-dimethylchromone-4-one.

Compound **4** was definitively identified as previously reported borrelidin on the basis of HR-ESI-MS, ^{1}H NMR, ^{13}C NMR, optical rotation and comparisons with previously reported data (Liu et al., 2012).

Borrelidin F (**5**) was also analyzed and identified comparing with the data of HR-ESI-MS, ^{1}H NMR, ^{13}C NMR and optical rotation provided in the reported literature (Sun et al., 2018).

Antimicrobial Screening

The antibacterial activities assay revealed that compounds **1** and **2** exhibited potent activity against methicillin-resistant *S. aureus* with the MICs of 0.195 and 1.563 μg/mL, respectively (**Table 4**). Compound **1** showed stronger activity than ciprofloxacin while **4** and **5** were inactive, indicating that the cleavage of the ester bond of the macrolides enhanced the antibacterial activity. It could be supposed that co-culture activated the silencing metabolic potential of the actinomycete and produced more potent

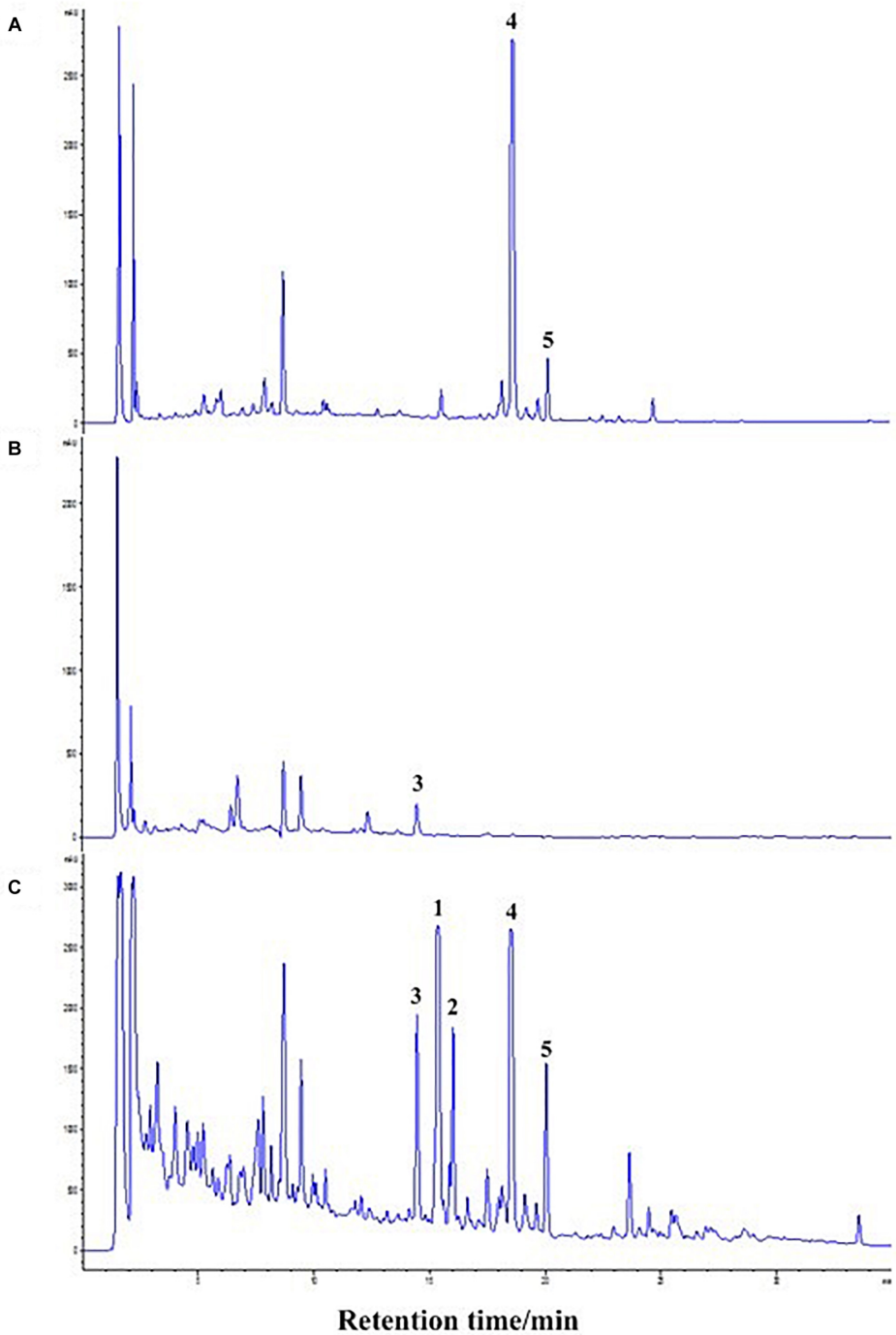

FIGURE 2 | High-performance liquid chromatography (HPLC) profiles of the EtOAc extracts of different culture approaches. **(A)** Pure culture of *Streptomyces rochei* MB037; **(B)** pure culture of *Rhinocladiella similis* 35; **(C)** co-culture of *S. rochei* MB037 and *R. similis* 35.

metabolites of antibacterial activity against microorganism, such as fungal attack.

Formation Mechanism Inference

Based on the structural characteristics of **1**, **2** and **4**, combined with literature reports (Uyama et al., 1995; Kobayashi, 2006), we proposed a plausible biosynthesis pathway for new compounds **1** and **2** (**Figure 6**). Compound **4** may be hydrolyzed to produce **1** by the lipase catalyst due to the mutual stimulation-inducing effect during the co-culture process of the actinomycete *S. rochei* MB037 and the fungus *R. similis* 35. Meanwhile, compound **2** was obtained when the C-23 carboxyl group of **1** was

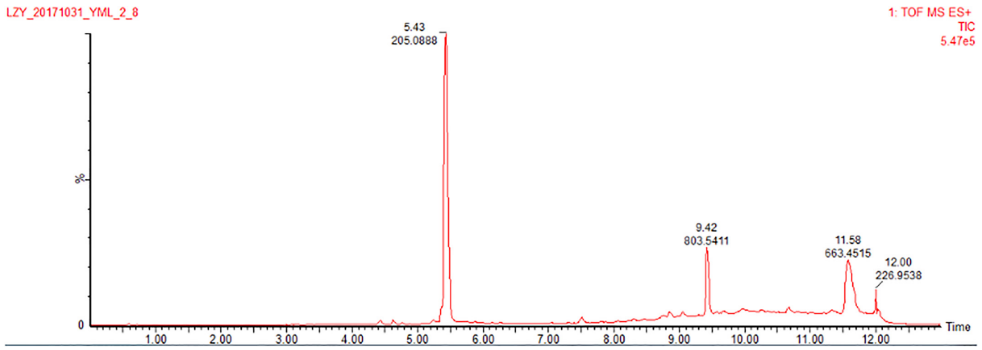

FIGURE 3 | HR-ESI-MS of the EtOAc extracts (positive ion mode) of pure culture of *R. similis* 35.

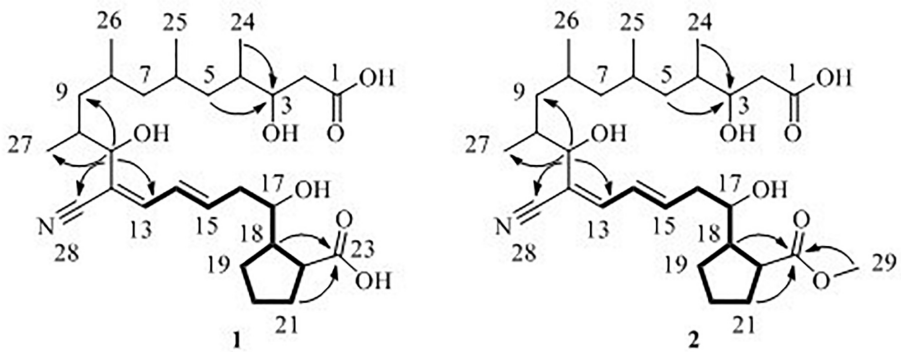

FIGURE 4 | ^{1}H–^{1}H COSY (—), key HMBC (⌒) correlations of compounds **1** and **2**.

methyl esterification. Consequently, it could be concluded that compounds **1** and **2** were derived from the actinomycete *S. rochei* MB037.

To investigate the cause for the enhancement of the production of compound **3**, we measured the pH value during the growths of pure and co-cultivation firstly. It was found that the pH value of the pure culture medium of the actinomycete began to decrease on the 2nd day (**Figure 7**), indicating that the actinomycete began to produce secondary metabolites. Subsequently, the fungus and actinomycete were cultured separately for 3 days and then sterilized. The inactivated cells of fungus or actinomycete were co-cultured with actinomycete or fungus for 3 days (**Figure 8**). As a result, it was found that when the fungus *R. similis* 35 was co-cultured with the sterilized actinomycete *S. rochei* MB037, compound **3** was still obtained with higher yield than pure culture (**Figure 8D**). It could be speculated that the secondary metabolites from actinomycete *S. rochei* MB037 stimulated the fungus *R. similis* 35 to produce **1**.

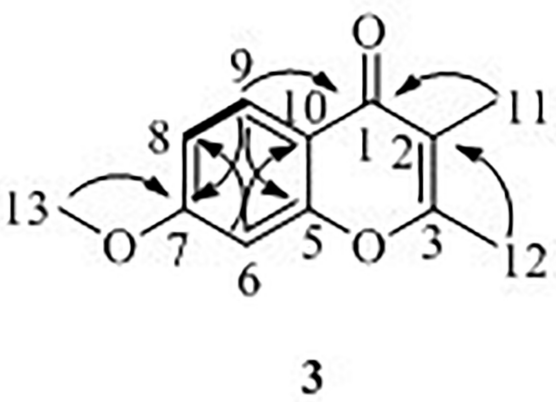

FIGURE 5 | ^{1}H–^{1}H COSY (—), key HMBC (⌒) correlations of compound **3**.

DISCUSSION

The fungus *R. similis* 35 was selected as the co-culture partner with *S. rochei* MB037 due to the stable emerging peaks in HPLC profiles of the co-cultures broth. 7-methoxy-2,3-dimethylchromone-4-one (**3**) showed a weak antibacterial activity against *P. aeruginosa* and *S. aureus*, therefore, the increased yield of compound **3** by fungus *R. similis* 35 may aim to inhibit the growth of *S. rochei* MB037. It is common for strains to compete with each other to obtain yield-increasing

TABLE 4 | Antibacterial activities of **1**–**5** (MIC, μg/ml).

	1	2	3	4	5	Ciprofloxacin
E. coli	>100	>100	>100	25	>100	0.156
P. aeruginosa	>100	50	25	>100	50	0.078
S. aureus	0.195	1.563	25	>100	>100	0.313
B. subtilis	>100	>100	>100	0.195	12.5	0.039
B. mycoides	>100	>100	>100	12.5	50	0.039

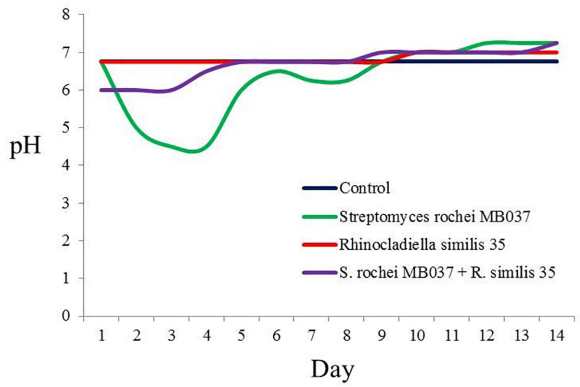

FIGURE 7 | pH trends of pure culture and co-culture of *S. rochei* MB037 and *R. similis* 35.

products in co-culture (Zuck et al., 2011; Sung et al., 2017). Co-culture is an effective method for inducing novel secondary metabolites from two interacted microbial strains. Recent studies revealed that many novel natural products were produced only from the interaction of two microbes (Scherlach and Hertweck, 2009; Hoshino et al., 2015; Wu et al., 2015). For example, the co-culture of two plant beneficial microbes *Trichoderma harzianum* M10 and *Talaromyces pinophilus* F36CF produced a novel harziaphilic acid (Vinale et al., 2017). From the perspective of microbial ecology, it is hypothesized that the production of borrelidin derivatives including two new compounds (**1** and **2**) and the increased yield of 7-methoxy-2,3-dimethylchromone-4-one (**3**) may be caused by the mutual competition for nutrition or space in the co-culture of these two strains.

Genomic sequencing has demonstrated that a large number of putative biosynthetic gene clusters encoding for secondary metabolites in many microorganisms are silent under classical cultivation conditions (Nett et al., 2009; Winter et al., 2011). A distinct fungal-bacterial interaction leads to the specific activation of fungal secondary metabolism genes, which has been demonstrated at the molecular level by microarray analyses, full-genome arrays, Northern blot, and quantitative RT-PCR analyses (Schroeckh et al., 2009). *Rhodococcus erythropolis* and *Corynebacterium glutamicum* were proved to change the biosynthesis of *Streptomyces* to produce new secondary metabolites (Onaka et al., 2011). Studies using chemical inhibitors disclosed that the activity of chromatin remodelers was the main factor for the interaction between *S. rapamycinicus* and *A. nidulans* to produce extra products (Nützmann et al., 2011). We speculated that compounds **1** and **2** were synthesized by the same biosynthetic gene clusters responsible for the biosynthesis of borrelidin according to the structure similarity. Compounds **1** and **2** were only produced by *S. rochei* MB037

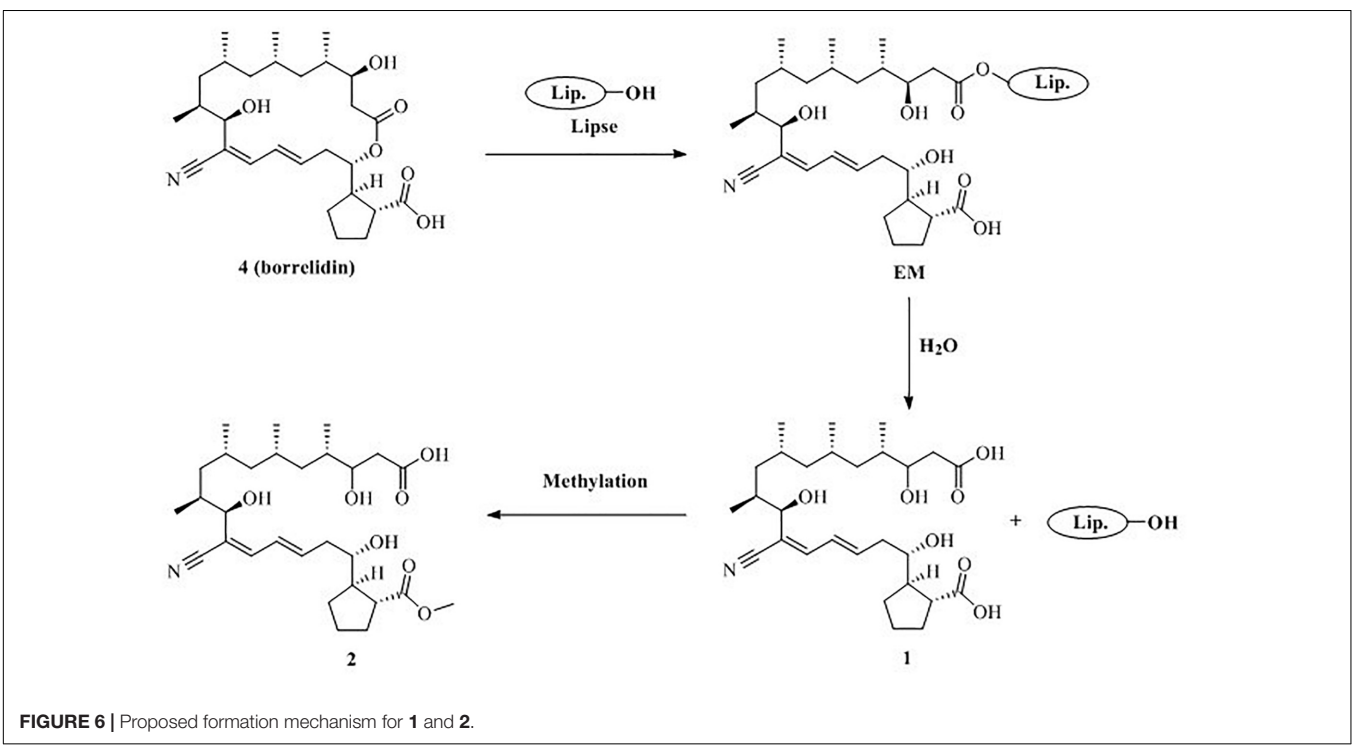

FIGURE 6 | Proposed formation mechanism for **1** and **2**.

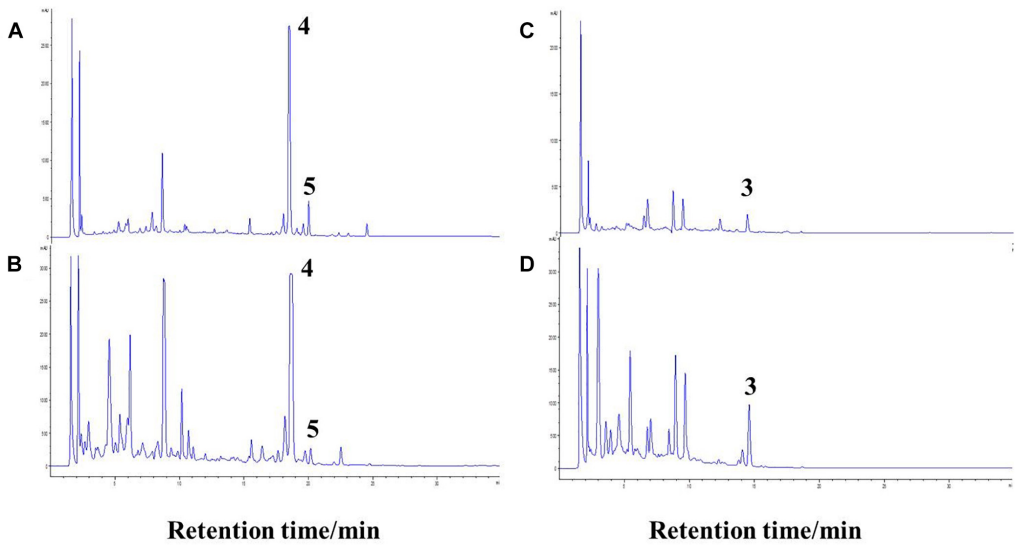

FIGURE 8 | Comparison of pure culture and co-culture. **(A)** Culture of *S. rochei* MB037; **(B)** co-culture of *S. rochei* MB037 and *R. similis* 35; **(C)** culture of *R. similis* 35; **(D)** co-culture of *R. similis* 35 and *S. rochei* MB037.

in co-culture condition indicating that the silent hydrolytic enzyme genes for hydrolyzing lactone in borrelidin could be activated by the co-culture of actinomycete *S. rochei* MB037 and fungus *R. similis* 35. The interaction between these two strains probably activate the expression of hydrolytic enzyme genes, harbored in actinomycete *S. rochei* MB037, and then led to hydrolytic action of lactone of borrelidin to generate compound **1**. Compound **2** is probably synthesized from compound **1** by methylation reaction since the only difference between **1** and **2** is the methyl ester group. During the extraction and purification processes, both of them were clearly detected in freshly prepared ethyl acetate extracts. Indeed, even if compound **1** was dissolved in methanol and stored at 28°C for 1 week, compound **2** was not detected in the solution. Therefore, compound **2** should be considered as a true natural product.

In the antibacterial bioassay, both compounds **1** and **2** exhibited stronger activities against *S. aureus* than **4** and **5**. Notably, the MIC value of compound **1** was 0.195 μg/ml, stronger than the positive control ciprofloxacin, indicating that **1** should be a potential antibacterial agent. It seems that the cleavage of the ester bond of the macrolide in borrelidin could enhance its antibacterial activity. Compounds **1** and **2** become the long-chain unsaturated fatty acid after the cleavage of the ester bond in borrelidin. Previous studies indicated that long-chain unsaturated fatty acid could exhibit strong activity against *S. aureus* by inhibiting the enoyl-acyl carrier protein reductase (FabI), which was the essential component in bacterial fatty acid synthesis (Zheng et al., 2005). However, the esterification of unsaturated fatty acid results in the loss of FabI-inhibitory activity, which is consistent with our results since compounds **4** and **5** exhibited no activity. The antibacterial activity of unsaturated fatty acid was very weak to the Gram negative bacteria due to the impermeability of their outer membrane. Consistently, compounds **1** and **2** showed weak antibacterial activity against *E. coli* and *P. aeruginosa*. Although *Bacillus subtilis* was the Gram positive bacteria, it has two kinds of enoyl-acyl carrier protein reductases, FabI and FabL, which may escape the inhibition of unsaturated fatty acid by alternative enoyl-acyl carrier protein reductase in fatty acid synthesis (Kim et al., 2011). The cytotoxicity of these compounds was not conducted, but their analogs exhibited cytotoxicity to mammalian cells (Zheng et al., 2005; Wilkinson et al., 2006; Chen et al., 2012; Sun et al., 2018).

CONCLUSION

This study demonstrated that microbial co-culture was an effective approach to explore the natural products. Based on a series of screening of co-cultivation of marine-derived microbes, a co-cultured combination of a sponge-derived actinomycete *S. rochei* MB037 and a gorgonian-derived fungus *R. similis* 35 was proved to induce the production of related polyketides with antibacterial activities successfully. The two new metabolites (**1** and **2**) produced by co-culture of marine-derived actinomycete and fungus represent the nitrogen-containing fatty acids which are rare in the nature. Future investigation should be focused on unveiling the mechanisms of action in molecular biology of the new produced compounds.

AUTHOR CONTRIBUTIONS

MY performed the experiments, data analyses, and wrote the draft manuscript. YL, LL, and CS assisted the bioactivity analysis and revised the manuscript. SB revised the manuscript.

ZL and CW supervised the whole work and edited the manuscript. All authors reviewed 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.2019.00915/full#supplementary-material

REFERENCES

Anderson, B. F., Herlt, A. J., Rickards, R. W., and Robertson, G. B. (1989). Crystal and molecular structures of two isomorphous solvates of the macrolide antibiotic borrelidin: absolute configuration determination by incorporation of a chiral solvent in the crystal lattice. *Aust. J. Chem.* 42, 717–730. doi: 10.1071/ch9890717

Appendino, G., Gibbons, S., Giana, A., Pagani, A., Grassi, G., Stavri, M., et al. (2008). Antibacterial cannabinoids from *Cannabis sativa*: a structure-activity study. *J. Nat. Prod.* 71, 1427–1430. doi: 10.1021/np8002673

Chen, Y., Liu, H. R., Liu, H. S., Cheng, M., Xia, P., Qian, K., et al. (2012). Antitumor agents 292. Design, synthesis and pharmacological study of S- and O-substituted 7-mercapto- or hydroxy-coumarins and chromones as potent cytotoxic agents. *Eur. J. Med. Chem.* 49, 74–85. doi: 10.1016/j.ejmech.2011.12.025

Hoshino, S., Wakimoto, T., Onaka, H., and Abe, I. (2015). Chojalactones A–C, cytotoxic butanolides isolated from *Streptomyces* sp. cultivated with mycolic acid containing bacterium. *Org. Lett.* 17, 1501–1504. doi: 10.1021/acs.orglett.5b00385

Ishiyama, A., Iwatsuki, M., Namatame, M., Nishihara-Tsukashima, A., Sunazuka, T., Takahashi, Y., et al. (2011). Borrelidin, a potent antimalarial: stage-specific inhibition profile of synchronized cultures of *Plasmodium falciparum*. *J. Antibiot.* 64, 381–384. doi: 10.1038/ja.2011.6

Karuppiah, V., Li, Y., Sun, W., Feng, G., and Li, Z. (2015). Functional gene-based discovery of phenazines from the actinobacteria associated with marine sponges in the South China Sea. *Appl. Microbiol. Biotechnol.* 99, 5939–5950. doi: 10.1007/s00253-015-6547-8

Kim, K. H., Ha, B. H., Kim, S. J., Hong, S. K., Hwang, K. Y., and Kim, E. E. (2011). Crystal structures of enoyl-ACP reductases I (FabI) and III (FabL) from *B. subtilis*. *J. Mol. Biol.* 406, 403–415. doi: 10.1016/j.jmb.2010.12.003

Kobayashi, S. (2006). Enzymatic ring-opening polymerization of lactones by lipase catalyst: mechanistic aspects. *Macromol. Symp.* 240, 178–185. doi: 10.1002/masy.200650822

Li, Y. X., Zhang, F. L., Banakar, S., and Li, Z. Y. (2018). Comprehensive optimization of precursor-directed production of BC194 by *Streptomyces rochei* MB037 derived from the marine sponge *Dysidea arenaria*. *Appl. Microbiol. Biotechnol.* 102, 7865–7875. doi: 10.1007/s00253-018-9237-5

Liu, C. X., Zhang, J., Wang, X. J., Qian, P. T., Wang, J. D., Gao, Y. M., et al. (2012). Antifungal activity of borrelidin produced by a *Streptomyces* strain isolated from soybean. *J. Agric. Food Chem.* 60, 1251–1257. doi: 10.1021/jf2044982

Mirando, A. C., Fang, P., Williams, T. F., Baldor, L. C., Howe, A. K., Ebert, A. M., et al. (2015). Aminoacyl-tRNA synthetase dependent angiogenesis revealed by a bioengineered macrolide inhibitor. *Sci. Rep.* 5, 13160–13176. doi: 10.1038/srep13160

Nett, M., Ikeda, H., and Moore, B. S. (2009). Genomic basis for natural product biosynthetic diversity in the actinomycetes. *Nat. Prod. Rep.* 26, 1362–1384. doi: 10.1039/B817069J

Nützmann, H. W., Reyes-Dominguez, Y., Scherlach, K., Schroeckh, V., Horn, F., Gacek, A., et al. (2011). Bacteria-induced natural product formation in the fungus *Aspergillus nidulans* requires Saga/Ada-mediated histone acetylation. *Proc. Nati. Acad. Sci. U.S.A.* 108, 14282–14287. doi: 10.1073/pnas.1103523108

Onaka, H., Mori, Y., Igarashi, Y., and Furumai, T. (2011). Mycolic acid-containing bacteria induce natural-product biosynthesis in *Streptomyces* species. *Appl. Environ. Microb.* 77, 400–406. doi: 10.1128/AEM.01337-10

Scherlach, K., and Hertweck, C. (2009). Triggering cryptic natural product biosynthesis in microorganisms. *Org. Biomol. Chem.* 7, 1753–1760. doi: 10.1039/B821578B

Schroeckh, V., Scherlach, K., Nützmann, H. W., Shelest, E., Schmidt-Heck, W., Schuemann, J., et al. (2009). Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *Proc. Nati. Acad. Sci. U.S.A.* 106, 14558–14563. doi: 10.1073/pnas.0901870106

Sun, J. B., Shao, J. L., Sun, C. L., Song, Y. X., Li, Q. L., Lu, L. C., et al. (2018). Borrelidins F–I, cytotoxic and cell migration inhibiting agents from mangrove-derived *Streptomyces rochei* SCSIO ZJ89. *Bioorgan. Med. Chem.* 26, 1488–1494. doi: 10.1016/j.bmc.2018.01.010

Sung, A. A., Gromek, S. M., and Balunas, M. J. (2017). Upregulation and identification of antibiotic activity of a marine-derived *Streptomyces* sp. via co-cultures with human pathogens. *Mar. Drugs* 15, 250–261. doi: 10.3390/md15080250

Uyama, H., Takeya, K., and Kobayashi, S. (1995). Enzymatic ring-opening polymerization of lactones to polyesters by lipase catalyst: unusually high reactivity of macrolides. *Bull. Chem. Soc. Jpn.* 68, 56–61. doi: 10.1246/bcsj.68.56

Vinale, F., Nicoletti, R., Borrelli, F., Mangoni, A., Parisi, O. A., Marra, R., et al. (2017). Co-culture of plant beneficial microbes as source of bioactive metabolites. *Sci. Rep.* 7, 14330–14341. doi: 10.1038/s41598-017-14569-5

Wagenaar, M. M., Corwin, J., Strobel, G., and Clardy, J. (2000). Three new cytochalasins produced by an endophytic fungus in the genus *Rhinocladiella*. *J. Nat. Prod.* 63, 1692–1695. doi: 10.1021/np0002942

Wilkinson, B., Gregory, M. A., Moss, S. J., Carletti, I., Sheridan, R. M., Kaja, A., et al. (2006). Separation of anti-angiogenic and cytotoxic activities of borrelidin by modification at the C17 side chain. *Bioorg. Med. Chem. Lett.* 16, 5814–5817. doi: 10.1016/j.bmcl.2006.08.073

Winter, J. M., Behnken, S., and Hertweck, C. (2011). Genomics-inspired discovery of natural products. *Curr. Opin. Chem. Biol.* 15, 22–31. doi: 10.1016/j.cbpa.2010.10.020

Wu, C., Zacchetti, B., Ram, A. F. J., van Wezel, G. P., Claessen, D., and Hae Choi, Y. (2015). Expanding the chemical space for natural products by *Aspergillus-Streptomyces* co-cultivation and biotransformation. *Sci. Rep.* 5, 10868–10877. doi: 10.1038/srep10868

Zhang, D. B., Yang, Z. D., Xue, P. H., Zhi, K. K., Shi, Y., and Wang, M. G. (2014). Two new cyclic dipeptides from *Rhinocladiella* sp. *lgt*-3, a fungal endophyte

isolated from *Tripterygiun wilfordii* Hook. *Nat. Prod. Res*. 28, 1760–1764. doi: 10.1080/14786419.2014.945176

Zheng, C. J., Yoo, J. S., Lee, T. G., Cho, H. Y., Kim, Y. H., and Kim, W. G. (2005). Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. *FEBS Lett*. 579, 5157–5162. doi: 10.1084/jem.193.3.393

Zuck, K. M., Shipley, S., and Newman, D. J. (2011). Induced production of N-formyl alkaloids from *Aspergillus fumigatus* by co-culture with *Streptomyces peucetius*. *J. Nat. Prod.* 74, 1653–1657. doi: 10.1021/np200255f

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Community Composition and Metabolic Potential of Endophytic Actinobacteria From Coastal Salt Marsh Plants in Jiangsu, China

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The diversity and functional roles of the plant associated endophytic actinobacteria in unique habitats remain poorly understood. In this paper, we examined the phylogenetic diversity and community composition of endophytic actinobacteria associated with native coastal salt marsh plants in Jiangsu, China using a combination of cultivation and 16S rRNA gene-based high-throughput sequencing (HTS) methods. Further, we evaluated the antifungal, fibrinolytic activities and the secondary metabolite biosynthesis potential of isolates via gene screening. A total of 278 actinobacterial isolates were isolated from 19 plant samples. 16S rRNA gene sequencing revealed that the isolates were highly diverse and belonged to 23 genera within the *Actinomycetales* order, with *Streptomyces*, *Saccharopolyspora*, and *Pseudonocardia* comprising the most abundant genera. In addition, more than 10 of the isolates were novel actinobacterial taxa distributed across eight genera. HTS analyses of seven representative plant root samples revealed that Actinobacteria phylum constituted 0.04–28.66% of root endophytic bacterial communities. A total of four actinobacterial classes, 14 orders, 35 families, and 63 known genera were detected via HTS, and these communities were found to be dominated by the members of the order *Actinomycetales* including the genera *Streptomyces*, *Mycobacterium*, *Arthrobacter*, *Nocardioides*, and *Micromonospora*. In addition, 30.4% of the representative isolates exhibited antifungal activities, 40.5% of them showed fibrinolytic activities, while 43.0% of the strains harbored secondary metabolite biosynthesis genes. These results demonstrated that coastal salt marsh plants in the Jiangsu Province represented an underexplored new reservoir of diverse and novel endophytic actinobacteria that may be of potential interest in the discovery of bioactive compounds with potential as biocontrol agents and for fibrinolytic enzyme production.

Keywords: endophytic actinobacteria, coastal salt marsh, diversity, high throughput sequencing, metabolic potential, gene screening

INTRODUCTION

The Actinobacteria phylum comprises Gram-positive and high guanine+cytosine (G+C) content bacterial taxa. Actinobacterial species are the main producers of active microbial natural products, are ubiquitously distributed among environments, and have been widely used in industrial and agricultural applications (Bèrdy, 2005; Qin et al., 2016). Recently, endophytic actinobacteria have particularly attracted significant attention, with increasing documentation of isolates from a wide range of plants, including various crop plants like wheat, rice, banana, apple, and tea plants, in addition to medicinal plants such as *Artemisia annua*, *Tripterygium wilfordii* Hook, *Glycyrrhiza inflata*, and *Jatropha curcas* (Conn and Franco, 2003; Tian et al., 2007; Qin et al., 2011, 2015b; Li et al., 2012; Miao et al., 2015; Álvarez-Pérez et al., 2017; Wei et al., 2018; Zhao et al., 2018). *Streptomyces*, *Micromonospora*, *Micrococcus*, *Pseudonocardia*, and *Microbacterium* are the most predominant endophytic actinobacterial genera cultivated, and many novel species from these genera have been identified in diverse host plants (Kaewkla and Franco, 2013; Golinska et al., 2015; Dinesh et al., 2017). Further, recent cultivation-independent analyses using 16S rRNA gene-based methods like denaturing gradient gel electrophoresis (DGGE), clone library analyses, and high-throughput sequencing have also revealed abundant endophytic actinobacterial communities within plants, including novel and uncultured taxa (Qin et al., 2012, 2018; Purushotham et al., 2018). For example, the high-throughput sequencing (HTS) of 16S rRNA genes from banana shoot tips yielded the detection of more than 50 known actinobacterial genera, which included many genera rarely identified as endophytes such as *Actinomycetospora*, *Actinoallomurus*, *Kibdelosporangium*, and *Kitasatospora* (Du et al., 2018).

In addition to the high species diversity of endophytic actinomycetes, increasing attention has been directed toward their ability to produce abundant bioactive metabolites. Several endophytic actinobacteria from medicinal plants produce novel bioactive compounds that have favorable antimicrobial and antitumor activities, besides showing a good potential for pharmaceutical development and biotechnological applications (Qin et al., 2011; Golinska et al., 2015; Dinesh et al., 2017). For example, the recently identified hamuramicins A and B obtained from the endophytic actinomycete *Allostreptomyces* sp. K12-0794 exhibited both antimicrobial activity and human cell line toxicity (Suga et al., 2018). In addition to their therapeutic uses, endophytic actinomycetes have a broad potential and value in agricultural application. The endophytes can promote plant growth via direct and indirect mechanisms, including phosphorus solubilization, production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, siderophores, induction of pathogen resistance, and aiding resistance to abiotic stresses like those imposed by high salinity and drought (Conn et al., 2008; Qin et al., 2014, 2017; Trujillo et al., 2015; Zeng et al., 2018).

When compared with the well-studied crop plants and endophytic actinobacteria within common environments, little is known about naturally occurring plant-associated actinobacteria within harsh habitats like hot and cold deserts, saline and acidic soils, and marine habitats. Coastal salt marshes are one of the most biologically productive habitats on Earth, and are one such example of a unique and harsh environment (Pennings and Bertness, 2001). Recent studies from coastal sediments and mangrove forests have revealed high levels of actinobacterial diversity (Hong et al., 2009; Hamedi et al., 2013; Jiang et al., 2018). Surprisingly, however, there is still little information related to the isolation, diversity and their biological activity of endophytic actinobacteria within coastal salt marsh ecosystems, despite the fact that such environments are likely to contain unique and phylogenetically diverse endophytes. Our recent analyses have indicated that there were abundant Actinobacteria and novel taxa in the rhizosphere soils associated with halophytes collected from coastal salt marshes within the Jiangsu Province of China (Wang et al., 2015; Bai et al., 2016; Ding et al., 2018). In particular, the phylum Actinobacteria comprised 2.8%–43.0% of rhizosphere bacterial communities, as evidenced by HTS on the Illumina MiSeq platform (Gong et al., 2018). Importantly, rhizosphere soils can be a source of endophytes (Long et al., 2010). There are abundant halophyte resources in the Jiangsu coastal region. Consequently, we speculated that plants within the Jiangsu coastal zone also contained abundant endophytic actinobacteria.

Thus, the aims of this study were to (i) survey the diversity of culturable endophytic actinobacteria from plants within coastal salt marshes in the Jiangsu province, (ii) analyze root endophytic actinobacterial communities from representative plants using 16S rRNA gene-based HTS technique, (iii) evaluate the anti-phytopathogenic fungal and fibrinolytic activity and analyze secondary metabolite biosynthetic gene profiles of isolates. We expect to provide a preliminary basis for the further discovery of biological active metabolites from coastal salt marsh plants endophytic actinobacteria and their further utilization for biocontrol of crops and thrombosis treatment for human beings.

MATERIALS AND METHODS

Sample Collection

Plant samples were collected from the coastal salt marsh region of the Jiangsu Province in Eastern China (32°26′09–34°45′14 N, 119°14′16–121°19′30 S) between 2010 and 2014. Nearly 20 healthy plant samples were collected, including roots, stems, and leaf samples from herbs, grasses, and woody trees. Representative plants sampled in the study included *Tamarix chinensis* Lour., *Dendranthema indicum*, *Salicornia europaea* Linn., *Sesbania cannabina*, *Spartina alterniflora*, and *Suaeda glauca*. Samples were placed in sterile plastic bags and transported to the laboratory within 24 h, where they were either stored at 4°C for analysis within one to 3 days or at −80°C for DNA extraction that was conducted within 2 weeks.

Isolation of Endophytic Actinobacterial Isolates

Plant surface sterilization and evaluation of sterilization effectiveness were conducted following our previously described

five-step procedure (Qin et al., 2009). The sterilization time used for each sample varied based on the specific plant tissues and organ types. Surface-sterilized samples were then aseptically grinded into smaller fragments using a commercial blender and then spread onto seven different Actinobacteria-selective media types: (A) modified TWYE medium supplemented with 20% (v/v) plant extracts (Qin et al., 2009); (B) starch-casein agar (Xing et al., 2012); (C) sodium propionate agar (Qin et al., 2009); (D) ISP 5 agar (Shirling and Gottlieb, 1966); (E) trehalose-proline agar (Qin et al., 2009); (F) MOPS-amino acid agar [MOPS 2.0 g, $(NH_4)_2SO_4$ 1.0 g, $CaCl_2$ 2.0 g, K_2HPO_4 0.5 g, amino acid mixture 1.0 g, agar 15.0 g, H_2O 1000 mL, pH 7.2]; and (G) CMC agar (Kaewkla and Franco, 2013). All media were supplemented with 3% (w/v) NaCl, nystatin antibiotic (50 mg l^{-1}), and nalidixic acid (50 mg l^{-1}). Cultures were incubated at 28°C for 2 to 8 weeks. Colonies obtained after incubation were purified and then maintained on slants at 4°C and also as 30% (v/v) glycerol suspensions stored at −80°C.

Isolates Identification

Isolates were preliminarily identified via morphologic and culture characteristics that were observed when growing on the International *Streptomyces* Project media ISP 2 and ISP 4 (Shirling and Gottlieb, 1966) that contained 3% NaCl. Diaminopimelic acid isomer analyses were used to distinguish *Streptomycetes* from other spore-forming actinomycetes following the methods described previously (Lechevalier and Lechevalier, 1980). The extraction of genomic DNA and PCR amplification of 16S rRNA genes for each representative isolate were conducted using the methods described previously (Li et al., 2007). The 16S rRNA gene was amplified using universal primer 27f (5′-CAGAGTTTGATCCTGGCT-3′) and 1492r (5′-AGGAGGTGATCCAGCCGCA-3′), and the similarities were determined by comparison of sequences against the EzTaxon-e database (Kim et al., 2012). Neighbor-joining phylogenetic trees of 16S rRNA gene sequences were constructed using the molecular evolutionary genetics analysis (MEGA) software program, version 6.0 (Tamura et al., 2013). A detailed identification of the potentially novel taxa was conducted using the conventionally used polyphasic taxonomic procedures (Stackebrandt et al., 2002).

High Throughput Sequencing Analysis of Endophytic Actinobacteria

After surface sterilization, seven representative plant root samples of *Phragmites australis* (HR1), *Sesbania cannabina* (HR2), *Chrysanthemum indicum* (HR3), *Metaplexis japonica* (HR4), *Suaeda glauca* (HR5), *Lycium* Linn (HR6), and *Spartina alterniflora* (HR7) collected at the same time were subjected to total genomic DNA extraction by Shanghai Majorbio Biotechnology, Co., Ltd. (Shanghai, China). The V5-V7 hypervariable region of bacterial 16S ribosomal RNA genes were then amplified by PCR using an amplification procedure consisting of 95°C for 3 min, followed by 27 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 10 min. PCRs incorporated the bacteria-specific primers 799F (5′-AACMGGATTAGATACCCKG-3′) (Chelius and Triplett, 2001) and 1115R (5′-AGGGTTGCGCTCGTTG-3′) (Reysenbach and Pace, 1995). PCR amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer's instructions, followed by quantification using the QuantiFluor™-ST system (Promega, United States). Purified amplicons were pooled in equimolar concentrations and paired-end sequenced (2 × 250) on the Illumina MiSeq platform at Majorbio Biotech, Co., Ltd. (Shanghai, China), according to standard protocols.

Raw fastq sequence files were demultiplexed and quality-filtered using QIIME version 1.17 (Caporaso et al., 2010). Operational taxonomic units (OTUs) were clustered at the 97% nucleotide similarity cutoff using the UPARSE software package version 7.1 (Edgar, 2013) while chimeric sequences were identified and removed using the UCHIME software package version 4.2.40 (Edgar et al., 2011). The phylogenetic affiliation of each 16S rRNA gene sequence was assessed using the RDP Classifier (Wang et al., 2007) and comparison against the SILVA 16S rRNA gene database while using a confidence threshold of 70% (Quast et al., 2013). Community alpha diversity values were evaluated using the mothur software version 1.30.1 (Schloss et al., 2009). The sequence data have been submitted to the NCBI Sequence Read Archive (SRA) database under the Accession No. PRJNA507498.

Antifungal and Fibrinolytic Activity Assays of Endophytic Actinobacterial Isolates

Actinobacterial strains were screened *in vitro* for the growth inhibition of eight different fungi that were pathogenic to crops and trees: *Rhizoctonia solani*, *Pyricularia grisea*, *Verticillium dahlia* Kleb, *Marssonina brunnea* YH1, *Lasiodiplodia theobromae* YB3, *Sclerotium* sp. YF2, *Fusarium graminearum,* and *Botryosphaeria berengeriana*. All pathogens were maintained at the Key Laboratory of Biotechnology for Medicinal Plants within the Jiangsu province, China. The fermentation capacity of the isolates and their antimicrobial activities were determined using soybean mannitol liquid medium (Qin et al., 2009) containing 3% NaCl and the agar diffusion assay method (Romero et al., 2004). Briefly, each tested strain was cultured in soybean mannitol liquid medium (shaken at 160 rpm, 28°C) for 5 days. Then, the fermentation broth was used for antimicrobial activity screening. Ketoconazole was used as the positive control, and sterile water was used as the negative control. The fibrinolytic activity was determined by using the fibrin plate method according to Astrup and Müllertz (1952). Fibrinolytic activity was evaluated by measuring the diameter of the transparent zone. Urokinase was used as the positive control, and uninoculated medium was used as the negative control.

Screening for Secondary Metabolite Genes

The potential for biosynthesis and production of secondary metabolites was evaluated for the isolates using PCR. The specific primer pairs K1F (5′-TSAAGTCSAACATCGGBCA-3′)/M6R (5′-CG

CAGGTTSCSGTACCAGTA-3′), KSα (5′-TSGCSTGCTTGGAY GCSATC-3′)/KSβ (5′-TGGAANCCG CCGAABCCTCT-3′), A3F (5′-GCSTACSYSATSTACACSTCSGG-3′)/A7R (5′-SASGTCVC CSGTSCGGTAS-3′), and Halo-B4-FW (5′-TTCCCSCGSTACC ASATCGGSGAG-3′)/Halo-B7-RV (5′-GSGGGATSWMCCAG WACCASCC-3′) were used to determine the presence of polyketide synthase I (PKS I), polyketide synthase II (PKS II), non-ribosomal peptide synthetase (NRPS), and $FADH_2$-dependent halogenase (Halo) genes, respectively (Metsä-Ketelä et al., 1999; Ayuso-Sacido and Genilloud, 2005; Hornung et al., 2007). PCRs were conducted following the methods described previously (Gao and Huang, 2009; Qin et al., 2009). Amplification products were determined by 1.0% agarose gel electrophoresis, and bands of size 1,200–1,400, 600, 700–800 and about 550 bp were considered positive PCR amplifications of PKS I, PKS II, NRPS, and Halo genes, respectively.

GenBank Accession Numbers

16S rRNA gene sequences for the isolates obtained in this study were deposited at GenBank under the Accession Nos. JQ819252–JQ819262 and JX982699–JX982766.

RESULTS

Isolation of Endophytic Actinobacteria

A total of 278 isolates were cultured from sampled plant tissues and the most abundant genus of isolates based on preliminary identifications was *Streptomyces* (∼60% of isolates). From the screening based on morphological criteria and cultural characteristics, 79 representative strains were selected for further investigation. The starch-casein and sodium propionate media cultures yielded the most isolates and the highest diversity of non-*Streptomyces* endophytic actinobacteria, with nine different genera cultivated including several unique rare genera. The ISP 5 medium yielded the next highest diversity of endophytic actinobacteria, while the other four media types (A, E, F, G) yielded only three to five genera each (**Table 1**). The majority of isolates were obtained from root samples (42 isolates, 53.2%), followed by leaves (20 isolates, 25.3%) and stems (17 isolates, 21.5%) (**Table 2**).

TABLE 1 | The abundance and diversity of isolates belonging to different actinobacterial genera that were recovered with different media.

Isolation Medium	Total isolates	Different genera obtained using different medium	Number of genera
A	5	*Kocuria*, *Saccharopolyspora*, *Streptomyces*	3
B	23	*Amycolatopsis*, *Brevibacterium*, *Kocuria*, *Micrococcus, Micromonospora*, *Nocardiopsis, Pseudonocardia*, *Saccharopolyspora*, *Streptomyces*, *Tamaricihabitans*	10
C	19	*Glycomyces*, *Gordonia*, *Kocuria*, *Micrococcus*, *Modestobacter*, *Mycobacterium*, *Nocardiopsis*, *Pseudokineococcus*, *Saccharopolyspora*, *Streptomyces*	10
D	12	*Citricoccus*, *Janibacter*, *Kineococcus*, *Micrococcus*, *Prauserella*, *Pseudonocardia*, *Saccharopolyspora*, *Streptomyces*	8
E	6	*Nesterenkonia*, *Pseudonocardia*, *Rhodococcus*, *Saccharopolyspora*, *Streptomyces*	5
F	6	*Glycomyces*, *Janibacter*, *Nocardiopsis*, *Streptomyces*	4
G	8	*Dietzia*, *Glycomyces*, *Kocuria*, *Nocardia*, *Streptomyces*	5

Medium: A, modified TWYE agar supplemented with plant extract (plant extracts were made from three different plants: Tamarix chinensis Lour., Suaeda glauca, and Spartina alterniflora); B, starch-casein agar; C, sodium propionate agar; D, ISP 5 agar; E, trehalose-proline medium; F, MOPS-amino acid agar; G, CMC agar.

Diversity of Isolated Endophytic Actinobacteria

16S rRNA gene sequence analysis of the 79 isolates revealed significant genetic diversity distributed among nine suborders within the *Actinobacteria* class: *Streptomycineae*, *Pseudonocardineae*, *Streptosporangineae*, *Micromonosporineae*, *Micrococcineae*, *Glycomycineae*, *Frankineae*, *Corynebacterineae*, and *Kineosporiineae*. The suborders comprised 14 families and 22 known genera in addition to the novel genus, *Tamaricihabitans* (**Figures 1**, **2** and **Table 2**). Except the *Streptomycineae* (19 isolates, one genus), rare actinobacterial strains of the family *Pseudonocardineae* (25 isolates, 5 genera), *Microococcineae* (15 isolates, six genera), and *Corynebacterineae* (9 isolates, 5 genera) were the most prominent among the isolates. *Streptomyces* was the most frequently isolated genus (24%, 19 isolates), followed by *Saccharopolyspora* (14 isolates) and *Pseudonocardia* (eight isolates). Fifteen genera including those rarely detected as endophytes such as *Dietzia*, *Kineococcus*, *Citriococcus,* and *Janibacter* were isolated less frequently and were each represented by only one or two strains. Genera *Prauserella*, *Modestobacter*, *Pseudokineococcus*, *Glycomyces*, and *Nesterenkonia* have only been rarely reported as culturable endophytes previously, and their presence in these samples underscores the high level of diversity within the plants of this environment. The root tissues yielded the majority of the genera (20), while stem and leaf tissues only yielded seven and nine genera, respectively (**Table 2**). The 16S rRNA gene sequence nucleotide identities ranged from 95.1 to 100% between strains and the available type strains. Interestingly, several strains were most closely related to other validly published endophytic actinobacteria from marine environments, but exhibited distinct phenotypic characteristics when compared with their marine relatives. For example, strain KLBMP 1279 shared 99.5% 16S rRNA gene sequence identity to *Modestobacter marinus* 42H12-1^T, a marine obtained actinobacterium isolated from a deep-sea sediment sample (2,983 m depth) from the Atlantic Ocean (Xiao et al., 2011). However, KLBMP 1279 exhibited culture characteristics differing from *M. marinus* and thus represents a new *Modestobacter* species, named as *Modestobacter roseus* (Qin et al., 2013b). Moreover, several strains exhibited relatively lower

TABLE 2 | Taxonomic distribution of endophytic actinobacterial isolates from different host plant tissues and their associated bioactivities, and the presence of secondary metabolite biosynthetic genes.

Taxonomic group	Sample tissues and number of isolates				No. of strains with antifungal activity	No. of strains with biosynthetic genes	No. of strains with fibrinodlytic activity
	Root	Stem	Leaf	Total			
Streptomycineae:				19			
Streptomyces	5	7	7	19	10	12	12
Pseudonocardineae:				25			
Pseudonocardia	5	1	2	8	7	2	2
Saccharopolyspora	6	4	4	14	4	4	8
Amycolatopsis		1		1		1	1
Prauserella	1			1	1		
Tamaricihabitans	1			1		1	1
Micromonosporineae:				1			
Micromonospora	1			1			1
Microococcineae:				15			
Brevibacterium			1	1			
Janibacter	2			2	1		
Kocuria	4		1	5		1	1
Nesterenkonia	1			1		1	
Micrococcus	1	2	2	5		2	
Citriococcus			1	1		1	
Streptosporangineae:				3			
Nocardiopsis	2	1		3			3
Corynebacterineae:				9			
Gordonia	3			3		2	
Dietzia	1			1			
Mycobacterium	1			1		1	
Nocardia	2			2			
Rhodococcus	2			2	1	2	
Frankineae:				2			
Modestobacter	2			2		1	
Kineosporiineae:				2			
Kineococcus			1	1		1	
Pseudokineococcus	1			1		1	
Glycomycineae:				3			
Glycomyces	1	1	1	3		1	3
Total	42	17	20	79	24	34	32

16S rRNA gene identities (<98.7%) to characterized isolates and represented novel species (**Table 3**). For example, two *Streptomyces* isolates, KLBMP 1440 and KLBMP 1330, shared only 98.5 and 98% 16S rRNA gene nucleotide similarities to their nearest characterized relatives, respectively. These values are lower than the suggested cutoff (98.7%) for species delineation, as suggested by Chun et al. (2018). Further, the 16S rRNA genes of these isolates also comprised distinct clades in the phylogenetic reconstructions (**Supplementary Figures S1**, **S2**). Consequently, the two strains were considered novel species of the genus *Streptomyces*. 16S rRNA gene sequence analysis led to the selection of 13 strains (16.5%) for detailed identification via standard polyphasic taxonomic identification methods, and eight of these strains (KLBMP 1356, KLBMP 1279, KLBMP 1305, KLBMP 1262, KLBMP 1269, KLBMP 1274, KLBMP 1282, and KLBMP 1284) were validated published as novel genus/species

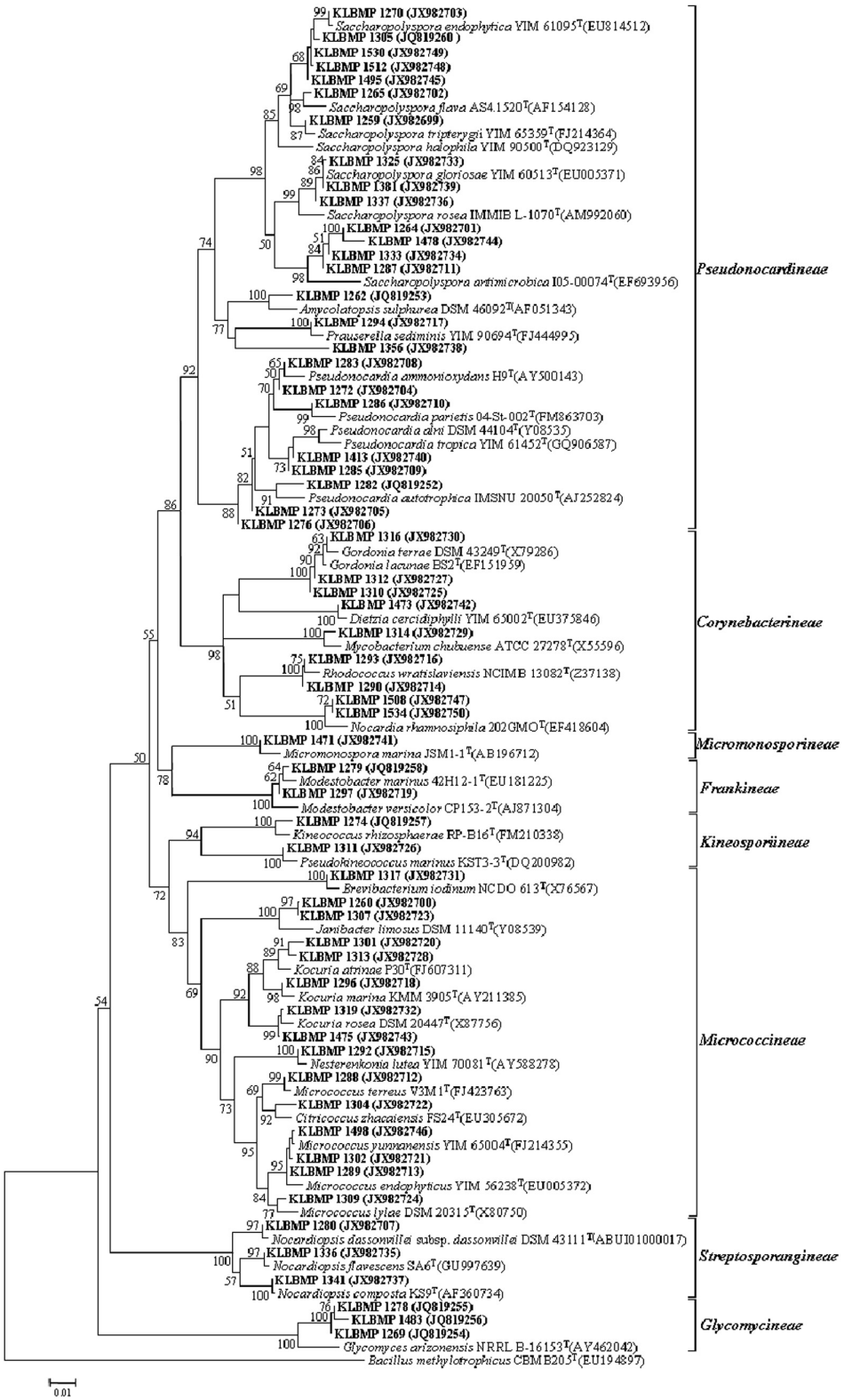

FIGURE 1 | Neighbor-joining phylogenetic tree of 16S rRNA gene sequences obtained from non-*Streptomyces* isolates in this study and their phylogenetic neighbors. Only bootstrap values above 50% are shown at nodes and are expressed as percentages of 1,000 replicates. Bar represents 0.01 substitutions per nucleotide position. GenBank accession numbers of reference 16S rRNA gene sequences are shown next to isolate names. *Bacillus methylotrophicus* CBMB205^T (EU194897) was used as the outgroup.

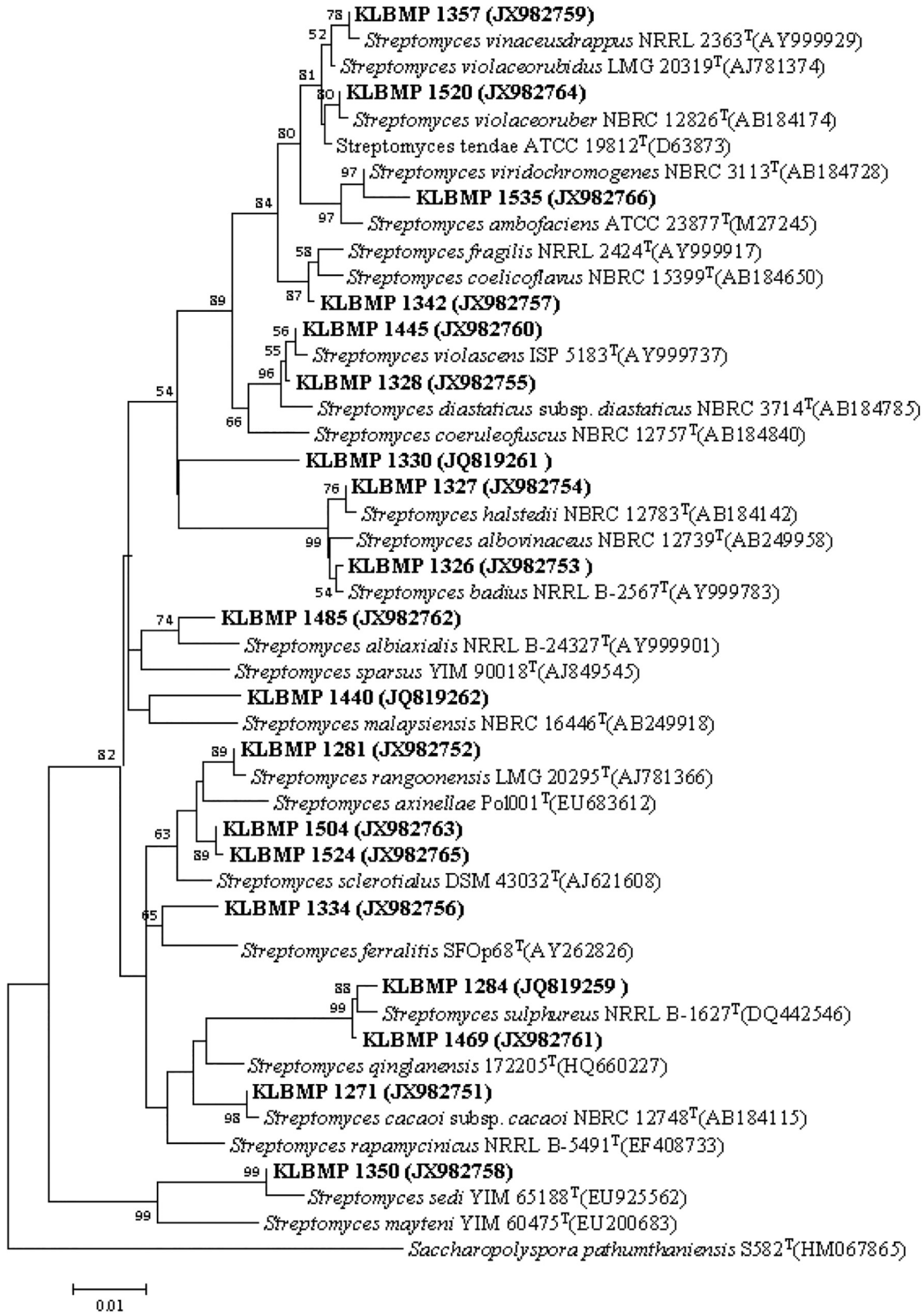

FIGURE 2 | Neighbor-joining phylogenetic tree of 16S rRNA gene sequences obtained from *Streptomyces* isolates in this study and their phylogenetic neighbors. Only bootstrap values above 50% are shown at nodes and are expressed as percentages of 1,000 replicates. Bar represents 0.01 substitutions per nucleotide position. GenBank accession numbers of reference 16S rRNA gene sequences are shown next to isolate names. *Saccharopolyspora pathumthaniensis* S582^T (HM067865) was used as the outgroup.

(**Table 3**). Overall, these results demonstrate the presence of abundant and diverse endophytic actinobacteria within plants that are native to coastal salt marshes.

HTS of Endophytic Actinobacterial Diversity

High-throughput sequencing on the Illumina MiSeq sequencing platform was conducted to explore the endophytic actinobacterial community within roots (where diversity was highest) of seven typical coastal plants. A total of 228,091 16S rRNA gene sequences were considered high quality, with 22,450–49,221 sequences obtained per sample. At the 97% nucleotide identity threshold for OTU identification, the number of OTUs ranged from 131 to 566 within samples (**Table 4**). An analysis of the coverage indicated that greater than 99% of the total diversity within the communities was sampled, while rarefaction analyses indicated that measured diversity was nearly saturated in most samples (**Figure 3**). Alpha diversity indices including the Chao, ACE, Shannon, and Simpson metrics were calculated and are provided in **Table 4**. These results indicated an abundant diversity of endophytic bacteria in the seven root samples.

Taxonomic assignment of OTUs revealed the presence of 12 phyla, wherein the Proteobacteria were the most abundant in all samples, followed by the Actinobacteria, Firmicutes, and Bacteroidetes. Actinobacterial relative abundances ranged between 0.04 and 28.66% in each sample. The phylum was in lowest abundance in *Phragmites australis* (HR1) roots (0.04%), and highest in *Metaplexis japonica* (HR4) and *Spartina alterniflora* (HR7) roots, comprising 28.66 and 23.63% of the communities, respectively (**Figure 4A**). At the class level, most of the endophytic bacteria belonged to the *Gammaproteobacteria*, *Alphaproteobacteria*, *Actinobacteria*, *Bacilli*, *Betaproteobacteria*, and *Acidimicrobiia* clades (**Figure 4B**). Four classes of Actinobacteria were detected, including the *Actinobacteria*, *Acidimicrobiia*, *Nitriliruptoria*, and *Thermoleophilia*, of which *Actinobacteria* and *Acidimicrobiia* were the most abundant. In addition, 14 known actinobacterial orders were detected, with *Acidimicrobiales*, *Streptomycetales*, and *Micrococcales* comprising the most abundant orders. A total of 35 known actinobacterial families were also detected, with variation of families among samples (**Figure 5A**). At the genus level, 63 actinobacterial genera were observed, with *Streptomyces*, *Nocardioides*, *Mycobacterium*, *Micromonospora*, *Ilumatobacter*, *Herbiconiux*, *Arthrobacter*, and *Actinoplanes* representing the most abundant taxa within root tissues, but with varying abundances among samples. For example, *Streptomyces* was most abundant in the HR3, HR4, HR6, and HR7 samples while *Arthrobacter* was most abundant in the HR5 sample. The genera *Arthrobacter*, *Mycobacterium*, and *Micrococcus* were observed in all samples. Most root tissues harbored numerous actinobacterial genera (>30), and the HR7 sample contained the highest diversity (47 genera), while the HR1 and HR2 samples only harbored 4 and 21 genera, respectively. In addition to characterized taxa, a high proportion of populations classified as *Acidimicrobiales*, *Kineosporiaceae*, and *Micromonosporaceae* could not be assigned to a known genus (**Figure 5B**), indicating the presence of numerous novel

TABLE 3 | 16S rRNA gene sequence nucleotide similarities between novel endophytic isolates and the most closely related type strain of a validly described species.

Strains	Host plant	Tissues type	The closest phylogenetic type strains	16S rRNA gene sequence similarity (%)	References
KLBMP 1356 (Novel genus)	*Tamarix chinensis* Lour.	Root	*Prauserella marina* MS498^T	95.1	Qin et al., 2015a
KLBMP 1279 (Novel species)	*Salicornia europaea* Linn.	Root	*Modestobacter marinus* 42H12-1^T	99.5	Qin et al., 2013b
KLBMP 1305 (Novel species)	*Dendranthema indicum* (Linn.) Des Moul	Stem	*Saccharopolyspora pathumthaniensis* S582^T	99.3	Zhang et al., 2013
KLBMP 1262 (Novel species)	*Dendranthema indicum* (Linn.) Des Moul	Stem	*Amycolatopsis sulphurea* DSM 46092^T	97.9	Xing et al., 2013
KLBMP 1274 (Novel species)	*Limonium sinense* (Girard) Kuntze	Leave	*Kineococcus rhizosphaerae* RP-B16^T	98.7	Bian et al., 2012
KLBMP 1282 (Novel species)	*Tamarix chinensis* Lour.	Leave	*Pseudonocardia kongjuensis* LM 157^T	98.3	Xing et al., 2012
KLBMP 1284 (Novel species)	*Tamarix chinensis* Lour.	Stem	*Streptomyces sulphureus* NRRL B-1627^T	99.4	Qin et al., 2013a
KLBMP 1469 (Novel species)	*Tamarix chinensis* Lour.	Leave	*Streptomyces sulphureus* NRRL B-1627^T	99.5	
KLBMP 1330 (Novel species)	*Aster tataricus* L. F.	Root	*Streptomyces malachitospinus* NBRC 101004^T	98.0	
KLBMP 1440 (Novel species)	*Tamarix chinensis* Lour.	Root	*Streptomyces yogyakartensis* NBRC 100779^T	98.5	
KLBMP 1269 (Novel species)	*Limonium sinense* (Girard) Kuntze	Leave	*Glycomyces arizonensis* NRRL B-16153^T	97.0	Xing et al., 2014
KLBMP 1483 (Novel species)	*Dendranthema indicum* (Linn.) Des Moul.	Stem	*Glycomyces arizonensis* NRRL B-16153^T	96.7	
KLBMP 1278 (Novel species)	*Sesbania cannabina* (L.) Pers.	Root	*Glycomyces arizonensis* NRRL B-16153^T	97.0	

TABLE 4 | Alpha diversity estimates for bacterial communities within the seven plant root samples.

Sample ID	16S rRNA gene reads	OTUs (97%)	Coverage	Community richness		Community diversity	
				Chao*	Ace	Simpson	Shannon
HR1	49,221	131	0.9993	183.000	184.5747	0.275	1.8406
HR2	35,125	350	0.9969	522.575	566.0036	0.312	2.0495
HR3	38,071	543	0.9982	587.652	601.6451	0.155	3.3998
HR4	26,919	506	0.9971	604.154	585.4462	0.024	4.5645
HR5	29,885	499	0.9985	555.447	563.5344	0.052	4.3357
HR6	26,420	566	0.9960	726.660	784.6813	0.032	4.5812
HR7	22,450	530	0.9966	617.000	593.0172	0.118	3.7663

**Chao and ACE indices reflect species richness, Shannon and Simpson indices reflect species diversity.*

taxa. HTS cultivation-independent analyses clearly led to the detection of more diverse endophytic Actinobacteria than did the cultivation-based methods. Of the 63 genera detected by cultivation-independent sequencing, 11 genera were also detected by cultivation, and *Streptomyces* were abundant among the communities identified using both methods (**Figure 6**).

Antifungal and Fibrinolytic Activities of Isolates

Twenty-four of the 79 strains (30.4%) exhibiting antifungal activity against at least one of the pathogenic fungi that were tested. Twelve strains (15.2%) inhibited half of the pathogens that were tested, while eight strains exhibited even wider inhibition spectra. Two *Streptomyces* strains (KLBMP 1445 and KLBMP 1357), one *Pseudonocardia* strain (KLBMP 1272), and one *Saccharopolyspora* strain (KLBMP 1287) showed antifungal activity against a total of six to eight pathogens. The antifungal activity against *Marssonina brunnea* YH1 was by far the most prevalent (18 isolates, 22.8%) (**Supplementary Table S1**). Overall, six different genera exhibited antifungal activities, including the *Streptomyces* (10 isolates), *Pseudonocardia* (seven isolates), *Saccharopolyspora* (four isolates), *Prauserella* (one isolate), *Janibacter* (one isolate), and *Rhodococcus* (one isolate). We found that 40.5% (32 strains) of the representative strains showed fibrinolytic activities, which were distributed into nine genera, including *Glycomyces*, *Saccharopolyspora*, *Streptomyces*, *Amycolatopsis*, *Pseudonocardia*, *Tamaricihabitans*, *Nocardiopsis*, *Micromonospora*, and *Kocuria* (**Table 2**). However, 31.9% of the active strains only displayed weak fibrinolytic activity. Two *Glycomyces* strains (KLBMP 1269 and KLBMP 1483), two *Saccharopolyspora* strains (KLBMP 1270 and KLBMP 1337), and two *Streptomyces* strains (KLBMP 1361 and KLBMP 1469) showed highest fibrinolytic activity (**Supplementary Table S2**).

Detection of Natural Product Biosynthesis Gene

A total of 34 of the 79 strains (43%) exhibited the presence of one or more types of natural product biosynthetic genes (PKS I, PKS II, NRPS, and Halo). NRPS genes were most frequently detected (16 strains; 20.3%), followed by Halo (12 strains; 15.2%). PKS I and PKS II genes were only detected in five and four of the 79 strains, respectively. The strains exhibiting the presence of biosynthetic genes were distributed among diverse genera (**Table 2**). The presence of PKS I genes was only observed in strains from the *Saccharopolyspora* and *Streptomyces* genera. In contrast, strains harboring NRPS and Halo genes were extensively distributed among nine and eight different genera, respectively. Both NRPS and Halo genes were only observed in five strains within the genus *Streptomyces*. Two strains, *Saccharopolyspora* sp. KLBMP 1333 and *Streptomyces* sp. KLBMP 1357, both exhibited the presence of two genes (PKS I and NRPS for KLBMP 1333, PKS II, and NRPS for KLBMP 1357; **Supplementary Table S1**). The positive amplification products of biosynthetic genes of some representative strains were sequenced, and the results confirmed that the amplification of these genes indeed encode the corresponding biosynthetic enzymes (data not shown).

DISCUSSION

Diverse endophytic actinomycetes have been found in many crops, model plants (e.g., *Arabidopsis thaliana*), and other plants from numerous typical environments (Conn and Franco, 2003; Qin et al., 2009; Golinska et al., 2015; Dinesh et al., 2017). The method used to isolate endophytic actinobacteria is an important factor that affects the acquisition and diversity of pure cultures. For example, plant surface sterilization, use of different selective media types, and plant treatment methods can all influence cultivation-dependent outcomes. In this study, we used a previously described five-step sterile sample processing protocol, longer incubation times, and various selective media to obtain pure endophytic actinobacterial cultures (Qin et al., 2009). Starch-casein agar and sodium propionate agar that were supplemented with 3% (w/v) NaCl yielded the greatest diversity of endophytic actinobacterial isolates, which comprised 10 different genera, including those that have rarely been reported as endophytes such as *Glycomyces*, *Modestobacter*, and *Pseudokineococcus* (**Table 1**). Starch-casein agar has previously been suggested to be effective in the isolation of Actinobacteria from saline environments (Abbas, 2006). Similarly, sodium propionate medium is seen to be effective for isolating endophytic actinobacteria from various plant hosts (Qin et al., 2009; Li et al., 2012). Our recent investigation also found that these two

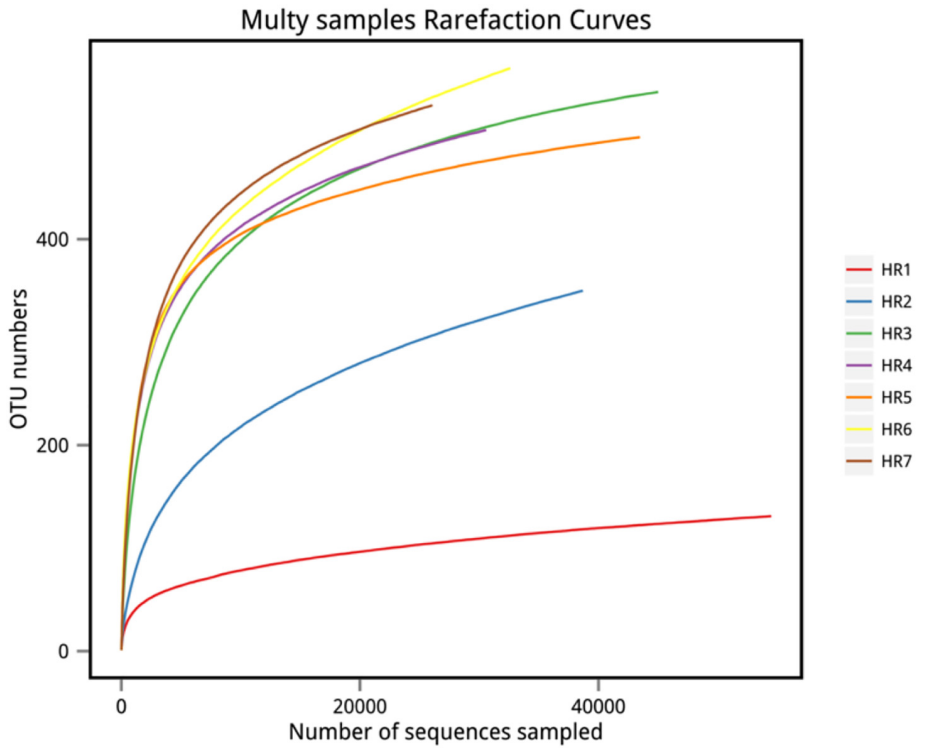

FIGURE 3 | Rarefaction curves of OTU richness among samples. OTU richness was determined via sequencing of the V5–V7 hypervariable regions of 16S rRNA genes.

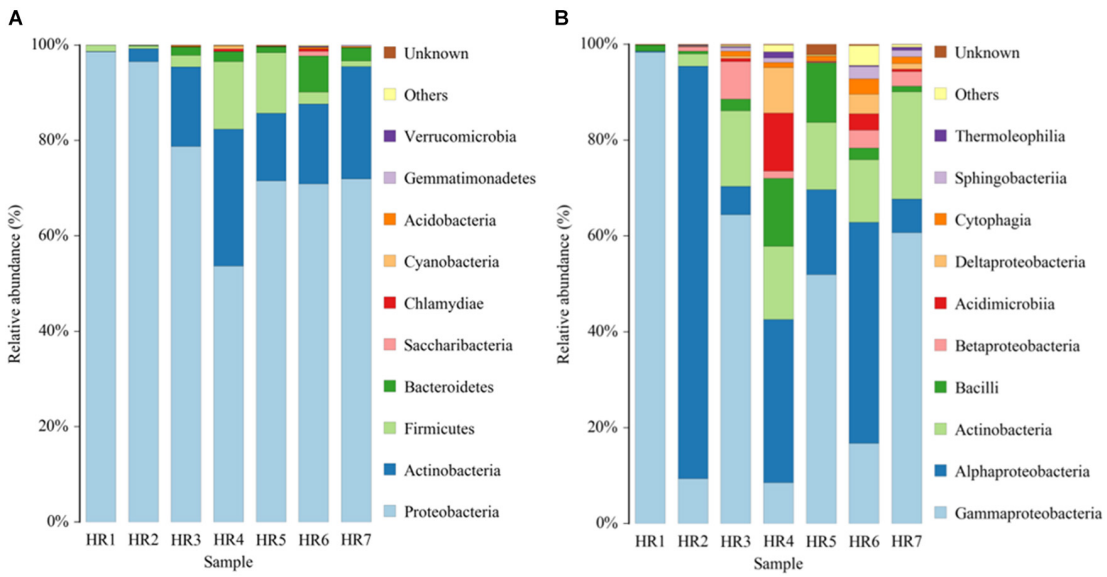

FIGURE 4 | Relative abundances of bacterial communities at the phylum **(A)** and class **(B)** levels in different plant root samples, as determined by culture-independent analyses.

types of media supplemented with 3% NaCl were suitable for isolating rare actinomycetes from rhizosphere soils associated with coastal halophytes (Gong et al., 2018). CMC medium has also been suggested as suitable for isolating rare endophytic actinobacteria (Kaewkla and Franco, 2013). Although only eight strains were obtained in this study using the CMC medium, they were distributed among five genera, which included *Dietzia*, *Glycomyces*, and *Kocuria*, and are all rarely reported as

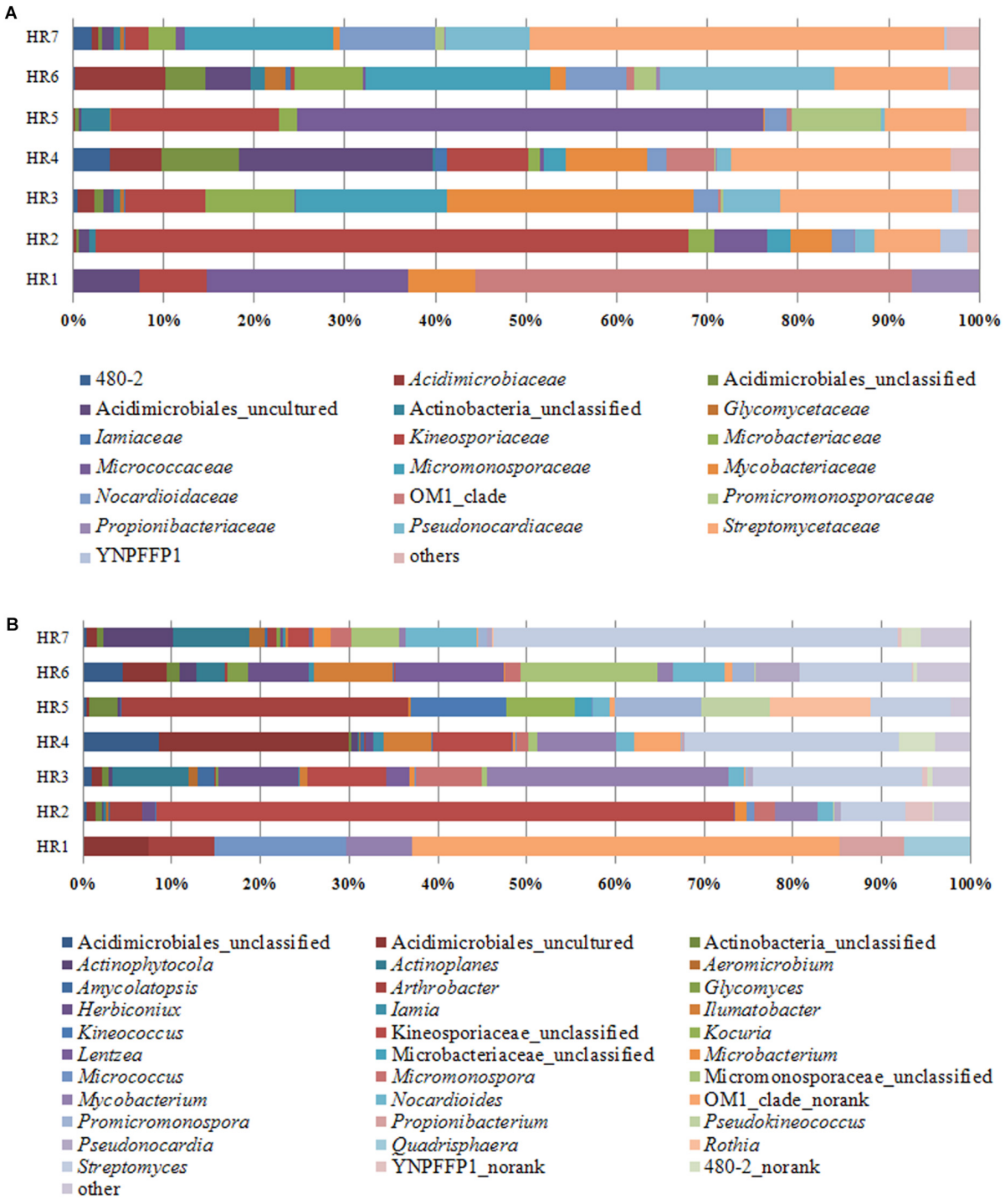

FIGURE 5 | Relative abundances of bacteria at the actinobacterial family **(A)** and genus **(B)** levels in different plant root samples, as determined by culture-independent analyses.

endophytes. These results suggest that further investigations of endophytic actinobacteria should strongly consider the in-depth exploration of the cultivation methods that are to be employed.

Marine environments host a diversity of rare actinobacteria (Pennings and Bertness, 2001; Hassan et al., 2017). Because coastal salt marshes are characterized by both terrestrial and marine characteristics, abundant and diverse endophytic actinobacterial resources are likely present in these environments. A total of 23 different actinobacterial genera were identified in the present study, including many novel species, suggesting

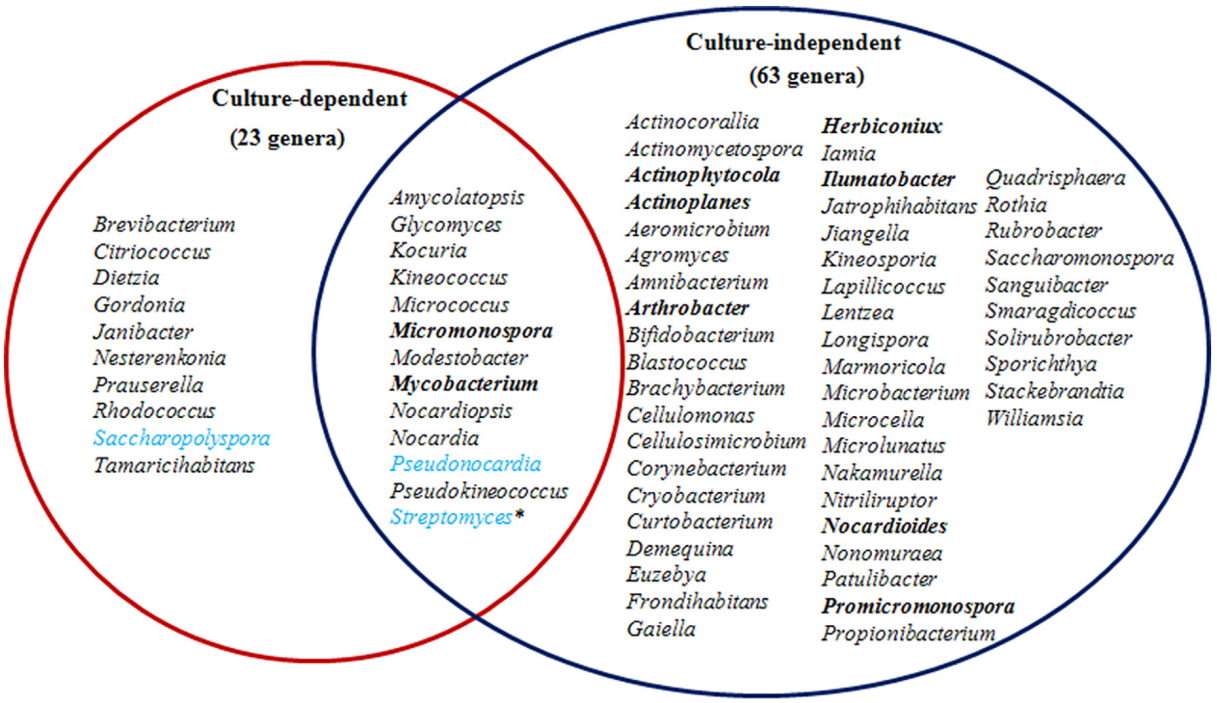

FIGURE 6 | A comparison of endophytic actinobacterial genera detected in roots using culture-dependent and -independent methods. The most abundant genera detected by culture-independent analysis are indicated in bold and for the culture-dependent analysis are indicated in light blue. Asterisks indicate that the genus was abundant using both methodologies.

a high diversity of actinobacteria within the native plants of the salt marsh investigated here. To our knowledge, such high diversity of culturable endophytic actinobacteria is yet to be demonstrated from such habitats. Tian and Zhang (2017) analyzed the endophytic and rhizosphere bacterial diversity of the Chinese coastal halophyte *Messerschmidia sibirica* using high-throughput sequencing method, and they revealed that the relative abundance of the phylum *Actinobacteria* accounts for more than 6.2% across all leaf, stem, and root tissues. Yamamoto et al. (2018) also found that *Actinobacteria* in the coastal halophyte *Glaux maritima* root tissues collected from the eastern part of Japan was extremely high using the Illumina MiSeq sequencing technique. However, none of these studies reported pure culture isolation of endophytic actinobacteria and their diversity. Recently, Jiang et al. (2018) obtained diverse endophytic actinobacterial strains (101 isolates from 15 families and 28 genera) from five mangrove plants collected from the Guangxi Zhuang Autonomous Region of China. Taken together with the above results, our study confirms that coastal plants contain abundant and diverse endophytic actinomycete resources. Interestingly, in this study, most of the culturable endophytic actinobacterial genera, such as *Streptomyces*, *Saccharopolyspora*, *Pseudonocardia* and *Micromonospora*, were also successfully cultured from their corresponding host plants rhizosphere soils previously (Gong et al., 2018). This result supports the previous conclusion that rhizosphere microorganisms are the main origin of endophytic bacteria (Mora-Ruiz Mdel et al., 2016).

A total of 72 known actinobacterial genera were detected from seven root samples using a combination of cultivation-dependent and -independent techniques (**Figure 6**). To our knowledge, this is the first investigation of coastal plant-associated endophytic actinobacterial diversity using both cultivation- and culture-independent methods. The majority of the isolates were obtained from plant roots (**Table 2**), which is consistent with the results of other studies, indicating that endophytic actinomycetes primarily colonized root tissues (Qin et al., 2009; Kaewkla and Franco, 2013; Tian and Zhang, 2017). The community composition of actinobacterial taxa at the phylum, family, and genus levels varied among different root samples (**Figures 4**, **5**). Recent investigations of plant microbiomes have suggested that plant genotypes significantly affect the composition of symbiotic root microbiota (Wintermans et al., 2016; Li et al., 2018). Therefore, the root-associated actinobacterial compositional differences observed here could be explained by differences in host plant species. At the genus level of actinomycetes, differences were observed in the number of genera detected by cultivation-dependent and -independent methods (**Figure 6**). Specifically, high-throughput 16S rRNA gene sequencing resulted in the detection of 63 known genera, including many genera that have rarely been reported as endophytes such as *Herbiconiux*, *Actinoplanes*, *Ilumatobacter*, and *Actinophytocola* (Pérez et al., 2016). Furthermore, HTS within root samples resulted in the detection of much higher levels of taxa, which could not be classified into known taxonomic groups. These results indicated that coastal salt marsh plants harbored a high level

of rare endophytic actinobacteria and novel taxa. Only 11 of the 63 genera detected by 16S rRNA gene sequencing were also detected by cultivation, while nine cultivated genera were not detected by sequence analysis (**Figure 6**). However, some genera that are widely distributed among different environments and that display diverse ecological functions associated with plants, including *Arthrobacter* (Prum et al., 2018; Qin et al., 2018), were not detected by cultivation. This result highlights the impetus to continue developing cultivation methods and strategies to culture greater diversity in future investigations. These disparities in the detected taxa could potentially be explained by the variable efficiency of genomic DNA extraction among species, taxonomic biases of primers used for PCR amplification, and the recalcitrance of certain taxa to cultivation under laboratory conditions. These explanations are consistent with the previous reports (Qin et al., 2012; Chen et al., 2016; Eevers et al., 2016). Therefore, it is critical to use a combined cultivation-dependent and -independent approach to accurately evaluate the composition of endophytic actinobacterial communities.

Streptomyces are the most prevalent genus observed among endophytic actinobacteria (Conn and Franco, 2003; Kaewkla and Franco, 2013; Qin et al., 2015b; Dinesh et al., 2017; Passari et al., 2017). In addition, many rare endophytic actinomycetes have also been recently reported (Qin et al., 2011; Dinesh et al., 2017). Our observation that *Streptomyces* were the most dominant species within coastal salt marsh plants is consistent with these previous studies. Endophytic *Streptomyces* spp. are well-known for their capacity to produce many types of bioactive metabolites, and several species have been reported as beneficial toward plant development and health (Mitra et al., 2008; Vurukonda et al., 2018). The highest proportions of secondary metabolite biosynthetic genes observed in this study were in *Streptomyces* spp. strains (63.2%), with at least 12 strains harboring PKS, NRPS, and/or Halo genes (**Table 2**). This indicates that the strains screened possess diverse secondary metabolite gene clusters, as evidenced by the presence of these genes. PCR-based screening of biosynthetic genes is a useful and efficient method to conduct bioprospecting of certain type of natural products producing actinobacteria. For example, based on the $FADH_2$-dependent halogenase genes screening from the mangrove-derived actinomycetes, Li et al. (2013) obtained a new enduracidin producer, *Streptomyces atrovirens* MGR140, which was further identified and confirmed by gene disruption and HPLC analysis. The presence of halo genes here is particular interesting, because more than 4,700 halogenated compounds have been reported, which are important sources for new drugs, due to their high diversity in structure and activity (Liao et al., 2016). Moreover, *Streptomyces* strains exhibited significant antagonistic activities toward fungal pathogens, with 52.6% strains displaying antifungal activities against at least one of the pathogentic fungi that were tested. One isolate, KLBMP 1357, exhibited broad-spectrum antifungal activity and contained both the PKS II and NRPS genes (**Supplementary Table S1**), meriting further research of its bioactive secondary metabolites. Nevertheless, antifungal activities and the presence of certain secondary metabolite functional genes did not appear to be directly correlated in this study. For example, functional secondary metabolite genes were not detected in the *Pseudonocardia* strain KLBMP 1272, despite the fact that it exhibited broad-spectrum antifungal activity. This result could potentially be due to one of two reasons. First, the fermentation medium used to cultivate the strain may not promote the production of antifungal secondary metabolites. Second, the degenerate primer pairs that were used to amplify the functional genes may not be suitable for amplification of these genes within certain strains. Alternatively, the strains considered here may actually lack the biosynthetic genes tested, but possess other secondary metabolite biosynthetic genes (Schneemann et al., 2010). Moreover, we have only carried out antifungal screening, and the results of gene screening may be more relevant to antibacterial and anti-tumor activity. The results reported here are consistent with those from a recent screening of culturable actinomycetes from hot springs, lichens, and tea plants (Liu et al., 2016, 2017, Wei et al., 2018). Considering that many actinomycetes were isolated from root tissues, and showed strong antagonistic activity against plant pathogenic fungi, we may speculate that these endophytic actinomycetes have potential for biological control and potential contribution to their ecological adaptation within roots.

We also observed that some strains of *Pseudonocardia* and *Saccharopolyspora* that are rarely described as endophytes exhibited good antifungal activity, including strains KLBMP 1272, KLBMP 1413, and KLBMP 1287. *Pseudonocardia* have been previously observed as dominant endophytes within plants; in addition, some new endophytic species have been discovered recently (Qin et al., 2009; Li et al., 2012; Zhao et al., 2012). In contrast, endophytic *Saccharopolyspora* spp. isolates have been rarely reported (Qin et al., 2008; Li et al., 2009). Recently, endophytic *Pseudonocardia* isolates were identified that were associated with mangrove trees and sea cucumbers. The isolates displayed antimicrobial and cytotoxic activities, and subsequently yielded novel bioactive compounds (Mangamuri et al., 2015; Ye et al., 2016). Microorganism is an important source of thrombolytic agents for thrombosis treatment, and lots of fibrinolytic enzymes were successively discovered from different microorganisms, including marine actinomycetes (Verma et al., 2018). However, non-*Streptomyces* rare actinobacteria and endophytes origin fibrinolytic enzymes were rarely reported (Lu et al., 2010; Mohanasrinivasan et al., 2017). Thus, we carried out the fibrinolytic activity screening for the endophytic actinobacterial isolates. Interestingly, we found that more than 40% of the strains secreted extracellular proteases that exhibited fibrinolytic activity in this study. It is noteworthy that some new species (KLBMP 1269, KLBMP 1483, KLBMP 1305, and KLBMP 1278) displayed strong and moderate fibrinolytic activity (**Supplementary Table S2**), meriting further purification and characterization of their fibrinolytic enzymes and exploring the thrombolytic effect. In addition, to our knowledge, this is the first time that strains of endophytic actinobacteria and the rare actinobacteria genera *Glycomyces*, *Saccharopolyspora*, *Amycolatopsis*, *Pseudonocardia*, and *Tamaricihabitans* have been reported to have fibrinolytic activity. Taken together, these results

indicate the coastal marsh halophytes-associated endophytic actinobacterial yet-to-be discovered bioactive metabolites and functions warrant further exploration.

CONCLUSION

Endophytic actinobacterial community structure was analyzed among coastal salt marsh halophyte plants within the Jiangsu Province of Eastern China using both culture-dependent and -independent methods. Coastal salt marsh plants harbored abundant and diverse communities of endophytic actinobacteria, including many rare actinobacteria and novel taxa. These results also indicate that halophyte plants have an important role in shaping the community composition of endophytic microbiota within roots. Taken together, the results of this study suggest that coastal salt marsh plants-associated endophytic actinobacteria represent a promising, yet underexplored, new resource for discovering bioactive natural products with potential as biocontrol agents and for fibrinolytic enzyme production.

AUTHOR CONTRIBUTIONS

CZ, KX, and SQ designed the experiments. PC, XJ, YX, and SQ performed the experiments. PC, CZ, YX, and SQ analyzed the data and prepared 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.2019.01063/full#supplementary-material

REFERENCES

Abbas, I. H. (2006). A biological and biochemical studies of actinomycetes isolated from Kuwait saline soil-Kuwait. *J. Appl. Sci. Res.* 2, 809–815.

Álvarez-Pérez, J. M., González-García, S., Cobos, R., Olego, M. Á, Ibañez, A., Díez-Galán, A., et al. (2017). Use of endophytic and rhizosphere actinobacteria from grapevine plants to reduce nursery fungal graft infections that lead to young grapevine decline. *Appl. Environ. Microbiol.* 83:e1564-17. doi: 10.1128/AEM.01564-17

Astrup, T., and Müllertz, S. (1952). The fibrin plate method for estimating fibrinolytic activity. *Arch. Biochem. Biophys.* 40, 346–351.

Ayuso-Sacido, A., and Genilloud, O. (2005). New PCR primers for the screening of NRPS and PKS-I systems in actinomycetes: detection and distribution of these biosynthetic gene sequences in major taxonomic groups. *Microb. Ecol.* 49, 10–24.

Bai, J. L., Wang, Y., Qin, S., Ding, P., Xing, K., Yuan, B., et al. (2016). *Nocardia jiangsuensis* sp. nov., an actinomycete isolated from coastal soil. Int. J. Syst. Evol. Microbiol. 66, 4633–4638. doi: 10.1099/ijsem.0.001402

Bèrdy, J. (2005). Bioactive microbial metabolites. *J. Antibiot.* 58, 1–26.

Bian, G. K., Feng, Z. Z., Qin, S., Xing, K., Wang, Z., Cao, C. L., et al. (2012). *Kineococcus endophytica* sp. nov., a novel endophytic actinomycete isolated from a coastal halophyte in Jiangsu, China. *Antonie van Leeuwenhoek.* 102, 621–628. doi: 10.1007/s10482-012-9757-4

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336.

Chelius, M. K., and Triplett, E. W. (2001). The diversity of archaea and bacteria in association with the roots of *Zea mays* L. *Microb. Ecol.* 41, 252–263.

Chen, P., Zhang, L., Guo, X., Dai, X., Liu, L., Xi, L., et al. (2016). Diversity, biogeography, and biodegradation potential of actinobacteria in the deep-sea sediments along the southwest Indian ridge. *Front. Microbiol.* 7:1340. doi: 10.3389/fmicb.2016.01340

Chun, J., Oren, A., Ventosa, A., Christensen, H., Arahal, D. R., da Costa, M. S., et al. (2018). Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int. J. Syst. Evol. Microbiol.* 68, 461–466. doi: 10.1099/ijsem.0.002516

Conn, V. M., and Franco, C. M. (2003). Isolation and identification of actinobacteria from surface-sterilized wheat roots. *Appl. Environ. Microbiol.* 69, 5603–5608.

Conn, V. M., Walker, A. R., and Franco, C. M. (2008). Endophytic actinobacteria induce defense pathways in *Arabidopsis thaliana*. *Mol. Plant Microbe. Interact.* 21, 208–218. doi: 10.1094/MPMI-21-2-0208

Dinesh, R., Srinivasan, V., Sheeja, T. E., Anandaraj, M., and Srambikkal, H. (2017). Endophytic actinobacteria: diversity, secondary metabolism and mechanisms to unsilence biosynthetic gene clusters. *Crit. Rev. Microbiol.* 43, 546–566. doi: 10.1080/1040841X.2016.1270895

Ding, P., Bai, J. L., Wang, T. T., Sun, Y., Cao, C. L., Jiang, J. H., et al. (2018). *Nocardia rhizosphaerihabitans* sp. nov., a novel actinomycete isolated from a coastal soil. Int. J. Syst. Evol. Microbiol. 68, 192–197. doi: 10.1099/ijsem.0.002481

Du, X., Zhai, Y., Deng, Q., Tan, H., and Cao, L. (2018). Illumina-based sequencing analysis directed selection for actinobacterial probiotic candidates for banana plants. *Probiotics. Antimicrob. Proteins* 10, 284–292. doi: 10.1007/s12602-017-9293-7

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity andspeed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Eevers, N., Beckers, B., Op de Beeck, M., White, J. C., Vangronsveld, J., and Weyens, N. (2016). Comparison between cultivated and total bacterial communities associated with *Cucurbita pepo* using cultivation-dependent techniques and 454 pyrosequencing. *Syst. Appl. Microbiol.* 39, 58–66. doi: 10.1016/j.syapm.2015.11.001

Gao, P., and Huang, Y. (2009). Detection, distribution, and organohalogen compound discovery implications of the reduced flavin adenine dinucleotide-dependent halogenase gene in major filamentous actinomycete taxonomic groups. *Appl. Environ. Microbiol.* 75, 4813–4820. doi: 10.1128/AEM.02958-08

Golinska, P., Wypij, M., Agarkar, G., Rathod, D., Dahm, H., and Rai, M. (2015). Endophytic actinobacteria of medicinal plants: diversity and bioactivity. *Antonie van Leeuwenhoek* 108, 267–289.

Gong, Y., Bai, J. L., Yang, H. T., Zhang, W. D., Xiong, Y. W., Ding, P., et al. (2018). Phylogenetic diversity and investigation of plant growth-promoting traits of actinobacteria in coastal salt marsh plant rhizospheres from Jiangsu. China. *Syst. Appl. Microbiol.* 41, 516–527. doi: 10.1016/j.syapm.2018.06.003

Hamedi, J., Mohammadipanah, F., and Ventosa, A. (2013). Systematic and biotechnological aspects of halophilic and halotolerant actinomycetes. *Extremophiles* 17, 1–13. doi: 10.1007/s00792-012-0493-5

Hassan, S. S., Anjum, K., Abbas, S. Q., Akhter, N., Shagufta, B. I., Shah, S. A., et al. (2017). Emerging biopharmaceuticals from marine actinobacteria. *Environ. Toxicol. Pharmacol.* 49, 34–47. doi: 10.1016/j.etap.2016.11.015

Hong, K., Gao, A. H., Xie, Q. Y., Gao, H., Zhuang, L., Lin, H. P., et al. (2009). Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China. *Mar. Drugs* 7, 24–44. doi: 10.3390/md7010024

Hornung, A., Bertazzo, M., Dziarnowski, A., Schneider, K., Welzel, K., Wohlert, S. E., et al. (2007). A genomic screening approach to the structure-guided identification of drug candidates from natural sources. *Chembiochem* 8, 757–766.

Jiang, Z. K., Tuo, L., Huang, D. L., Osterman, I. A., Tyurin, A. P., Liu, S. W., et al. (2018). Diversity, novelty, and antimicrobial activity of endophytic actinobacteria from mangrove plants in Beilun Estuary national nature reserve of Guangxi. China. *Front. Microbiol.* 9:868. doi: 10.3389/fmicb.2018.00868

Kaewkla, O., and Franco, C. M. (2013). Rational approaches to improving the isolation of endophytic actinobacteria from Australian native trees. *Microb. Ecol.* 65, 384–393. doi: 10.1007/s00248-012-0113-z

Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., et al. (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.* 62, 716–721. doi: 10.1099/ijs.0.038075-0

Lechevalier, M. P., and Lechevalier, H. A. (1980). "The chemotaxonomy of actinomycetes," in *Actinomycete Taxonomy*, eds A. Dietz and D. W. Thayer (Arlington, VA: Society for Industrial Microbiology), 22–291.

Li, J., Zhao, G. Z., Huang, H. Y., Qin, S., Zhu, W. Y., Zhao, L. X., et al. (2012). Isolation and characterization of culturable endophytic actinobacteria associated with *Artemisia annua* L. *Antonie van Leeuwenhoek* 101, 515–527. doi: 10.1007/s10482-011-9661-3

Li, J., Zhao, G. Z., Qin, S., Huang, H. Y., Zhu, W. Y., Xu, L. H., et al. (2009). *Saccharopolyspora tripterygii* sp. nov., an endophytic actinomycete isolated from the stem of *Tripterygium hypoglaucum*. Int. J. Syst. Evol. Microbiol. 59, 3040–3044. doi: 10.1099/ijs.0.011734-0

Li, W. J., Xu, P., Schumann, P., Zhang, Y. Q., Pukall, R., Xu, L. H., et al. (2007). *Georgenia ruanii* sp. nov., a novel actinobacterium isolated from forest soil in Yunnan (China) and emended description of the genus Georgenia. *Int. J. Syst. Evol. Microbiol.* 57, 1424–1428.

Li, X. G., Tang, X. M., Xiao, J., Ma, G. H., Xu, L., Xie, S. J., et al. (2013). Harnessing the potential of halogenated natural product biosynthesis by mangrove-derived actinomycetes. *Mar. Drugs* 11, 3875–3890. doi: 10.3390/md11103875

Li, Y., Wu, X., Chen, T., Wang, W., Liu, G., Zhang, W., et al. (2018). Plant phenotypic traits eventually shape its microbiota: a common garden test. *Front. Microbiol.* 9:2479. doi: 10.3389/fmicb.2018.02479

Liao, L., Chen, R., Jiang, M., Tian, X., Liu, H., Yu, Y., et al. (2016). Bioprospecting potential of halogenases from Arctic marine actinomycetes. *BMC Microbiol.* 16:34. doi: 10.1186/s12866-016-0662-2

Liu, C., Jiang, Y., Wang, X., Chen, D., Chen, X., Wang, L., et al. (2017). Diversity, antimicrobial activity, and biosynthetic potential of cultivable actinomycetes associated with lichen symbiosis. *Microb. Ecol.* 74, 570–584. doi: 10.1007/s00248-017-0972-4

Liu, L., Salam, N., Jiao, J. Y., Jiang, H. C., Zhou, E. M., Yin, Y. R., et al. (2016). Diversity of culturable thermophilic actinobacteria in hot springs in Tengchong, China and studies of their biosynthetic gene profiles. *Microb. Ecol.* 72, 150–162. doi: 10.1007/s00248-016-0756-2

Long, H. H., Sonntag, D. G., Schmidt, D. D., and Baldwin, I. T. (2010). The structure of the culturable root bacterial endophyte community of *Nicotiana attenuata* is organized by soil composition and host plant ethylene production and perception. *New Phytol.* 185, 554–567. doi: 10.1111/j.1469-8137.2009.03079.x

Lu, F., Lu, Z., Bie, X., Yao, Z., Wang, Y., Lu, Y., et al. (2010). Purification and characterization of a novel anticoagulant and fibrinolytic enzyme produced by endophytic bacterium *Paenibacillus polymyxa* EJS-3. *Thromb Res.* 126, e349–e355. doi: 10.1016/j.thromres.2010.08.003

Mangamuri, U. K., Vijayalakshmi, M., Poda, S., Manavathi, B., Bhujangarao, C. H., and Venkateswarlu, Y. (2015). Bioactive metabolites produced by *Pseudonocardia* endophytica VUK-10 from mangrove sediments: isolation, chemical structure determination and bioactivity. *J. Microbiol. Biotechnol.* 25, 629–636.

Metsä-Ketelä, M., Salo, V., Halo, L., Hautala, A., Hakala, J., Mäntsälä, P., et al. (1999). An efficient approach for screening minimal PKS genes from *Streptomyces*. *FEMS Microbiol. Lett.* 180, 1–6.

Miao, G. P., Zhu, C. S., Feng, J. T., Han, L. R., and Zhang, X. (2015). Effects of plant stress signal molecules on the production of wilforgine in an endophytic actinomycete isolated from *Tripterygium wilfordii* Hook.f. *Curr. Microbiol.* 70, 571–579. doi: 10.1007/s00284-014-0758-6

Mitra, A., Santra, S. C., and Mukherjee, J. (2008). Distribution of actinomycetes, their antagonistic behaviour and the physico-chemical characteristics of the world's largest tidal mangrove forest. *Appl. Microbiol. Biotechnol.* 80, 685–695.

Mohanasrinivasan, V., Yogesh, S., Govindaraj, A., Naine, S. J., and Devi, C. S. (2017). In vitro thrombolytic potential of actinoprotease from marine *Streptomyces violaceus* VITYGM. *Cardiovasc. Hematol. Agents Med. Chem.* 14, 120–124. doi: 10.2174/1871525715666161104112553

Mora-Ruiz Mdel, R., Font-Verdera, F., Orfila, A., Rita, J., and Rosselló-Móra, R. (2016). Endophytic microbial diversity of the halophyte *Arthrocnemum macrostachyum* across plant compartments. *FEMS Microbiol. Ecol.* 92:fiw145. doi: 10.1093/femsec/fiw145

Passari, A. K., Mishra, V. K., Singh, G., Singh, P., Kumar, B., Gupta, V. K., et al. (2017). Insights into the functionality of endophytic actinobacteria with a focus on their biosynthetic potential and secondary metabolites production. *Sci. Rep.* 7:11809. doi: 10.1038/s41598-017-12235-4

Pennings, S. C., and Bertness, M. D. (2001). "Salt marsh communities," in *Marine Community Ecology*, eds M. D. Bertness, S. D. Gaines, and M. E. Hay (Sunderland, MA: Sinauer Associates, Inc.).

Pérez, M. L., Collavino, M. M., Sansberro, P. A., Mroginski, L. A., and Galdeano, E. (2016). Diversity of endophytic fungal and bacterial communities in *Ilex paraguariensis* grown under field conditions. *World J. Microbiol. Biotechnol.* 32:61. doi: 10.1007/s11274-016-2016-5

Prum, C., Dolphen, R., and Thiravetyan, P. (2018). Enhancing arsenic removal from arsenic-contaminated water by *Echinodorus cordifolius*-endophytic *Arthrobacter creatinolyticus* interactions. *J. Environ. Manage.* 213, 11–19. doi: 10.1016/j.jenvman.2018.02.060

Purushotham, N., Jones, E., Monk, J., and Ridgway, H. (2018). Community structure of endophytic actinobacteria in a New Zealand native medicinal plant *Pseudowintera colorata* (Horopito) and their influence on plant growth. *Microb. Ecol.* 76, 729–740. doi: 10.1007/s00248-018-1153-9

Qin, S., Bai, J. L., Wang, Y., Feng, W. W., Yuan, B., Sun, Y., et al. (2015a). *Tamaricihabitans halophyticus* gen. nov., sp. nov., an endophytic actinomycete of the family *Pseudonocardiaceae*. *Int. J. Syst. Evol. Microbiol.* 65, 4662–4668. doi: 10.1099/ijsem.0.000628

Qin, S., Miao, Q., Feng, W. W., Wang, Y., Zhu, X., Xing, K., et al. (2015b). Biodiversity and plant growth promoting traits of culturable endophytic actinobacteria associated with *Jatropha curcas* L. growing in Panxi dry-hot valley soil. *Appl. Soil. Ecol.* 93, 47–55.

Qin, S., Bian, G. K., Tamura, T., Zhang, Y. J., Zhang, W. D., Cao, C. L., et al. (2013a). *Streptomyces halophytocola* sp. nov., an endophytic actinomycete isolated from the surface-sterilized stems of a coastal halophyte *Tamarix chinensis* Lour. *Int. J. Syst. Evol. Microbiol.* 63, 2770–2775. doi: 10.1099/ijs.0.047456-0

Qin, S., Bian, G. K., Zhang, Y. J., Xing, K., Cao, C. L., Liu, C. H., et al. (2013b). *Modestobacter roseus* sp. nov., an endophytic actinomycete isolated from the coastal halophyte *Salicornia europaea* Linn., and emended description of the genus *Modestobacter*. *Int. J. Syst. Evol. Microbiol.* 63, 2197–2202. doi: 10.1099/ijs.0.044412-0

Qin, S., Chen, H. H., Zhao, G. Z., Li, J., Zhu, W. Y., Xu, L. H., et al. (2012). Abundant and diverse endophytic actinobacteria associated with medicinal plant *Maytenus austroyunnanensis* in Xishuangbanna tropical rainforest revealed by culture-dependent and culture-independent methods. *Environ. Microbiol. Rep.* 4, 522–531. doi: 10.1111/j.1758-2229.2012.00357.x

Qin, S., Feng, W. W., Wang, T. T., Ding, P., Xing, K., and Jiang, J. H. (2017). Plant growth-promoting effect and genomic analysis of the beneficial endophyte *Streptomyces* sp. KLBMP 5084 isolated from halophyte *Limonium sinense*. *Plant Soil* 416, 117–132.

Qin, S., Feng, W. W., Zhang, Y. J., Wang, T. T., Xiong, Y. W., and Xing, K. (2018). Diversity of bacterial microbiota of coastal halophyte *Limonium sinense* and amelioration of salinity stress damage by symbiotic plant growth-promoting actinobacterium *Glutamicibacter halophytocola* KLBMP 5180. *Appl. Environ. Microbiol.* 84:e1533-18. doi: 10.1128/AEM.01533-18

Qin, S., Li, J., Chen, H. H., Zhao, G. Z., Zhu, W. Y., Jiang, C. L., et al. (2009). Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna. China. *Appl. Environ. Microbiol.* 75, 6176–6186. doi: 10.1128/AEM.01034-09

Qin, S., Li, J., Zhao, G. Z., Chen, H. H., Xu, L. H., and Li, W. J. (2008). *Saccharopolyspora endophytica* sp. nov., an endophytic actinomycete isolated from the root of *Maytenus austroyunnanensis*. *Syst. Appl. Microbiol.* 31, 352–357. doi: 10.1016/j.syapm.2008.08.001

Qin, S., Li, W. J., Dastager, S. G., and Hozzein, W. N. (2016). Editorial: actinobacteria in special and extreme habitats: diversity, function roles, and environmental adaptations. *Front. Microbiol.* 7:1415. doi: 10.3389/fmicb.2016.01415

Qin, S., Xing, K., Jiang, J. H., Xu, L. H., and Li, W. J. (2011). Biodiversity, bioactive natural products and biotechnological potential of plant-associated endophytic actinobacteria. *Appl. Microbiol. Biotechnol.* 89, 457–473. doi: 10.1007/s00253-010-2923-6

Qin, S., Zhang, Y. J., Yuan, B., Xu, P. Y., Xing, K., Wang, J., et al. (2014). Isolation of ACC deaminase-producing habitat-adapted symbiotic bacteria associated with halophyte *Limonium sinense* (Girard) Kuntze and evaluating their plant growth-promoting activity under salt stress. *Plant Soil* 374, 753–766.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Reysenbach, A., and Pace, N. (1995). "Reliable amplification of hyperthermophilic archaeal 16S rRNA genes by the polymerase chain reaction," in *Archaea: A Laboratory Manual*, ed. F. Robb (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), 101–107.

Romero, D., Pérez-García, A., Rivera, M. E., Cazorla, F. M., and de Vicente, A. (2004). Isolation and evaluation of antagonist bacteria towards the cucurbit powdery mildew fungus *Podosphaera fusca*. *Appl. Microbiol. Biotechnol.* 64, 263–269.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Schneemann, I., Nagel, K., Kajahn, I., Labes, A., Wiese, J., and Imhoff, J. F. (2010). Comprehensive investigation of marine actinobacteria associated with the sponge *Halichondria panicea*. *Appl. Environ. Microbiol.* 76, 3702–3714. doi: 10.1128/AEM.00780-10

Shirling, E. B., and Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* 16, 313–340.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A., Kämpfer, P., Maiden, M. C., et al. (2002). Report of the ad hoc committee for the reevaluation of the species definition in bacteriology. *Int. J. Syst. Evol. Microbiol.* 52, 1043–1047.

Suga, T., Kimura, T., Inahashi, Y., Iwatsuki, M., Nonaka, K., Také, A., et al. (2018). Hamuramicins A and B, 22-membered macrolides, produced by an endophytic actinomycete *Allostreptomyces* sp. K12-0794. *J. Antibiot.* 71, 619–625. doi: 10.1038/s41429-018-0055-x

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

Tian, X., Cao, L., Tan, H., Han, W., Chen, M., Liu, Y., et al. (2007). Diversity of cultivated and uncultivated actinobacterial endophytes in the stems and roots of rice. *Microb. Ecol.* 53, 700–707.

Tian, X. Y., and Zhang, C. S. (2017). Illumina-based analysis of endophytic and rhizosphere bacterial diversity of the coastal halophyte *Messerschmidia sibirica*. *Front. Microbiol.* 8:2288. doi: 10.3389/fmicb.2017.02288

Trujillo, M. E., Riesco, R., Benito, P., and Carro, L. (2015). Endophytic actinobacteria and the interaction of *Micromonospora* and nitrogen fixing Plants. *Front. Microbiol.* 6:1341. doi: 10.3389/fmicb.2015.01341

Verma, P., Chatterjee, S., Keziah, M. S., and Devi, S. C. (2018). Fibrinolytic protease from marine *Streptomyces rubiginosus* VITPSS1. *Cardiovasc. Hematol. Agents Med. Chem.* 16, 44–55. doi: 10.2174/1871525716666180226141551

Vurukonda, S. S. K. P., Giovanardi, D., and Stefani, E. (2018). Plant growth promoting and biocontrol activity of *Streptomyces* spp. as endophytes. *Int. J. Mol. Sci.* 19:E952. doi: 10.3390/ijms19040952

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267.

Wang, Y., Liu, W., Feng, W. W., Zhou, X. Q., Bai, J. L., Yuan, B., et al. (2015). *Nocardia rhizosphaerae* sp. nov., a novel actinomycete isolated from the coastal rhizosphere of *Artemisia* Linn., China. *Antonie van Leeuwenhoek* 108, 31–39. doi: 10.1007/s10482-015-0460-0

Wei, W., Zhou, Y., Chen, F., Yan, X., Lai, Y., Wei, C., et al. (2018). Isolation, diversity, and antimicrobial and immunomodulatory activities of endophytic actinobacteria from tea cultivars Zijuan and Yunkang-10 (*Camellia sinensis* var. *assamica*). *Front. Microbiol.* 9:1304. doi: 10.3389/fmicb.2018.01304

Wintermans, P. C., Bakker, P. A., and Pieterse, C. M. (2016). Natural genetic variation in *Arabidopsis* for responsiveness to plant growth-promoting rhizobacteria. *Plant Mol. Biol.* 90, 623–634. doi: 10.1007/s11103-016-0442-2

Xiao, J., Luo, Y., Xu, J., Xie, S., and Xu, J. (2011). *Modestobacter marinus* sp. nov., a psychrotolerant actinobacterium from deep-sea sediment, and emended description of the genus Modestobacter. Int. J. Syst. Evol. Microbiol. 61, 1710–1714. doi: 10.1099/ijs.0.023085-0

Xing, K., Liu, W., Zhang, Y. J., Bian, G. K., Zhang, W. D., Tamura, T., et al. (2013). *Amycolatopsis jiangsuensis* sp. nov., a novel endophytic actinomycete isolated from a coastal plant in Jiangsu. China. *Antonie van Leeuwenhoek* 103, 433–439. doi: 10.1007/s10482-012-9823-y

Xing, K., Qin, S., Bian, G. K., Zhang, Y. J., Zhang, W. D., Dai, C. C., et al. (2012). *Pseudonocardia nantongensis* sp. nov., a novel endophytic actinomycete isolated from the coastal halophyte *Tamarix chinensis* Lour. Antonie van Leeuwenhoek 102, 659–667. doi: 10.1007/s10482-012-9764-5

Xing, K., Qin, S., Zhang, W. D., Cao, C. L., Ruan, J. S., Huang, Y., et al. (2014). *Glycomyces phytohabitans* sp. nov., a novel endophytic actinomycete isolated from the coastal halophyte in Jiangsu. East China. *J. Antibiot.* 67, 559–563. doi: 10.1038/ja.2014.40

Yamamoto, K., Shiwa, Y., Ishige, T., Sakamoto, H., Tanaka, K., Uchino, M., et al. (2018). Bacterial diversity associated with the rhizosphere and endosphere of two halophytes: *Glaux maritima* and *Salicornia europaea*. *Front. Microbiol.* 9:2878. doi: 10.3389/fmicb.2018.02878

Ye, X., Anjum, K., Song, T., Wang, W., Yu, S., Huang, H., et al. (2016). A new curvularin glycoside and its cytotoxic and antibacterial analogues from marine actinomycete *Pseudonocardia* sp. HS7. *Nat. Prod. Res.* 30, 1156–1161. doi: 10.1080/14786419.2015.1047775

Zeng, J., Xu, T., Cao, L., Tong, C., Zhang, X., Luo, D., et al. (2018). The role of iron competition in the antagonistic action of the rice endophyte *Streptomyces sporocinereus* OsiSh-2 against the pathogen *Magnaporthe oryzae*. *Microb. Ecol.* 76, 1021–1029. doi: 10.1007/s00248-018-1189-x

Zhang, Y. J., Zhang, W. D., Qin, S., Bian, G. K., Xing, K., Li, Y. F., et al. (2013). *Saccharopolyspora dendranthemae* sp. nov. a halotolerant endophytic actinomycete isolated from a coastal salt marsh plant in Jiangsu. China. *Antonie van Leeuwenhoek* 103, 1369–1376. doi: 10.1007/s10482-013-9917-1

Zhao, G. Z., Li, J., Zhu, W. Y., Wei, D. Q., Zhang, J. L., Xu, L. H., et al. (2012). *Pseudonocardia xishanensis* sp. nov., an endophytic actinomycete isolated from the roots of Artemisia annua L. Int. J. Syst. Evol. Microbiol. 62, 2395–2399. doi: 10.1099/ijs.0.037028-0

Zhao, K., Li, J., Zhang, X., Chen, Q., Liu, M., Ao, X., et al. (2018). Actinobacteria associated with *Glycyrrhiza inflata* Bat. are diverse and have plant growth promoting and antimicrobial activity. *Sci. Rep.* 8:13661. doi: 10.1038/s41598-018-32097-8

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