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Original article

Diversity, novelty, antimicrobial activity, and new antibiotics of cultivable endophytic actinobacteria isolated from psammophytes collected from Taklamakan Desert

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ABSTRACT

Three hundred and twenty endophytic actinobacterial strains were isolated from psammophytes collected from Taklamakan Desert and identified. Among them, three strains already had been identified as new species of two genera and sixteen isolates showed relatively low 16S rRNA similarities < 98.6% to validly described species. Seventy-five of the isolates were selected as representative strains to screen antibacterial activity and mechanism. Forty-seven strains showed antagonistic activity against at least one of the indicator bacteria. Two *Streptomyces* strains produced bioactive compounds inducing DNA damage, and two *Streptomyces* strains produced bioactive compounds with inhibitory activity on protein biosynthesis. Notably, the strain *Streptomyces* sp. 8P21H-1 that demonstrated both strong antibacterial activity and inhibitory activity on protein biosynthesis was prioritized for exploring new antibiotics. Under the strategy of integrating genetics-based discovery program and MS/MS-based molecular networking, two new streptogramin-type antibiotics, i.e., acetyl-griseoviridin and desulphurizing griseoviridin, along with known griseoviridin, were isolated from the culture broth of strain 8P21H-1. Their chemical structures were determined by HR-MS, and 1D and 2D NMR. Desulphurizing griseoviridin and griseoviridin exhibited antibacterial activities by inhibiting translation.

1. Introduction

Actinobacteria residing in special environments have a unique capacity to produce bioactive novel compounds and become irreplaceable sources to inhibit antibiotic-resistant pathogens [1,2]. Facing the dilemma of rediscovery of known antibiotics, novel strategies and innovative techniques have been developed to speed up the discovery of new and efficient antibiotics [3,4], such as integrating new and classical biological screening model [5], the "bottom-up" genetics-based bioinformatics tools on behalf of genome mining [6,7], as well as "top-down" approach of molecular networking and other cheminformatics techniques. In this study, the diversity, novelty, antimicrobial activity and potential for producing new secondary metabolites from cultivable actinobacteria from plants of Taklamakan Desert were investigated. Culture-dependent method utilizing a variety of media was employed to select actinomyces. Apart from screening antibacterial activities against "ESKAPE" strains (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), high-throughput screening using

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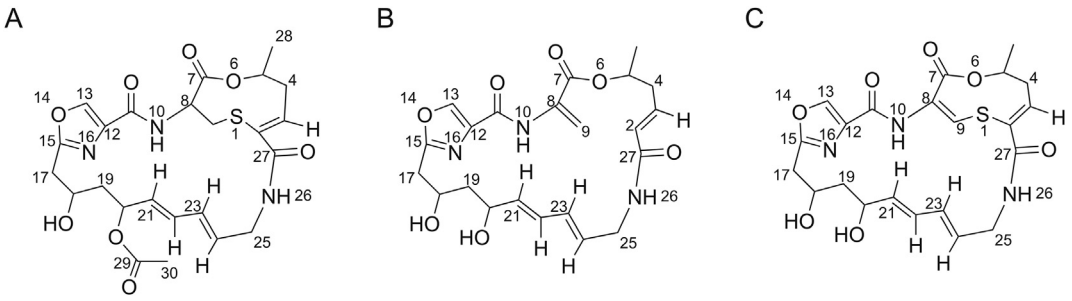

Fig. 1. Chemical structure of the isolated compounds. (A) Acetyl-griseoviridin, (B) desulphurzing griseoviridin, and (C) griseoviridin.

a double fluorescence reported system was also implemented to recognize strains producing inhibitors of ribosome or/and DNA biosynthesis [8,9]. Dereplication strategies which integrated antiSMASH platform analysis and MS/MS-based molecular networking were applied to find new antibiotics from *Streptomyces* sp. 8P21H-1. Finally, two new streptogramin-type antibiotics, i.e., acetyl-griseoviridin and desulphurizing griseoviridin, along with known griseoviridin, were structurally elucidated by HR-MS, and 1D and 2D NMR (Fig. 1).

2. Materials and methods

2.1. Collection of plant samples

A total of fifteen plant samples were collected in Taklamakan Desert, located in Xinjiang, Uygur Autonomous Region, China. The sampling information in detail is listed in Table S1. All the samples were packed in sterilized polyethylene bags and carried back to the laboratory at the earliest possible time.

2.2. Surface sterilization and processing

All samples were immediately processed for surface sterilization after being air-dried. The samples were subjected to a six-step surface sterilization procedure [10]. In order to check the surface sterilization results, the 200 μL of last rinse solution was spread on ISP2 agar plates and incubated at 28 °C for 1 week to check the microbial growth. After being dried in the laminar flow hood for 2 days, the successfully surface-sterilized samples were ground into powders by using a micromill.

Table 1
The ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data for acetyl-griseoviridin and desulphurzing griseoviridin.

Position	Acetyl-griseoviridin				Desulphurzing griseoviridin			
	Methanol-d_4		DMSO-d_6		Methanol-d_4		DMSO-d_6	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
2		132.4		130.3	5.95, d (15.7)	125.4	5.89–5.81, m[a]	126.1
3	7.40–7.33, m	146.4	7.39–7.33, m	144.9	6.73, dt (15.2, 7.4)	139.7	6.61–6.54, m	138.5
4	3.09–3.02, m; 2.41, dd (12.5, 7.3)	39.0	2.87–2.83, m; 2.42, dd (11.9, 7.5)	37.5	2.56, dd (14.9, 6.7); 2.47, dt (15.1, 9.2)	39.1	2.53, dd (15.0, 6.2); 2.43–2.33, m	38.6
5	5.29–5.22, m	72.6	5.15–5.08, m	70.9	5.42–5.34, m	72.1	5.29–5.22, m	72.5
7		172.4		170.9		163.0		162.8
8	4.71, dd (10.9, 7.0)	52.0	4.60–4.51, m	50.2		131.2		131.1
9	3.38, dd (14.4, 7.0); 2.76, dd (14.4, 11.0)	39.6	3.45, dd (14.4, 7.0); 2.70, dd (14.3, 11.0)	38.6	6.61, s; 5.97, s	108.2	6.54, s; 5.89–5.81, m[a]	108.0
10-NH			7.39–7.33, m				9.05, s	
11		161.6		158.8		159.6		158.6
12		136.1		134.4		135.7		135.2
13	8.29, s	142.6	8.59, s	141.5	8.33, s	141.6	8.65, s	142.4
15		164.2		162.5		163.5		163.9
17	2.98, dd (16.7, 4.4); 2.91, dd (16.7, 8.9)	36.5	2.95–2.90, m; 2.90–2.87, m	35.3	2.98–2.87, m	36.0	2.87, dd (14.8, 5.0); 2.80, dd (14.9, 8.3)	36.4
18	4.17–4.10, m	66.7	4.01–3.90, m[a]	64.5	3.94, d (3.9)	67.0	3.74–3.62, m[a]	67.0
18-OH			5.17, d (5.8)				4.86, d (6.3)	
19	1.92–1.87, m 1.82–1.76, m	42.2	1.70–1.65, m	41.0	1.91–1.81, m	43.6	1.72–1.61, m	44.7
20	5.49–5.43, m	74.6	5.40–5.34, m	72.5	4.23, td (9.2, 4.5)	70.2	4.06–3.98, m	69.0
20-OH							4.79, d (4.2)	
21	5.61, dd (15.1, 8.9)	131.5	5.47, dd (15.1, 8.8)	129.9	5.68, dd (9.7, 4.7)	134.9	5.56, q (14.5, 7.9)	136.8
22	6.44, dd (15.1, 10.5)	135.9	6.42–6.35, m	134.2	6.24–6.15, m[a]	130.9	6.11–6.01, m[a]	129.3
23	6.19, dd (15.3, 10.6)	130.1	5.96–5.89, m	127.6	6.24–6.15, m[a]	130.6	6.11–6.01, m[a]	131.0
24	5.90–5.83, m	131.6	5.89–5.83, m	131.8	5.71, d (6.3)	128.8	5.64–5.58, m	129.6
25	4.03, dd (17.4, 4.3); 3.95, dd (17.4, 2.8)	41.7	4.01–3.90, m[a] 3.82–3.75, m	40.3	3.91, dt (16.4, 5.4); 3.72, dd (16.1, 6.5)	39.6	3.74–3.62, m[a]	40.1
26-NH			8.32, t (5.9)				8.14, t (6.0)	
27		166.0		162.2		167.0		164.6
28	1.46, d (6.4)	20.7	1.39, d (6.4)	20.3	1.40, d (6.3).	19.9.	1.33, d (6.4)	20.9
29		171.9		169.4				
30	1.99, s	21.2	1.96, s	21.1				

[a] Signals partially overlapped and may be interchanged.

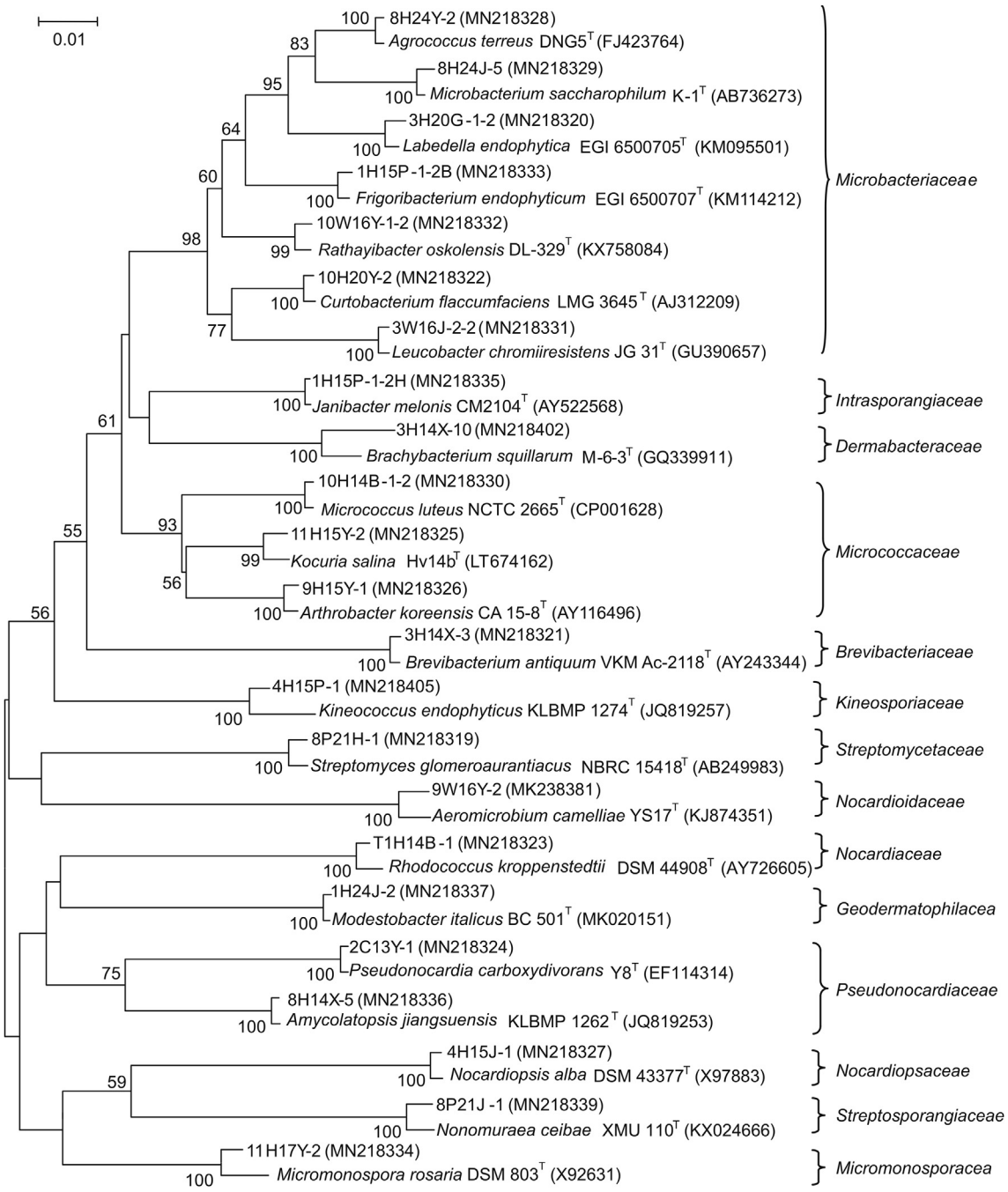

Fig. 2. Phylogenetic tree based on the 16S rRNA gene sequences using neighbor-joining method for 23 representative actinobacterial strains and their closely related type strains. Numbers at nodes indicate the level of bootstrap support > 50% based on 1000 replications. Bar, 2 nt substitutions per 100 nt.

2.3. Endophytic actinobacteria isolation and maintenance

For the selective isolation of actinobacteria, 12 isolation media were prepared (Table S2) and supplemented with nalidixic acid (20 mg/L), cycloheximide (50 mg/L) and potassium dichromate (50 mg/L) to prevent the growth of Gram-stain negative bacteria and fungi. After the plant powders were dispersed in the isolation media, the plates were incubated at 28 °C for 2 months. Single colony was picked up and streaked on the freshly prepared YIM38 medium [11] to obtain the pure isolates. The pure cultures were preserved in glycerol suspensions (20%, *V/V*) at −80 °C.

2.4. Identification of endophytic actinobacteria

Extractions of genomic DNA and PCR amplification of 16S rRNA gene were performed as described by Jiang et al. [12]. The PCR products were purified and sequenced on the ABI PRISM™ 3730XL DNA Analyzer (Thermo Fisher Scientific, USA). The obtained sequences were compared with available 16S rRNA gene sequences in the EzBioCloud (https://www.ezbiocloud.net/) to determine an approximate phylogenetic affiliation of each strain [13]. Phylogenetic tree was constructed by the algorithms of neighbor-joining in MEGA 7.0 [14–16]. Evolutionary distance was estimated by the

Table 2
Information on genera distribution of actinobacterial strains in this study.

Genus	No. of isolates	No. of strains for assay	No. of strains with antibacterial activity
Streptomyces	119	27	20
Curtobacterium	53	4	2
Brachybacterium	22	3	2
Leucobacter	19	2	1
Microbacterium	19	7	3
Labedella	13	3	3
Nocardiopsis	12	5	5
Amycolatopsis	8	1	1
Agrococcus	7	2	1
Kineococcus	7	2	0
Kocuria	6	4	1
Aeromicrobium	6	2	1
Arthrobacter	5	1	1
Rathayibacter	4	1	0
Modestobacter	3	2	0
Janibacter	3	1	1
Frigoribacterium	2	1	0
Brevibacterium	2	1	0
Pseudonocardia	2	1	1
Micromonospora	3	2	2
Rhodococcus	1	1	1
Micrococcus	1	1	1
Nonomuraea	1	1	0
Total number	320	75	47

Kimura's two-parameter model [17]. The bootstrap analysis was performed with 1000 replications [18].

2.5. Antimicrobial screening

The agar well diffusion method was used to screen the antimicrobial activity of isolated actinobacterial strains. The indicator bacteria used for antibacterial assay consisted of 12 bacteria, which were divided into two groups based on consisted of drug-sensitive or drug-resistant of "ESKAPE" bacteria. The detailed information is listed below: *Enterococcus* (ATCC 33186, 310682), *Staphylococcus aureus* (ATCC 29213, ATCC 33591), *Klebsiella pneumoniae* (ATCC 10031, ATCC 700603), *Acinetobacter baumannii* (2799, ATCC 19606), *Pseudomonas aeruginosa* (ATCC 27853, 2774) and *Escherichia coli* (ATCC 25922, ATCC 35218). For each type of bacteria, the strain listed in the former was drug-sensitive strain and the latter was drug-resistant strain. All bacteria were obtained from American Type Culture Collection (ATCC), expect for *Enterococcus* 310682 (resistant to vancomycin) and *Pseudomonas aeruginosa* 2774 (resistant to aminoglycosides and carbapenems) from the clinic. Small-scale fermentation of selective actinobacterial strains and antibacterial assays were performed as described earlier [11].

2.6. High-throughput screening using a double fluorescent protein reporter system

Ribosome and DNA biosynthesis inhibitors were screened by the double fluorescent protein reporter system with reporter strain JW5503-pDualrep2 (also abbreviated as the pDualrep2 reported system) [8,9,19]. The reporter strain JW5503-pDualrep2 was constructed by the strain JW5503 (Δ*tolC*) of *E. coli* transformed by a report plasmid, pDualrep2, to screen compounds for antimicrobial activity with simultaneous classification by their mechanisms of action. Briefly, 0.1 mL of ethyl acetate extracts was dried in laminar flow hood and 100 μL of DMSO was added to each sample. 2 μL of each sample was applied to agar plate containing a lawn of the reporter strain. After overnight incubation at 37 °C, the plate was scanned by ChemiDoc (Bio-Rad) system with two channels consisting of "Cy3-blot" (553/574 nm, green pseudocolor) for RFP triggered by DNA biosynthesis inhibitors and "Cy5-blot" (588/633 nm, red pseudocolor) for Katushka2S triggered by ribosome inhibitors. Levofloxacin and erythromycin were used as positive controls for DNA biosynthesis and ribosome inhibitors, respectively. The procedure was also applied to Section 3.9.

2.7. Identification of biosynthesis gene clusters for secondary metabolite

The procedures of genomic DNA extraction, whole-genome sequencing and subsequently assembly were performed according to previously described methods by Li et al. [20]. The sequences were examined using the bioinformatics tool antiSMASH 5.0.0 (https://antismash.secondarymetabolites.org/). The bacterial database was set as the reference depository, and the FASTA file with unannotated nucleotide sequence was uploaded to antiSMASH platform (version 5.0.0). The gene cluster basic local alignment search tool (BLAST) comparative analysis and "Active Site Finder" function were selected with relaxed detection parameters that detect not only well-defined clusters containing all required parts but also partial clusters missing one or more genes. The characteristics of each predicted secondary metabolite were investigated by searching related literature. On the basis of the results of the high-throughput screening platform, the putative secondary metabolites that might target ribosome were prioritized for further analysis.

2.8. Molecular networking

The crude extract (1 mg) of *Streptomyces* sp. 8P21H-1 cultured in ISP2 broth was dissolved in methanol (1 mL) and filtered with 0.22 μm filter membrane. Then the sample was analyzed by UPLC-HRESI-MS/MS (Waters Xevo G2-XS QTof; ACQUITY UPLC BEH C_{18} column, 2.1 mm × 100 mm, 1.7 μm). MS method was acquired by data dependent acquisition performed in positive ion mode for

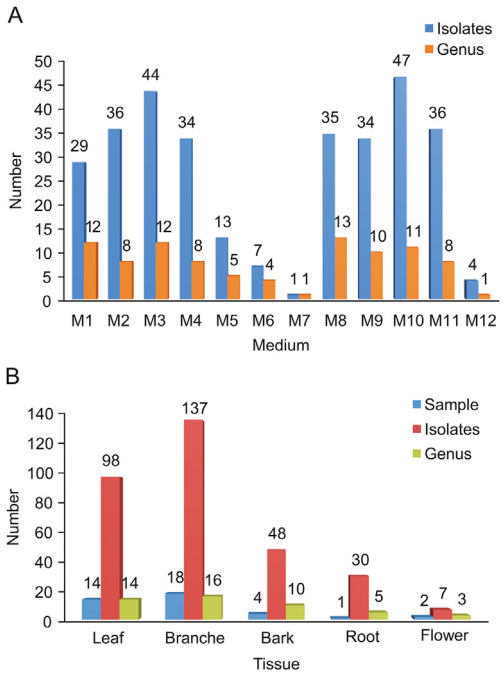

Fig. 3. Diversity of cultivable actinobacteria from psammophytes collected in Taklamakan Desert. (A) Number of actinobacterial isolates recovered from the different culture media; (B) number of actinobacterial isolates from different plant tissues.

Table 3
The sequence analyses based on almost full-length 16S rRNA gene of 16 potential new species and 3 new species.

Strain	Accession number	Closest type species	Similarity of 16S rRNA gene sequence (%)
3H14B-1-1	MN218394	*Brachybacterium sacelli* LMG 20345^T	98.5
6H14B-1	MN218395	*Brachybacterium sacelli* LMG 20345^T	98.5
1H14B-1	MN218396	*Brachybacterium sacelli* LMG 20345^T	98.6
3H14X-6	MN218397	*Brachybacterium sacelli* LMG 20345^T	98.6
8H14X-2	MN218398	*Brachybacterium sacelli* LMG 20345^T	98.5
8H14J-1-1	MN218399	*Brachybacterium sacelli* LMG 20345^T	98.5
10H14J-1	MN218400	*Brachybacterium sacelli* LMG 20345^T	98.5
2H14Y-4	MN218401	*Brachybacterium sacelli* LMG 20345^T	98.5
3H14X-10	MN218402	*Brachybacterium squillarum* M-6-3^T	97.8
1H15P-2	MN218403	*Kineococcus endophyticus* KLBMP 1274^T	98.6
8H15P-1	MN218404	*Kineococcus endophyticus* KLBMP 1274^T	98.6
4H15P-1	MN218405	*Kineococcus endophyticus* KLBMP 1274^T	98.6
3H15P-2	MN218406	*Kineococcus endophyticus* KLBMP 1274^T	98.6
10W25J-3	MN218407	*Microbacterium enclense* NIO-1002^T	98.1
10H14X-1-1	MN218408	*Microbacterium lacusdiani* JXJ CY 01^T	98.1
8H24J-3	MN218409	*Microbacterium testaceum* DSM 20166^T	98.6
8H24J-4-2	MK333281	*Labedella endophytica* CPCC 203961^T	99.0
11W25H-1	MK333282	*Labedella gwakjiensis* KCTC 19176^T	99.2
9W16Y-2	MK238381	*Aeromicrobium camelliae* CGMCC 1.12942^T	99.0

Global Natural Products Social Molecular Networking (GNPS) analysis. The full MS survey scan was performed for 0.1 s time in the range of 100–1600 Da, and MS/MS scanned over a mass range of 50–1600 Da with the same scan time. The raw data obtained were converted digitally to mzxml format files using freely available MSConvert software (http://www.proteowizard.sourceforge.net) [21] and were uploaded subsequently to the online workflow at GNPS platform (http://gnps.ucsd.edu). The MS/MS molecular networking was generated using the GNPS molecular networking workflow (METABOLOMICS-SNETS-V2), in which the MS-Cluster was activated. The generated molecular networking was visualized using Cytoscape 3.7.1 [22] and searched for clusters of *m/z* data that could be used to clarify the compounds generating the clusters.

2.9. Large-scale fermentation, extraction and isolation workflow

Streptomyces sp. 8P21H-1 was grown in seed broth (ISP2 medium) for two days at 28 °C, and then an aliquot (50 mL) was transferred to inoculate 1000 mL of fermentation medium in 5-L shake flasks, which were cultivated at 180 rpm and 28 °C for 6 days. The whole culture broth (20 L) was extracted with equal volumes of ethyl acetate three times (20 L/time) to afford a crude residue (0.96 g) after concentration in vacuo. In order to optimize the isolation workflow toward previously predicted compounds, 2 μL of the whole extract solution (1 mg/mL) was analyzed by UPLC-HRMS/MS in positive ion mode. All the other crude residue was subjected to Sephadex LH-20 column chromatography and eluted with MeOH to yield 35 fractions (Frs. 1–35). Frs. 1 to 4 containing targeted compounds were merged together and then chromatographed over an ODS column chromatography with a gradient system of MeOH–H_2O (20:80, 40:60, 60:40, 80:20, 95:5, *V/V*) to yield seven subfractions (Subfrs. A-G). Subfr. B was successively purified by Zorbax SB-C_{18} column (9.4 mm × 250 mm, 5 μm, Agilent Technologies Inc., Santa Clara, USA) with MeOH–H_2O (28:72, *V/V*) as mobile phase at the flow rate of 2 mL/min in semi-preparative HPLC (Agilent 1200, Agilent Technologies Inc., Santa Clara, USA) to obtain griseoviridin (30.5 mg, R_t = 22.2 min). Subfr. C was separated by semi-preparative HPLC with a gradient mobile phase (MeOH–H_2O, 47% MeOH isocratic conditions for 37 min, then 47%–90% linear gradient over 15 min; flow rate of 2 mL/min) to yield acetyl-griseoviridin (1.8 mg, R_t = 26.3 min) and desulphurzing griseoviridin (1.38 mg, R_t = 46.6 min), respectively.

2.9.1. Acetyl-griseoviridin

White, amorphous powder; ^{1}H NMR and ^{13}C NMR data, see Table 1; HR-ESI-MS *m/z* 542.1578 $[M+Na]^+$ (calcd. for $C_{24}H_{29}N_3O_8SNa$, 542.1573).

2.9.2. Desulphurzing griseoviridin

White, amorphous powder; ^{1}H NMR and ^{13}C NMR data, see Table 1; HR-ESI-MS *m/z* 468.1749 $[M+Na]^+$ (calcd. for $C_{22}H_{27}N_3O_7Na$, 468.1747).

2.10. Antibacterial activities of isolated compounds

The minimum inhibitory concentration (MIC) values of the compounds were evaluated for antibacterial activities using an agar dilution method according to the Clinical and Laboratory Standards Institute recommended guidlines [23]. Levofloxacin was used as the positive control (final concentration ranged from 0.03 μg/mL to 128 μg/mL). The final test concentrations of griseoviridin ranged from 0.03 μg/mL to 64 μg/mL. Final concentrations of acetyl-griseoviridin and desulphurzing griseoviridin ranged from 0.03 μg/mL to 32 μg/mL. The test bacteria were cultured in Mueller-Hinton (MH) broth medium at 35 °C for 8 h and then were adjusted to a turbidity of 0.5 using the McFarland standard. The bacterial suspensions were inoculated onto the drug-supplemented MH agar plates using a multipoint inoculator. After being incubated at 35 °C for 16 h, the MICs were defined as the lowest concentrations that prevented visible growth of the bacteria.

3. Results and discussion

3.1. Isolation of endophytic actinobacteria

In the present study, a total of 428 purified isolates were obtained from 15 samples collected from Taklamakan Desert. The 16S rRNA gene sequence analyses revealed that 320 strains belonged to actinobacteria, which were assigned to 23 genera in 14 families (Fig. 2). *Streptomyces* was the dominant genus (119 strains, 37.2%, Table 2), which was in line with the previous reports [24,25]. In addition to *Streptomyces*, other six genera, i.e., *Micromonospora*, *Nocardia*, *Nonomuraea*, *Nocardiopsis*, *Amycolatopsis* and *Modestobacter,* were widespread in desert. Besides, other rare actinobacterial genera from desert plants have been identified such as

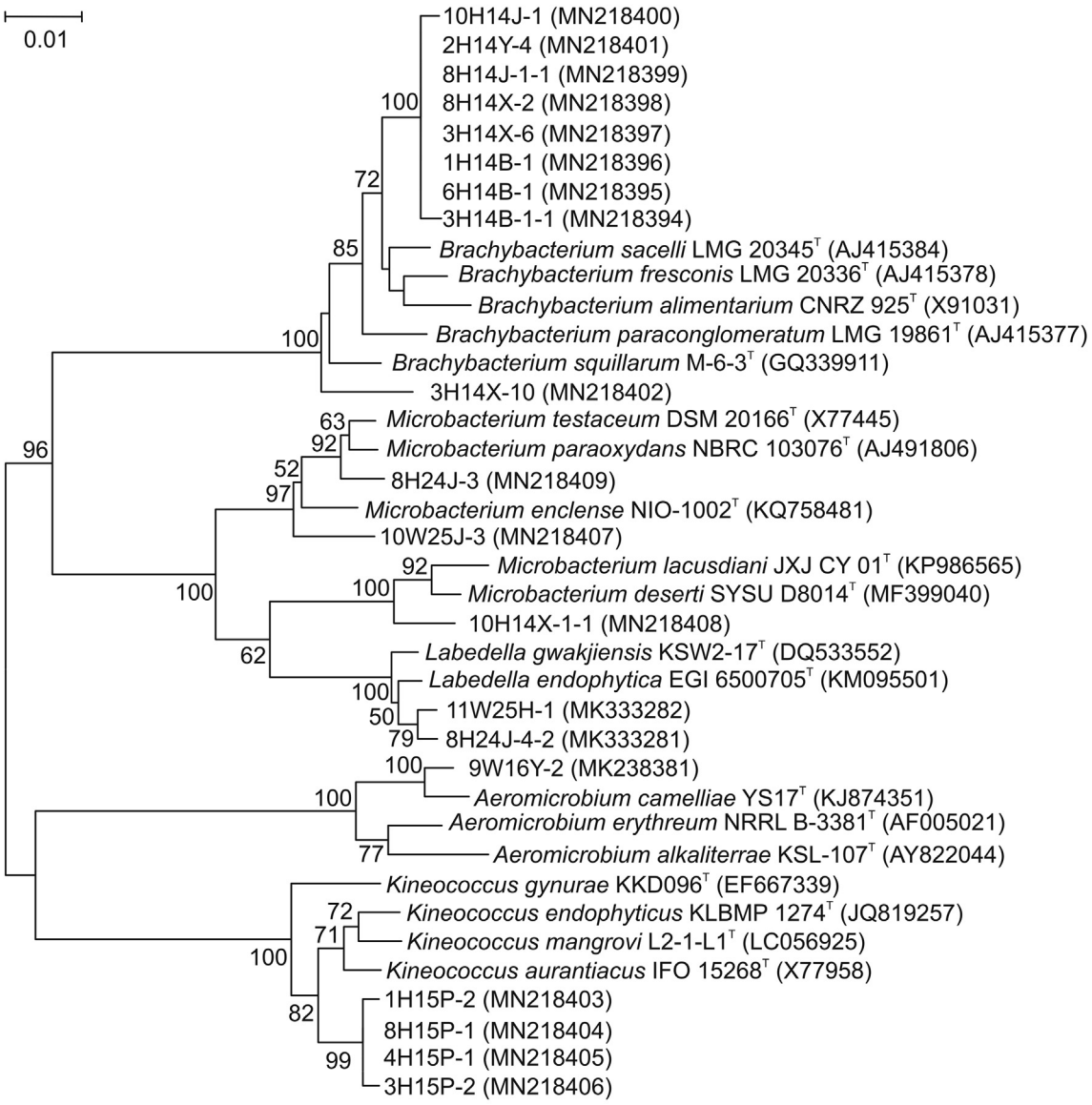

Fig. 4. Phylogenetic neighbor-joining tree of 16 potential novel strains and 3 new species based on almost full-length 16S rRNA gene sequences. Numbers at nodes indicate the level of bootstrap support > 50% based on 1000 replications. Bar, 1 nt substitutions per 100 nt.

Labedella, *Rathayibacter*, *Leucobacter*, *Frigoribacterium*, *Kineococcus* and *Aeromicrobium*.

The distributions of the 320 strains isolated from 12 different media and 5 different plant tissues are shown in Figs. 3A and B, respectively. Among the 12 different isolation media, both M10 and M3 were the most successful media for isolating actinobacterial strains. The highest number (65 strains, 20.3%) and the lowest number (2 strains, 0.6%) of the endophytic actinobacteria obtained were isolated from S6 (*Phragmites australis*) and S8 (*Reaumuria soongorica*), respectively. The result of calculating the average number of actinobacteria from each plant tissue showed that the actinobacteria isolated from roots had the highest diversity (30 strains in 5 genera from one sample). Although the diversity was relatively low, actinobacteria isolated from barks were relatively more than those from branches, leaves and flowers. Our findings supported the previous reports that many endophytes originate from the rhizosphere of plants [26].

3.2. Novelty analysis of endophytic actinobacteria

Among the 320 actinobacterial strains, 16 strains exhibited low sequence similarities < 98.65%, the threshold for differentiating two species [27] with validly described species based on the results of BLAST search in EzBiocloud database (http://www.ezbiocloud.net/) (Table 3). The results indicated that these isolates might represent hitherto unrecognized new species. In the phylogenetic dendrogram based on almost full-length 16S rRNA gene sequences (Fig. 4), these potential novel strains fell into three genera, i.e., *Brachybacterium* (9 strains), *Kineococcus* (4 strains), and *Microbacterium* (3 strains). Strains 10H14J-1, 2H14Y-4, 8H14J-1-1, 8H14X-

2, 3H14X-6, 1H14B-1, 6H14B-1 and 3H14B-1-1 were isolated from *Populus euphratica* at sample S4. These 8 potential novel strains formatted a monophyletic in clade, which may represent a novel strain of genus *Brachybacterium*. In the same way, strains 1H15P-2, 8H15P-1, 4H15P-1 and 3H15P-2 were isolated from *Populus euphratica* in sample S5. These 4 potential novel strains formatted a monophyletic clade, which may represent a novel strain of genus *Kineococcus*. These strains will be further identified with a polyphasic approach to determine their taxonomic positions. In addition, three strains with relatively high 16S rRNA sequence similarities, i.e., 11W25H-1^T, 8H24J-4-2^T, and 9W16Y-2^T, had been identified as type species and named as *Labedella phragmitis*, *Labedella populi*, and *Aeromicrobium endophyticum*, respectively [28,29]. In this study, novelty analysis suggested that desert plants have the potential as excellent sources of novel actinobacterial species.

3.3. Antimicrobial activities

Seventy-five strains were selected for antimicrobial assay based on analyses of partial 16S rRNA gene sequences and phenotypic characteristics. The antibacterial profiles of the selected strains against "ESKAPE" bacteria are shown in Fig. 5 and Table 2. Among the 75 strains selected for antimicrobial assay, 47 strains showed antagonistic activity against at least one of the indicator bacteria. They were distributed in 20 different genera, and the predominant active strains were affiliated to genus *Streptomyces*, which was in accordance with the reported data [30], since members of the genus *Streptomyces* usually possess a number of biosynthetic gene clusters that encode multifunctional biosynthetic enzymes [31,32]. Twenty *Streptomyces* strains showed inhibitory activity against at least one of Gram-positive bacteria, and seven of them also showed activity against at least one of Gram-negative bacteria (Table S3).

Rare actinobacteria are also important sources for the discovery of novel antibiotics [33]. Recently, saccharothriolides A-C [34] and saccharothriolides D-F [35], a group of new secondary metabolites, have been found from *Saccharothrix* sp. A1506 isolated from a soil sample, which further indicates the rare actinobacteria deserve to be studied extensively to find new antibiotics. In the present study, active strains distributed in 18 rare genera such as *Brachybacterium*, *Leifsonia*, *Arthrobacter*, *Labedella* and *Leucobacter* were obtained and have been rarely studied for their bioactive secondary metabolites. Notably, both strains 11W25H-1^T and 8H24J-4-2^T as two new species in rare genus *Labedella*, showed promising activity against *S. aureus* (Table S3). Thus, these rare actinobacteria deserved further studies on their bioactive secondary metabolites.

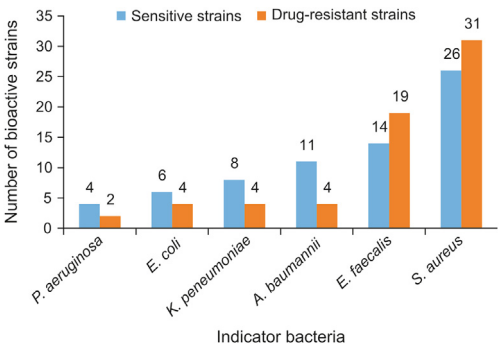

Fig. 5. The antibacterial profiles of the actinobacteria against "ESKAPE" bacteria.

3.4. High-throughput screening using a double fluorescent protein reporter system

The pDualrep2 reporter system is a very sensitive screening model for detection of compounds that inhibit protein translation and DNA biosynthesis. According to the results of primary screening, two *Streptomyces* strains, 3H14X-7 and 8P21H-1, induced Katushka2S expression as erythromycin did. Meanwhile, two *Streptomyces* strains, 2H20Y-2 and 3H20G-2, induced SOS-response as levofloxacin did (Fig. 6). Furthermore, taking results of antibacterial activities of these four *Streptomyces* strains into consideration, strain 8P21H-1 was prioritized as an example for chemical research to find new antibiotics targeted on ribosome.

3.5. Identification of biosynthesis gene clusters for secondary metabolite

The assembled draft genome of strain 8P21H-1 was 6.65 Mbp. A total of 71 biosynthetic gene clusters were identified by antiSMASH platform (https://antismash.secondarymetabolites.org/). Single gene clusters encoded the biosynthetic pathway for "griseoviridin/viridogrisein" non-ribosomal peptide synthases-polyketide synthases (NRPS-PKS), indole, nucleoside, lanthipeptide, lassopeptide, melanin, and LAP, respectively. There were four gene clusters for bacteriocins, four for siderophores, eight for terpenes, twenty-three for PKS, and twenty-four for NRPS. Furthermore, one gene cluster showed no predicted product family. AntiSMASH analysis also showed putative gene clusters previously identified for micromonolactam, nanchangmycin, bleomycin, griseoviridin/viridogrisein and diisonitrile antibiotic SF2768 biosynthesis in strain 8P21H-1. Among these putative compounds, especially for known antibiotics, the "griseoviridin/viridogrisein" demonstrated antibacterial activity by inhibiting ribosome [36]. Presumably, this group of antibiotics was the active components of the strain as the results of bioinformatics analysis indicated.

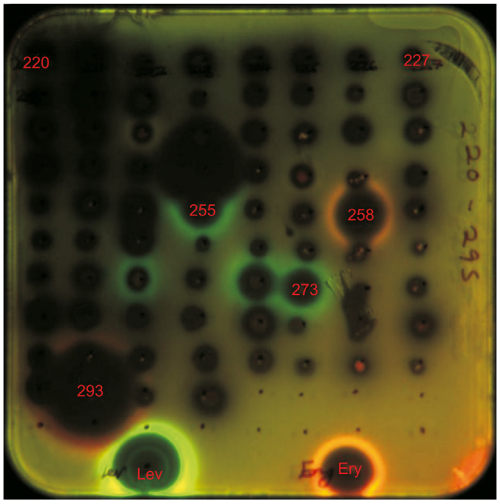

Fig. 6. Induction of a two-color dual reporter system sensitive to inhibitors of the ribosome progression or inhibitors of DNA replication, respectively. Spots of erythromycin (Ery), levofloxacin (Lev), and tested samples were placed on the surface of an agar plate containing *E. coli* Δ*tolC* cells transformed with the pDualrep2 reporter plasmid. The shown image is the fluorescence of the lawn of *E. coli* cells scanned at 553/574 nm (green pseudocolor) for red fluorecent protein fluorescence and 588/633 nm (red pseudocolor) for Katushka2S fluorescence. Induction of expression of Katushka2S was triggered by translation inhibitors, while RFP was upregulated by induction of DNA damage SOS response. 255: 2H20Y-2; 258: 3H14X-7; 273: 3H20G-2; 293: 8P21H-1.

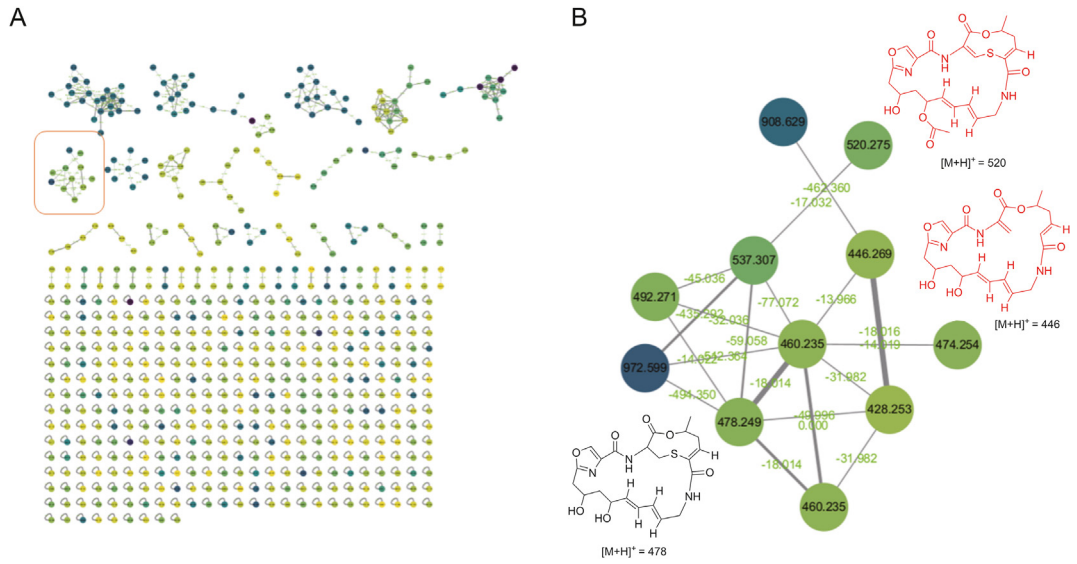

Fig. 7. (A) Molecular networking of *Streptomyces* sp. 8P21H-1. (B) Molecular networking of griseoviridin analogs.

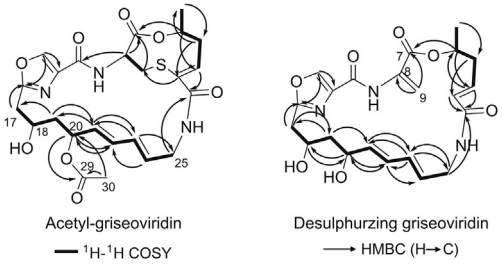

Fig. 8. Summary of ^{1}H-^{1}H COSY and HMBC experiments of acetyl-griseoviridin and desulphurzing griseoviridin in methanol-d_4.

3.6. Molecular networking

One major advantage of the molecular networking strategy is the function of identifying known compounds and potential analogs, thereby allowing for the prioritization of the isolation workflow and guiding the user toward unknown molecules [4]. A molecular networking was established to investigate the sample of *Streptomyces* sp. 8P21H-1 using the GNPS platform (https://gnps.ucsd.edu/). The crude extract was subjected to LC-MS/MS analysis, a major chromatographic fraction with nominal mass of 477 and similar UV characteristic spectrum profiled to that of griseorividin. Based on the analysis of molecular networking (Fig. 7A), the major component was identified to be griseoviridin and fell into the cluster (Fig. 7B). This result was consistent with that of the antiSMASH analysis that griseoviridin was a potential compound of one gene cluster. Other nodes in cluster (Fig. 7B) had clear connections with griseoviridin and were predicted as its analogs. These analogs were not identified to the previously reported compounds according to the dereplication tool at GNPS platform [37], thus suggesting that they were potential new compounds. Given the limit of the amounts of analogs present in the cluster (Fig. 7B), only the putative griseoviridin-like acetyl-griseoviridin ($[M+H]^+$ ion peak at *m/z* 520.275) and desulphurzing griseoviridin ($[M+H]^+$ ion peak at *m/z* 446.269) were suitable for further purified and structure elucidation by NMR analysis.

Table 4
Antimicrobial activities of acetyl-griseoviridin, desulphurzing griseoviridin, and griseoviridin.

Test organisms	MIC (μg/mL)			
	Acetyl-griseoviridin	Desulphurzing griseoviridin	Griseoviridin	Levofloxacin
Staphylococcus epidermidis ATCC 12228	> 32	32	8	0.12
Staphylococcus aureus ATCC 29213	> 32	> 32	> 64	0.12
Pseudomonas aeruginosa PAO1	> 32	32	64	4
Providencia rettgeri ATCC 31052	> 32	32	>64	≤ 0.03
Enterococcus faecium ATCC 700221	> 32	> 32	8	64
Escherichia coli ATCC 25922	> 32	> 32	> 64	≤ 0.03
Klebsiella pneumoniae ATCC 700603	> 32	> 32	> 64	0.5
Acinetobacter baumannii ATCC 19606	> 32	> 32	> 64	0.25

3.7. Structure elucidation of compounds

Acetyl-griseoviridin was isolated as a white amorphous powder. The molecular formula was determined as $C_{24}H_{29}N_3O_8S$ on the basis of HR-ESI-MS (Fig. S1) (*m/z* 542.1578 $[M+Na]^+$, calc. for 542.1573), requiring 12° of unsaturation. The 1D NMR data (Table 1) of acetyl-griseoviridin suggested the presence of seven quaternary carbons, ten methines, five methylenes, and two methyl groups. Careful analysis of the NMR data (Figs. S2–S12, Table 1) indicated that acetyl-griseoviridin was an analogue of griseoviridin [38,39] but with an acetoxy rather than a hydroxyl group attached to C-20 (δ_C 74.6). The heteronuclear multiple bond correlation (HMBC) (Fig. 8) from H-20 (δ_H 5.49–5.43) to C-29 (δ_C 171.9), and from H_3-30 (δ_H 1.99) to C-20 and C-29 indicated the presence of the acetoxy group linked to C-20 as well.

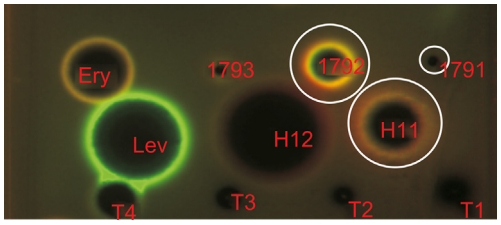

Fig. 9. Agar plate coated with a layer of reporter strain JW5503 (Δ*tolC*) of *E. coli* transformed by plasmid pDualrep2 with the tested compounds (1791; 1792; H11). Cells with tested compounds were identified by white circles and corresponding number. 1791 was on behalf of acetyl-griseoviridin; 1792 was on behalf of desulphurizing griseoviridin; H11 was on behalf of griseoviridin.

Desulphurizing griseoviridin was a white amorphous powder with a molecular formula $C_{22}H_{27}N_3O_7$ based on the HR-ESI-MS (Fig. S13) (*m/z* 468.1749 $[M+Na]^+$, calc. for 468.1747). The HR-ESI-MS showed the S atom was absent in desulphurizing griseoviridin. The NMR spectra (Figs. S14–S26, Table 1) of desulphurizing griseoviridin were similar to those of griseoviridin [38,39], suggesting the presence of a similar skeleton. The chemical shifts of C-9 (δ_C 39.5) and C-8 (δ_C 52.0) in griseoviridin were changed dramatically to downfield in desulphurizing griseoviridin (C-9 at δ_C 108.2; C-8 at δ_C 131.2), indicating that presence of an additional double bond in position C-8 and C-9 and absence of the S atom in position **2**, which was further confirmed by the HMBC correlations (Fig. 8) from H_2-9 (δ_H 6.61, 5.97) to C-7 (δ_C 163.0) and C-8 (δ_C 131.2).

Known griseoviridin was isolated as a white amorphous powder. In the HR-ESI-MS spectrum, a molecular ion peak at *m/z* 500.1632 $[M+Na]^+$ (calc. for $C_{22}H_{27}N_3O_7SNa$) provided the elemental formula $C_{22}H_{27}N_3O_7S$. The chemical structure was identified by comparison of the NMR data (Table S4) with those in the literature [38,39]. Griseoviridin as a representative member of polyunsaturated macrolactones was first discovered in the culture of *Streptomyces graminofaciens* in 1953 [40].

Both acetyl-griseoviridin and desulphurizing griseoviridin are classified as streptogramin antibiotics just like griseoviridin, which are a family of natural products isolated from *Streptomyces*. To the best of our knowledge [40–42], streptogramin family is generally divided into two structurally different subclasses of type A and type B. Acetyl-griseoviridin and desulphurizing griseoviridin are cyclic polyunsaturated macrolactones belonging to type A. Chemical modifications to various members of the streptogramins have been pursued, and the new natural compounds provide a new perspective for further structure modifications.

3.8. Antibacterial activities of isolated compounds

All isolated compounds were evaluated for their antibacterial activities by MIC assay. The results (Table 4) indicated that only desulphurizing griseoviridin and griseoviridin exhibited modest antibacterial activities against partial test strains. Griseoviridin showed antibacterial activities against *Staphylococcus epidermidis* ATCC 12228 and *Enterococcus faecium* ATCC 700221 with MIC value of 8 μg/mL. Desulphurizing griseoviridin showed antibacterial activities against *Staphylococcus epidermidis* ATCC 12228, *Pseudomonas aeruginosa* PAO1, and *Providencia rettgeri* ATCC 31052 with MIC value of 32 μg/mL. It had been reported that streptogramins could be inactivated by acetyl transferases, which could explain why acetyl-griseoviridin showed no antibacterial activity [41].

3.9. Mechanism of action determination for isolated compounds

The mechanism of action of griseoviridin (named as **H11** when tested), desulphurizing griseoviridin (named as **1792** when tested) and acetyl-griseoviridin (named as **1791** when tested) were tested with the pDualrep2 reported system. The results are shown in Fig. 9, and both desulphurizing griseoviridin and griseoviridin demonstrated antibacterial activity by inhibiting translation. This finding corresponded to the primarily screening results described in antibacterial activity and high-throughput screening. Remarkably, a thorough literature survey has revealed that this type of molecules is believed to prevent access of the active aminoacyl-tRNA to the ribosomal A site [41,42], thus prohibiting peptide bond formation. Importantly, members of these subclasses have shown excellent synergistically biological activities (100-fold than each individual compound) with the other subclass, cyclic octadepsipeptide. In consideration of the excellent activity and unique mechanism of action, the typical antibiotics are very valuable to further research for synergistical action and bioactivity.

4. Conclusions

As an illustratively systemic study, the pipeline from isolation and identification of actinobacteria to discover bioactive compounds was successfully accomplished. Among 320 endophytic actinobacteria, three strains were identified as new species, and 16 strains were considered as potential new taxa. The strain 8P21H-1 with strong antibacterial activity targeting ribosome was selected for exploring new antibiotics. Ultimately, two new streptogramin-type antibiotics, i.e., acetyl-griseoviridin and desulphurizing griseoviridin, along with known griseoviridin, were isolated from the culture broth of the strain. Acetyl-griseoviridin possessed an acetylated hydroxyl group and desulphurizing griseoviridin obtained an exocyclic double bond. Desulphurizing griseoviridin and griseoviridin exhibited antibacterial activities by inhibiting translation. A valuable integrated strategy for tracing new and/or bioactive compounds was established in this study, which is conducive to addressing the key bottleneck of rediscovery of known antibiotics and helping to find novel secondary metabolites.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2020.06.004.

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