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ORIGINAL ARTICLE

Culturable actinomycetes from desert ecosystem in northeast of Qinghai-Tibet Plateau

Dong Ding · Guichen Chen · Bochan Wang · Qilan Wang · Demei Liu · Min Peng · Ping Shi

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Abstract In this study, 53 actinomycetes strains were isolated from desert ecosystems located in northeast of Qinghai-Tibet Plateau and grouped into four RFLP patterns. Twenty-six actinomycetes with obvious morphology differences were chosen for phylogenetic diversity study and antimicrobial testing. As a result, the 16S rRNA gene sequencing showed that these strains belonged to *Streptomyces*, *Micromonospora*, *Saccharothrix*, *Streptosporangium* and *Cellulomonas*, and that most of the strains had antimicrobial bioactivity. The PKS and NRPS genes detection indicated diversified potential bioactive products of actinomycetes in this ecosystem. Among these strains, Sd-31 was chosen to study the bioactive products using HPLC-MS because of its optimum antimicrobial bioactivity. The result showed that it might produce Granatomycin A, Granatomycin C, and an unknown compound.

Keywords Qinghai-Tibet Plateau · Actinomycete · 16S rRNA-RFLP · PKS · NRPS · Desert ecosystem

Introduction

To exploit actinomycetes from unusual and underexplored environments is a way to develop bioactive products. The Qinghai-Tibet plateau is the highest plateau in the world. Its distinctive plateau climate and geographic position promote the formation of its unique ecological environment. Recent studies have focused on culturable actinomycetes from this environment and proved the potential of this resource (Chen et al. 2011; Zhang et al. 2005, 2007, 2010). However, the Qinghai-Tibet plateau possesses different types of ecosystems, which include desert, alpine shrub, alpine meadow, prairie, forest and wetland. In order to exploit more laudable actinomycetes, it is necessary to better understand the diversity of culturable actinomycetes in a single ecosystem of the Qinghai-Tibet plateau.

Qinghai-Lake Desert Island, as the desert ecosystem representative in the Qinghai-Tibet plateau, is located in the northeast (36°37′–37°5′N, 100°25′–100°55′E) and covers 950 km^2. Its windy climate contains high amounts of UV radiation. The mean annual temperature and elevation are 0.3°C and 3,265 m, respectively. While the mean annual rainfall is 378.2 mm, the ratio of mean annual evaporation to mean annual rainfall is 3.8. The plateu contains different types of sand, including aeolian, wandering dune, riverbed and lakeside. The dominant plants are *Artemisia sphaerocephala*, *Calamagrostis macrolepis, Carex qinghaiensis, Clematis tangutica*, *Corispermum declinatum, Hippophae rhamnoides, Orinus kokonorica* and *Sabina vulgaris*. With the environment having a high altitude desert ecosystem and a barren setting, we studied the diversity of culturable actinomycetes in this location and evaluated their potential capacity to synthesize bioactive secondary metabolites by using the antimicrobial property of actinomycetes, polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) genes detection as well as LC-MS analysis.

D. Ding · G. Chen · Q. Wang · D. Liu · M. Peng (✉)
Northwest Institute of Plateau Biology,
Chinese Academy of Sciences,
Xining 810008, People's Republic of China
e-mail: pengm@nwipb.ac.cn

B. Wang · P. Shi (✉)
State Key Laboratory of Bioreactor Engineering,
East China University of Science and Technology,
Shanghai 200237, People's Republic of China
e-mail: ship@ecust.edu.cn

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Materials and methods

Sample collection and pretreatment

Sample collection was performed during the middle of August 2010. The plot is located at 36°30′09.5″N, 100° 50′09.5″E, and at an elevation of 3,304 m. About 50 quadrat (20 cm×20 cm) soil samples covered by vegetation from the depth of 0–15 cm were randomly collected and mixed in a steriled Zip lock bag and then transported to the laboratory on the same day. According to the study on the best air-drying time for actinomycetes isolation from soil samples (Fan et al. 2010), part of the samples used to isolate the actinomycete were dried for 20 days at room temperature, while others were stored at −20°C.

Isolation and cultivation of actinomycetes

Gause′s medium No.1, ISP2 and HV were used to isolate strains. An amount of 1 g of dried sieved soil sample was pretreated for 1 h at 100 °C and soil suspension was sonicated for 40 s. Then, 0.1 mL of serial diluted supernatant was spread onto the surface of the medium and incubated at 27 °C for 5 days.

DNA extraction, amplification and purification

The purified single actinomycete was cultivated in liquid medium for 5 days and then the cell was harvested by centrifugation for DNA extraction. Genomic DNA was extracted by modified freeze–thaw procedure as previously described (Reischl et al. 1994): Briefly, the harvested cells were washed with PBS twice and dissolved in 1 mL double- distilled H_2O. Then, the sample was frozen and thawed about four times, and 2 mL absolute ethyl alcohol was added to the sample for the precipitation of DNA overnight at −20°C. The DNA was harvested by centrifugation and dissolved in 50 μL double-distilled H_2O for PCR. 16S rRNA gene was amplified using primers 27F (5′-AGAGTTTGATC (A/C) TGGCTCAG-3′) and 1492R (5′-TACGG (C/T) TACCTTGTTACGACTT-3′) (Heuer et al. 1997). The PCR reaction was performed by EmeraldAmp™ PCR Master Mix Kit (Takara Biotechnology, Dalian, China). The PCR program was pre-denaturation at 95 °C for 6 min, 30 cycles of denaturation at 95 °C for 45 s, annealing at 56 °C for 45 s, extension at 72 °C for 1 min, and delay at 72 °C for 10 min. PCR products were checked by 1 % agarose gels and purified by TIANgel Midi Purification Kit (Tiangen Biotech, Beijing, China) following the manufacturer's protocol.

16S rRNA gene restriction fragment length polymorphism analysis

Purified 16S rRNA genes were digested with *Taq*I (Takara Biotechnology) following the manufacturer's protocol. DNA fragments were checked by 1.5 % agrose gels.

DNA sequencing and phylogenetic analysis

Purified PCR products were sequenced by Sangon (Sangon Biotech, Shanghai, China). 16S rRNA sequences of isolates were compared with the data of NCBI BLAST to decide the relative phylogenetic positions. The closest type strains' 16S rRNA sequences were obtained from the Ribosomal Database Project (RDP). The neighbor-joining tree with the closest type strain 16S rRNA gene was constructed by Clustal X (Thompson et al. 1997) and MEGA 4 (Tamura et al. 2007) with bootstrap values using 1,000 replications.

Antimicrobial testing

Using the double-layer plate method, four indicator strains were chosen for antimicrobial testing: *Escherichia coli* (CGMCC 1.1369), *Staphyloccocus aureus* (CGMCC 1.128), *Bacillus subtilis* (CGMCC 1.1630), and *Aspergillus niger* (CGMCC 3.6479) (Alford and Steinle 1967). In order to inoculate the indicator strain at the stationary phase of testing actinomycetes, Sd-5 without diffusible pigment and Sd-15 with diffusible pigment were chosen to determine the growth curve of tested actinomycetes by GENios Pro (Tecan, Mannedorf, Switzerland). Media used for indicator strains were LB Agar medium for bacteria and Sabouraud's Agar medium for fungus.

Detection and analysis of NRPS, PKS-I and PKS-II

Genes encoding PKS-I, PKS-II and NRPS were amplified using primers K1F (5′-TSAAGTCSAACATCGGBCA-3′) and M6R (5′-CGCAGGTTSCSGTACCAGTA -3′) (Ayuso-Sacido and Genilloud 2005); IIPF6 (5′-TSGCSTGCTT CGAYGCSATC-3′) and IIPR6 (5′-TGGAANCCGCC GAABCCGCT-3′) (Ketela et al. 1999); A3F (5′-GCSTAC SYSATSTACACSTCSGG-3′) and A7R (5′-SASGTCVCCS GTSCGGTAS-3′) (Ayuso-Sacido and Genilloud 2005). PCR reactions were performed by EmeraldAmp™ PCR Master Mix Kit (Takara Biotechnology). The PCR programs for NRPS, PKS-I and PKS-II were described before (Ayuso-Sacido and Genilloud 2005; Ketela et al. 1999).

Secondary metabolites analysis

Cultures in 100-mL flasks of the strains showing antimicrobial activity and the blank medium were incubated on 27 °C

on a rotary shaker (Shiping, Shanghai, China) for 7 days. The supernatant was obtained by centrifuge (Thermo Fisher Scientific, San Jose, CA, USA) and evaporated by rotary evaporator (Senco, Shanghai, China) at 45 °C. The pellet was weighed and resuspended with 1 mL double- distilled H_2O. The antimicrobial activity of the secondary metabolites from the strains was analyzed by the paper-disc agar plate method (Edwin and Marion 1945) and the indicator strains used were same as the antimicrobial testing part.

The antimicrobial substance was prepared by thin-layer chromatography (Sunasia, Shanghai, China). Its detection was carried out using an HPLC system (Thermo Fisher Scientific), equipped with a DAD 3000 detector (Thermo Fisher Scientific), a MSQ PlusTM a single quadruple mass spectrometer (Thermo Fisher Scientific), and a M-path electro spray ionization (ESI) source operated in both negative and positive mode controlled by Xcalibur software (v.2.0; Thermo Fisher Scientific) for data acquisition and processing.

Sample solutions were separated on a reversed-phase Acclaim C18 analytical column (4.6 mm×150 mm, i.d. 5 μm). A linear gradient elution of water–formic acid (100:0.1, v/v) (A) and acetonitrile (B) was used for the separation of samples. The gradient programmer was as follows: 0–8 min, 95–20 % A; 8–10 min, 20 % A; 10 min–10.1 min, 20 %–95 % A; 10.1–25 min, 95 % A. The solvent flow rate was 1 ml/min and 10 μL of sample solution was injected in each run. Column temperature was maintained at 30 °C. The effluent was introduced into a DAD detector (scanning range 190–400 nm, resolution 1.2 nm) and subsequently into an electro spray ionization (ESI) source (desolvation temperature 400 °C, capillary voltage 3.0 kV, cone voltage 15 V). The split ratio of HPLC flow between DAD detector and MS detector was 2:1. Helium was used as collision gas (collision energy 15 V) and nitrogen as the disolving gas (500 L/h).

Nucleotide sequence accession number

16S rRNA sequences were deposited to Genbank under Accession numbers: JF833094–JF833096, JF833098–JF833105, JF833107, JF508411–JF508412, JF508414–JF508418, JF508422–JF508425, and JN641295–JN641297.

Results

16S rRNA-RFLP analysis

Fifty-three strains were isolated from the soil sample. RFLP patterns showed that all strains were grouped into four types. Thirty-two strains were grouped into type I, six strains were grouped into type II, six strains were grouped into type III, and nine strains were grouped into type IV. According to RFLP patterns, type I exihibited three bands at 1,000/400/150 bp, type II exhibited two bands at 1,000/600 bp, type III exhibited three bands at 500/400/150 bp and type IV exhibited two bands at 500/400 bp (Fig. 1). Finally, 26 isolates with obvious morphology differences were selected for further study.

Phylogenetic analysis

According to the similarity of 16S rRNA gene(about 1500 bp) analysis, 26 actinomycetes belonged to five genera: *Streptomyces*, *Micromonospora*, *Saccharothrix*, *Streptosporangium* and *Cellulomonas*. Seventeen strains belonged to *Streptomyces* and showed 100–96 % similarity to *Streptomyces umbrinus, Streptomyces griseobrunneus, Streptomyces kanamyceticus, Streptomyces venezuelae, Streptomyces cirratus, Streptomyces kurssanovii, Streptomyces exfoliatus, Streptomyces aureus, Streptomyces griseinus, Streptomyces avidinii, Streptomyces crystallinus, Streptomyces lateritius, Streptomyces bobili* and *Streptomyces tauricus*. Four strains belonged to *Micromonospora* and showed 100, 96.5, 96.1 and 96 % similarity to *Micromonospora chaiyaphumensis*, *Micromonospora saelicesensis* and *Micromonospora chokoriensis*, respectively. Three strains belonged to *Streptosporangium* and showed 99.2 and 99.1 % similarity to *Streptosporangium vulgare* and *Streptosporangium amethystogenes,* respectively. Two strains belonged to *Saccharothrix* and *Cellulomonas*, and showed 98.6 and 96 % similarity to *Saccharothrix texasensis* and *Cellulomonas flavigena*, respectively. The phylogenetic tree of 16S rRNA sequences combined with the closest type

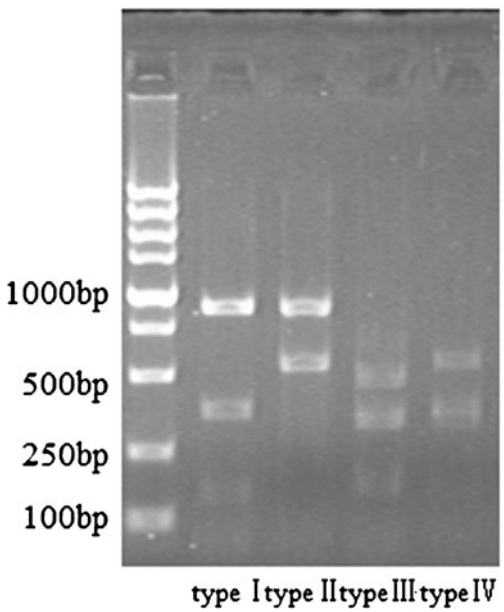

Fig. 1 16S rRNA-RFLP patterns of isolates from a desert ecosystem in the northeast of the Qinghai-Tibet plateau

strains' 16S rRNA was constructed and only values above 50 % are shown in Fig. 2. The accession number of isolates and similarity to the closest type strains are shown in Table 1.

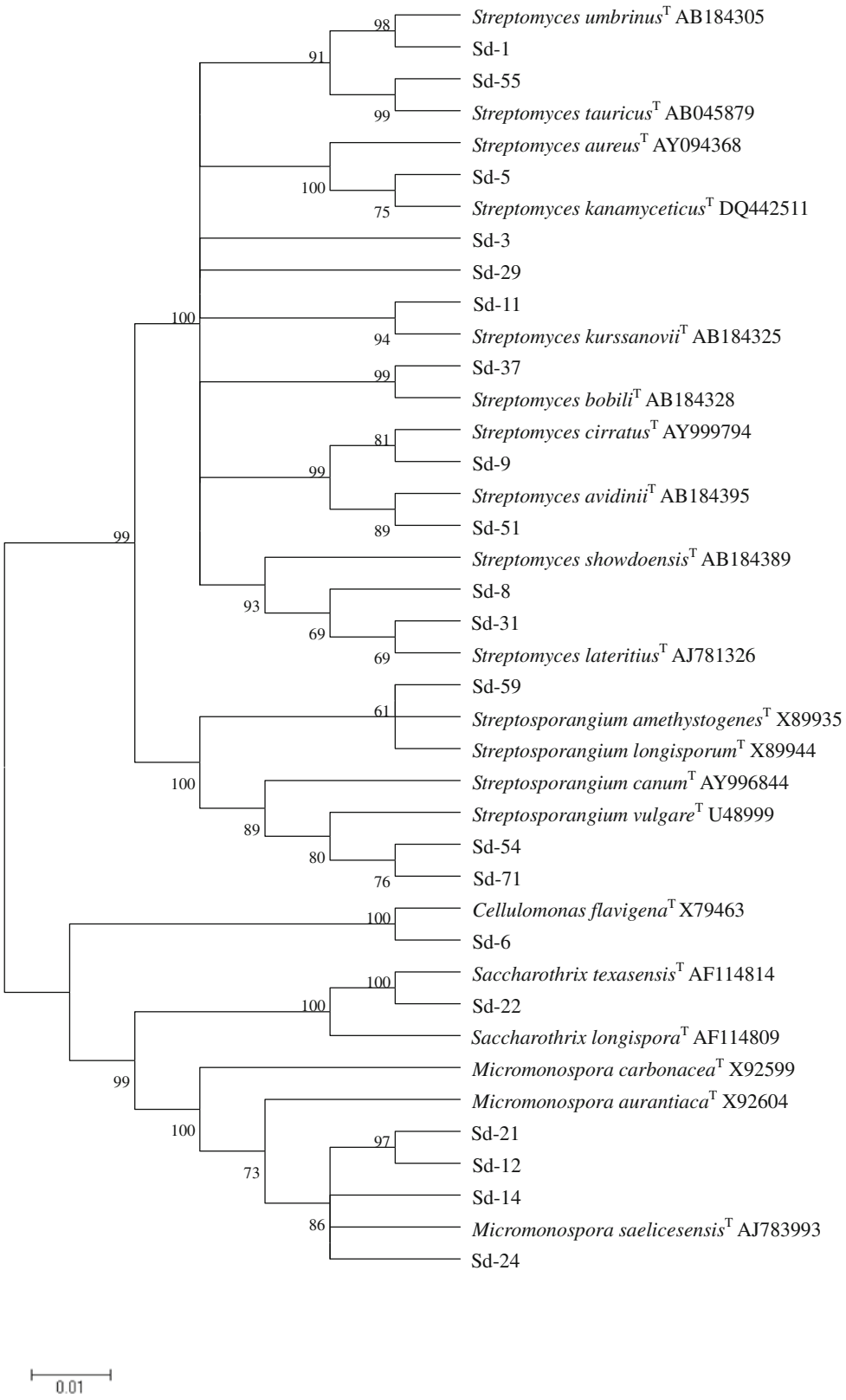

Fig. 2 Neighbor-joining tree of nearly complete 16SrRNA showing the phylogenetic position of actinomycetes isolated from a desert ecosystem in the northeast of the Qinghai-Tibet plateau with the closest type strains obtained from RDP. Numbers at the nodes are bootstrap values (percentages of 1,000 replicate) and only values above 50 % are given. *T* type strains. *Numbers in parentheses* are accession numbers in genebank. *Scale bar* 0.01 substitutions per nucleotide position

Table 1 Summary of culturable actinomycetes isolates from a desert ecosystem in the northeast of the Qinghai-Tibet plateau

Genus	Isolates	Aerial mycelium	Pigment[a]	RFLP Patterns	The closest type strain (similarity)	Accession number	Antimicrobial activity[b]				NRPS	PKS-I	PKS-II
							E.coli	*S.aureus*	*B.subtilis*	*A.niger*			
Streptomyces	Sd-1	Yellow	–	type II	*Streptomyces umbrinus*(100 %)	JF833104	−	−	−	−	−	−	−
	Sd-3	Yellow	–	type II	*Streptomyces griseobrunneus*(97.2 %)	JF833094	−	−	−	−	−	−	−
	Sd-5	White	–	type II	*Streptomyces kanamyceticus*(96.7 %)	JF508411	−	++	+	−	+	−	+
	Sd-8	White	–	type II	*Streptomyces venezuelae*(100 %)	JF508414	−	−	+	−	+	−	−
	Sd-9	White	–	type II	*Streptomyces cirratus*(98.6 %)	JF508415	−	−	−	−	−	−	−
	Sd-10	Pink	–	type II	*Streptomyces venezuelae*(100 %)	JF508416	−	−	−	−	+	−	−
	Sd-11	Grey	–	type II	*Streptomyces kurssanovii*(100 %)	JF833105	−	++	++	−	+	−	+
	Sd-13	Brown	–	type II	*Streptomyces exfoliates*(97.2 %)	JF508417	−	−	−	−	−	−	−
	Sd-15	Brown	Gray	type II	*Streptomyces aureus*(99.4 %)	JF833095	−	−	−	−	−	+	+
	Sd-23	Red	Pink	type II	*Streptomyces griseinus*(98 %)	JF833096	−	−	++	−	−	−	+
	Sd-28	Brown	Green	type II	*Streptomyces avidinii*(98.1 %)	JF508425	−	−	−	−	+	−	+
	Sd-29	Pink	–	type II	*Streptomyces crystallinus*(98.9 %)	JF833098	−	++	++	−	−	−	+
	Sd-31	Blue	Blue	type II	*Streptomyces lateritius*(100 %)	JF833099	−	++	++	−	+	−	+
	Sd-37	Black	–	type II	*Streptomyces bobili*(100 %)	JF833100	−	−	−	−	−	+	+
	Sd-51	Gray	–	type II	*Streptomyces avidinii*(98.8 %)	JF833101	−	++	++	−	+	−	+
	Sd-55	Orange	Orange	type II	*Streptomyces tauricus*(100 %)	JF833102	−	−	++	−	−	−	+
	Sd-56	White	–	type II	*Streptomyces aureus*(96.1 %)	JF833103	−	−	+	−	+	−	+
Cellulomonas	Sd-6	Green	–	type I	*Cellulomonas flavigena*(96 %)	JF508412	−	−	−	−	−	−	−
Micromonospora	Sd-12	Yellow	–	type IV	*Micromonospora chaiyaphumensis*(100 %)	JF833107	−	−	−	−	+	−	+
	Sd-14	White	–	type IV	*Micromonospora saelicesensis*(96.1 %)	JF508418	−	−	−	−	+	−	−
	Sd-21	Brown	–	type IV	*Micromonospora chokoriensis*(96 %)	JF508422	−	−	+	−	+	−	−
	Sd-24	White	–	type IV	*Micromonospora saelicesensis*(96.5 %)	JF508424	−	−	−	−	+	−	+
Saccharothrix	Sd-22	Gray	–	type III	*Saccharothrix texasensis*(98.6 %)	JF508423	−	−	−	−	−	−	+
Streptosporangium	Sd-54	White	–	type IV	*Streptosporangium vulgare*(99.2 %)	JN641295	−	−	−	−	−	−	+
	Sd-59	Red	–	type IV	*Streptosporangium amethystogenes*(99.1 %)	JN641297	−	−	+	−	−	+	+
	Sd-71	White	–	type IV	*Streptosporangium vulgare*(99.2 %)	JN641296	−	−	−	−	−	−	+

[a] Diffusible pigment of isolate, − no pigment

[b] ++ high inhibition(inhibition zone>15 mm); + inhibition(inhibition zone 6–15 mm); − without inhibition (inhibition zone 0–6 mm)

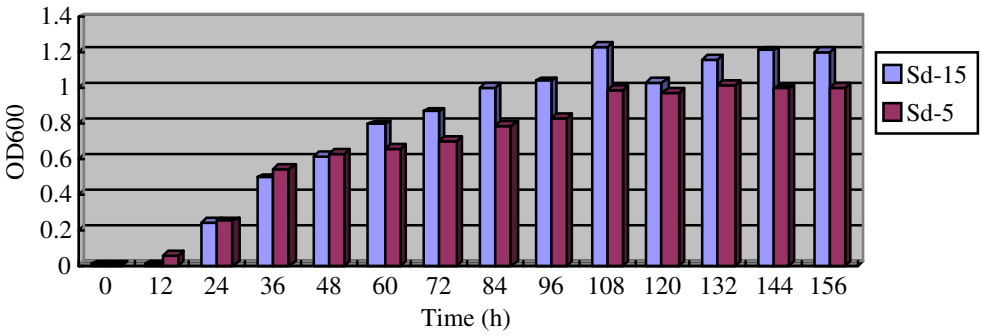

Fig. 3 Growth curves of Sd-5 and Sd-15

Antimicrobial properties of isolates

According to the growth curve of two representative strains (Fig. 3), indicator strains were inoculated after testing actinomycetes that had grown for 5 days. We classified the antimicrobial activity as high inhibition (inhibition zone >15 mm); inhibition(inhibition zone 6–15 mm); without inhibition (inhibition zone 0–6 mm). Among these strains, *Streptomyces* (nine strains) showed better antimicrobial properties, and mainly inhibited Gram-positive strains *S. aureus* and *B. subtilis* (Table 1). In addition, two of the five rare actinomycetes, which belonged to *Micromonospora* and *Streptosporangium*, had anti-Gram-positive microbial properties (Table 1).

Analysis of NRPS, PKS-I and PKS-II

As shown in Table 1, PCR analysis showed that most of the strains have genes encoding NRPS, PKS-I and/or PKS-II except Sd-1, Sd-3, Sd-6, Sd-9 and Sd-13. Sd-5, Sd-11, Sd-12, Sd-15, Sd-24, Sd-28, Sd-31, Sd-37, Sd-51, Sd-56 and Sd-59. From these, we could amplify at least two genes encoding NRPS, PKS-I and/or PKS-II, and most of them belong to *Streptomyces*. From the others, only one gene encoding NRPS, PKS-I or PKS-II could be amplified.

Secondary metabolites analysis

All of the strains showing antimicrobial activity could produce the secondary metabolites (Table 2). Among these strains, Sd-31 was chosen to study its secondary metabolites using HPLC-MS because of its optimum antimicrobial bioactivity. The antimicrobial fractions of Sd-31 were detected by HPLC-MS. As a result, three compounds were detected: peak 1 (retention time=11.183) exhibited the $[M+H]^+$ at 436.3, peak 2 (retention time=14.317) exhibited the $[M+H]^+$ at 445.1, and peak 3 (retention time=16.953) exhibited $[M+H]^+$ at 483.1 (Table 3; Fig. 4). After comparing the molecular weights of the secondary metabolites of *Streptomyces lateritius* (Fleck et al. 1980; Gilpin et al. 1988; Tuntiwachwuttikul et al. 2008), peak 1 might be an unknown compound, peak 2 and 3 might be Granatomycin A and C.

Table 2 The secondary metabolites assay followed the paper-disc agar plate method

Genus	Isolation	Mean concentration[a]	Mean inhibition zone[b]			
			E. coli	*S. aureus*	*B. subtilis*	*A. niger*
Streptomyces	Sd-5	32.6	–	25	15	–
	Sd-8	18.7	–	–	15	–
	Sd-11	17.7	–	40	16	–
	Sd-23	28.1	–	–	17	–
	Sd-29	41.5	–	30	38	–
	Sd-31	14.4	–	70	75	–
	Sd-51	12.8	–	30	40	–
	Sd-55	26.4	–	–	20	–
	Sd-56	27.5	–	–	15	–
Micromonospora	Sd-21	16.9	–	–	13	–
Streptosporangium	Sd-59	22.7	–	–	10	–

[a]The unit of Mean concentration is mg/mL

[b]The unit of Mean inhibition zone is mm

Table 3 Characterization of compounds in extract of Sd-31 by HPLC-MS

Peak no	Retention time (min)	Absorbance (nm)	Potential compound	Negative ions (m/z)	Positive ions (m/z)
1	11.183	306	Unknown	–	$[M+H]^+$: 436.3
2	14.317	306	Granatomycin A	–	$[M+H]^+$: 445.1
3	16.953	306	Granatomycin C	–	$[M+Na]^+$: 483.1

Discussion

16S rRNA-RFLP is a rapid method for actinomycetes classification at the genus level (Cook and Meyers 2003; Steingrube et al. 1997). Combined with morphological characteristics, 16S rRNA-RFLP can efficiently reduce the number of isolates for sequencing. However, restriction endonuclease is the key to RFLP analysis. *Taq* I is an efficient restriction endonuclease for actinomycetes analysis (Jing et al. 2008; Steingrube et al. 1997). In this study, *Taq* I was chosen for RFLP analysis, and all isolates were separated into four groups. However, *Taq*I failed to distinguish *Streptosporangium* from *Micromonospora*. According to 16S rRNA sequencing, five genera were obtained from this environment. *Streptomyces* (17 strains) was the largest group followed by *Micromonospora* (4 strains), *Streptosporangium* (3 strains), *Saccharothrix* (1 strain) and *Cellulomonas* (1 strain). Species diversity is significantly influenced by the ecological environment (Drenovsky et al. 2004; Kowalchuk et al. 2002; Marschner et al. 2001). Here, significant differences about species composition can be found with other desert ecosystems. (Okoro et al. 2009; Takahashi et al. 1996). Based on the 16S rRNA analysis, Sd-3(97.2 %), Sd-5 (96.7 %), Sd-6 (96 %), Sd-13 (97.2 %), Sd-14 (96.1 %) and Sd-56 (96.1 %) showed low similarity to the closest type strain, respectively, and these might be the promising strains. On the other hand, some isolates showed high similarity to the closest type strains, which have also been reported as worthy strains. Since the fungal cell wall is rich in chitin, chitinases have received increased attention due to their potential application in biocontrol of phytopathogenic fungi. Sd-11 showed 100 % similarity to *Streptomyces kurssanovii* which produce chitinase (Gomes et al. 2001; Stoyachenko and Varlamov 1994). In this study, Sd-31 showed 100 % similarity to *Streptomyces lateritius* which has proved to be a promising strain for producing secondary metabolites (Fleck et al. 1980; Gilpin et al. 1988; Tuntiwachwuttikul et al. 2008), and we studied its bioactive fraction by LC-MS. In order to obtain a good chromatogram, water–formic acid (100:0.1, v/v) (A) and acetonitrile (B) were found to be the optimal mobile phase in HPLC and MS analysis. As a result, Sd-31 might produce Granatomycin A and Granatomycin C which have proved to be the promising compounds (Fleck et al. 1980), and the unknown compound is worth further study.

Rare actinomycetes from different environments can represent a unique source of biotechnology; however, a low recovery rate has limited the developing progress (Baltz 2006; Bredholdt et al. 2007; Takahashi et al. 1996). Designing a medium fitted for a certain environment is a way to exploit more rare actinomycetes (Hozzein et al. 2008; Seong et al. 2001). In this study, about one-third of the strains were rare actinomycetes, and this result showed that Gause′s medium No.1 might be one medium fitted for recovering worthwhile strains from this environment. Additionally, prescreening NRPS, PKS-I and PKS-II genes is a simple way to evaluate the potential of active polyketide and peptide compounds (Hopwood 1997), and most strains had the potential ability to produce active compounds in this study.

In conclusion, the results suggest that the desert ecosystem in the northeast of the Qinghai-Tibetan plateau is a source of bioactive actinomycetes. It indicates that the culturable actinomycetes in this environment are an interesting and promising topic for further study.

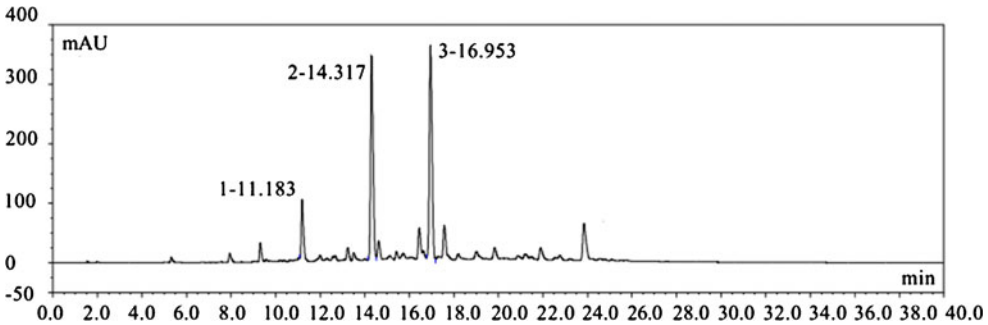

Fig. 4 HPLC chromatograms of extracts of Sd-31

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