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Research Article

STREPTOMYCES SPECIES FROM RED SEA HABITAT: ISOLATION, CHARACTERIZATION AND SCREENING FOR ANTIBACTERIAL COMPOUNDS

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ABSTRACT

In spite of the large number of antibacterial compounds that have been discovered till now, most pathogenic bacteria have become resistant to these compounds. Therefore, searching for new antibacterial compounds is of increasing urgency. The aims of this study were to isolate *Streptomyces* species from Red Sea sediments as well as to screen the isolate(s) for antibacterial compounds. Soil samples were collected from in-depth sediments in the Red Sea in the neighborhood of Port-Sudan. The samples were cultivated on Starch Casein Agar. Adoption of Overlay Method after overnight incubation, resultant in isolation of many promising strains. The strains have activities against both Gram-positive and Gram-negative bacteria. Molecular assay was carried out to confirm the identity of the isolated strains using 16s rDNA gene sequence. The sequence similarities of the isolates were found to be varying from 92% to 99% of the *Streptomyces* deposited in the NCBI GeneBank. This study provides recent information about bioactivity of the Red Sea *Streptomyces* and proved that this unexplored habitat may be a promising source for antibacterial compounds.

Keywords: Screening, *Streptomyces*, Antibacterial compounds, Red Sea, Sudan.

INTRODUCTION

Since the discovery of penicillin in 1928, antibiotics have been used effectively to control emerging pathogens, resulting in progress affecting against these pathogens and preventing life threating (Omulu*et al*., 2015).The search for new antibacterial compounds is become a global issue due to problem of bacterial resistance to existing antibacterial agents(Martins *et al*., 2015).This resistance has become a major health concern. Many opportunistic pathogens were becoming resistant to virtually every available antibacterial agent. The emergence of multi resistant pathogenic bacterial strains has caused a therapeutic problem of enormous proportions. In response, there is interest in discovering novel classes of antimicrobial agents that have different mechanisms of action (Greenberg, 2003).Natural products have been regarded as important sources of their compounds. Seas and marineswere considered as major niches of microorganisms promising for natural products (Gulve&Deshmukh, 2012; Kiruthika *et al*., 2013).Thus, the interest of investigators was directed towards such habitats as unusual sources to be explored for the development of new drugs. Significant part of this attention has been paid to marine microorganisms, which have become important in the study of novel compounds exhibiting antibacterial activities(Kokare *et al*., 2004).Recently, true indigenous marine *Streptomyces* have been described;hence they drew special attention as a

promising source of novel and unique metabolites (Maldonado *et al*., 2005 & Moore *et al*., 2005).

MATERIALS AND METHODS

Location and collection of samples

The Sudanese Red Sea coast (about 750 km) is located at the central part of the Red Sea. The area is to be found on the western side of the central Red Sea, at Lat. 19º38 N and Long. 37º13 E. It is particularly famous of its exceptionally unique and varied habitats with rich biological communities, especially coral reefs, its productive and highly sensitive coastal and marine habitats (coastal halophytes, mangroves, sea grasses / algal beds and coral reefs)(Rasul&stewart, 2015)Figure 1.Marine sediment samples were collected from Port Sudan (Falamingo area) coast, Sudan (Lat.19° 39N, Long. 37° 14E) Figure 2.Fifty (n=50) samples were collected from different depths. The depths of collection werevarying from 0.5 to 20 meterTable 1.Sterile containers were used for collection. All samples were air dried for preventing contaminations and ensuring a mass of spores, then store at 4°C until used.

Culture media

Starch casein agar (SCA): Casein powder, 0.3 g; Starch, 10.0 g; KNO3 2; NaCl 2; K2HPO4 2; MgSO4 7 H2O 0.05; CaCO3 0.02; FeSO4 7H2O 0.01; Agar, 18.0 g; Distilled water, 1000 ml; pH, 7.2. International Streptomyces Project No.7 (ISP No.7): L-Asparagine, 1.0 g; L-Tyrosine, 0.5 g; Dipotassium phosphate, 0.5 g; Magnesium sulphate. 7H2O, 0.5 g; Sodium chloride, 0.5 g; Trace salt solution (ml), 1.0 g; Agar, 20.0 g; Ferrous sulphate, 7H2O, 1.369 mg; Copper chloride, 2H2O, 0.027 mg; Cobalt chloride, 6H2O, 0.040 mg; Sodium molybdate, 2H2O, 0.025 mg; Zinc chloride, 0.020 mg; Boric acid, 2.850 mg; Manganese chloride, 4H2O, 1.800 mg; Sodium tartarate, 1.770 mg; Distilled water, 1000 ml; pH, 7.3. Mueller Hinton Agar (MH): Beef, infusion from, 300.0 g; Casein acid hydrolysate, 17.5 g; Starch, 1.5 g; Agar, 17.0 g; Distilled water, 1000 ml; pH, 7.3. Nutrient Agra (NA): Peptic digest of animal tissue, 5.0 g; Sodium chloride, 5.0 g; Beef extract, 1.5 g; Yeast extract, 1.5 g; Agar, 15.0 g; Distilled water, 1000 ml; pH, 7.4. Soyabean Casein Digest Medium (Tryptone Soya Broth) (SCDM): Pancreatic digest of casein, 17.0 g; Papaic digest of soyabean meal, 3.0 g; Sodium chloride, 5.0 g; Dextrose(Glucose), 2.5 g; Dipotassium hydrogen phosphate, 2.5 g; distilled water, 1000 ml; pH, 7.3. All media was obtained from HiMedia Laboratories Pvt. Limited, Mumbai, India.

Test microorganisms

The bacterial strains *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 used for screening of antibacterial activity.

Isolation of *Streptomyces*

One gram of soil was suspended in 4 ml of sterile seawater. The suspension was agitated vigorously, filtered and heated for 6 min at 55°C to reduce non-spore forming bacteria. The contents were diluted (1:4) in sterile seawater. Aliquots of 1 ml of the samples were spread onto the isolation media (SCA and ISP No.7 media were prepared and sterilized at 121 °C in 15 lbs pressure for 15 min)(Mincer *et al*., 2005; Jensen*et al*., 2005)Rifampicin 5µg/ml and Nystatin 25µg/ml were added to prevent bacterial and fungal growth respectively. Plates were incubated at 30°C for 7 days (Terli, 2011).
Screeningof the obtained coloniesfor antibacterial activities was done byAgar Overlay Technique. Five ml of molten nutrient agar (temp. ~ 43°C) with indicator bacteria that prepared according to 0.5 McFarland Standard was poured over the colonies that isolated. Zones of inhibition around the colonies were recorded after incubation at 37°C for 24 hrs.(Srinu*et al*., 2013).Only promising isolates with good inhibition zones were considered. Colonies of the promising isolates were picked up and sub-cultured on CSA and incubated at room temperature for 3 to 7days. The resultant growth was stored in refrigerator at 4°C for further investigations.

DNA extraction

Isolates were grown in Tryptone soya broth prepared with 75% seawater and 25% Distilled water. Genomic DNA was extracted using Jena Bioscience bacterial DNA isolation and purification kit (Jena Bioscience Laboratories, GmbH, Germany) according to the manufacturer instructions.

PCR primer

The primers for detection of isolated *Streptomyces* genes obtained from (Macrogene Inc., Korea) are shown in Table 2.

PCR amplification

The amplification was done using CONVERGYS® Ltd peltier thermal cycle (Germany). DNA amplifies of 16S rDNA gene was done using Maxime PCR PreMix kit (iNtRON, Korea).
DNA extract from the promising *Streptomyces* was amplified using two pairs of primers specific for the genus *Streptomyces*. All of the strains formed an amplification product of 519

bp with the primers StrepB/ StrepE, and of 1074 bp with the pair StrepB/StrepF.
The primer pairs StrepB/StrepF and StrepB/StrepE amplified 1070 and 520 bp fragments, nucleotides 139 -1212. The Multiplex PCR were programmed as follows: the initial denaturation for 5 min at 98°C, 30 cycles of denaturation (1 min at 95°C), primer annealing 40 s at 57°C and primer extension (2 min at 72°C) were performed. A final extension at 72°C for 10 min followed.

Gel electrophoresis
The purity of the extracted DNA was determined by running the DNA sample on 1% agarose gel(Sambrook*et al*., 1989).

Sequencing and phylogenetic analysis
The PCR products obtained were sent to Macrogene (Korea) for sequence determination. The sequenced 16S rRNA gene of the strains was aligned with the nucleotide sequences of *Streptomyces* genera in GenBank database using BLAST (Thenmozhi *et al*., 2010). A phylogenetic tree was generated using neighbor-joining method on NCBI.

Agar well diffusion method
Antimicrobial activity was determined using agar well diffusion method (Augustine *et al*., 2014), with the test pathogenic bacteria *S.aureus* and *E. coli*. *Streptomyces*isolates were cultured in CSDM prepared with 75% seawater. The cultures were incubated on a rotary shaker for 7 days at 30 °C. Test microorganism suspension, with a turbidity equivalent to that of 0.5 McFarland standards, were seeded on MH agar plates. Wells were made in these plates using alcohol sterilized cork-borer (7mm diameter), and filled with 50 µl of cell free filtrate. The plates were then incubated at 37 °C for 24 hours, and the inhibition zones around the wells were measured (mm).

Perpendicular streak method
Antimicrobial activity was done by perpendicular streak method on nutrient agar plates. The test organism for primary screening used *S. aureus*&*E. coli*.Each of the isolates was streaked as a straight line on nutrient agar medium and incubated at 30 °C for 7 days. After the 7th day, test pathogenic bacteria were streaked at right angle, but not touching each other, and then incubated at 30-37 °C for 24 hrs. If the organism is susceptible to the antibiotic compound produced by the *Streptomyces*, then it will not grow near the *Streptomyces*(Rana& Salam, 2014).

RESULTS
Among fifty isolates obtained from marine soil sediment an agar overlay technique revealed that,21 (42%)shoed antibacterial activities against both Gram-positive and Gram-negative bacteria.
Cultural characteristics including aerial mycelium growth & color of aerial mycelium of the identified *Streptomyces* were tabulated in Table 3.
Polymerase chain reaction (PCR) was done to determine the identities of the 21 promising isolates revealed that nine (n=9) (18%) were identified as*Streptomyces* species (PS 1, PS 5, PS 10, PS 13, PS 20, PS 21, PS 23, PS 24, and PS 28) Figure3.
The antibacterial activity of the nine *Streptomyces* were revealed that the strains PS 1 & PS 28 have the highly activity against Gram-positive,butlow activity against Gram-negative test organisms (*S. aureus*&*E. coli*)Table 4.
The 16S rRNA gene sequences of the nine strains were compared with the nucleotide sequences of other *Streptomyces* strains from the NCBI GenBank database (blstn). All the identifiedwere related to the genus *Streptomyces*.The percentage of similarity varies between 92% and 99% Table 5. The phylogenetic tree generated on the basis of 16S rRNA gene sequence of the strains PS 1 and PS 28 and the nucleotide sequences from closely related *Streptomyces* strains using neighbor-joining method is presented in Figure 4&5.

DISCUSSION
Antibiotics are the most important bioactive compounds for the treatment of infectious diseases. Emergence of multi-drug resistant pathogens, pose a great challenges for searching effective new antibacterial agent to treat of infectious diseases. *Streptomyces* have been recognized as producers of secondary metabolites such as antibiotics(Attimarad *et al*., 2012).Therefore, detection of new species from unexplored areas such as Port Sudan is one of the more efficient approaches for the development of new antibacterial compounds.The objectives of this study were to isolate antibacterial producing *Streptomyces* speciesfrom marine environment in Sudan as well as to evaluate the potency of the product. Currently the isolation and characterization of *Streptomyces* from virgin habitats given the chance for screening such organisms and increases the discovery of new natural products that can be developed as a resource for antibacterial compounds (Bredholt *et al*., 2008; Eccleston *et al*., 2008).

In this studythe isolated *Streptomyces* species from marine soil sedimentssamples was (9)18%. This result is less than that reported by Kokare and his colleagues (Kokare *et al*., 2004)who isolated43%, but more than reported by (Al-Hulu, 2013)as only 9%. Theidentified*Streptomyces*nine (n=9) have an activities against both Gram-positive and Gram-negative pathogenic bacteria. Similar result (17%) was obtained by (Mohan *et al*., 2013). Moreover(Bizuye *et al*., 2013) showed that 26% of the isolates exhibited activity against pathogenic bacteria which is more that reported in the present study. Of the broad spectrum *Streptomyces* isolates, only PS 1 & PS 28 showed activities against *S. aureus*and *E. coli*. These results are similar to that obtained by (Reddy *et al.,* 2011).

The differences in sensitivity between Gram-positive and Gram-negative bacteria may be attributed to morphological differences between these microorganisms; Gram-negative bacteria have an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic compounds; while the Gram-positive bacteriais more susceptible as they have only an outer peptidoglycan layer which is not affect permeability of cell wall (Singh *et al*., 2014).

CONCLUSION

Based on the results, the marine soil sediment collected from the Red Sea, Sudan teems with antibiotic-producing*Streptomyces* and may be one of the richestsources of antibacterial compounds. Further investigations are required to explore other bioactive compounds in the Red Sea coast in Sudan.

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Competing interests

The authors declare that they have no competing of interests.

Fig. 1: Showed the coast of Port Sudan

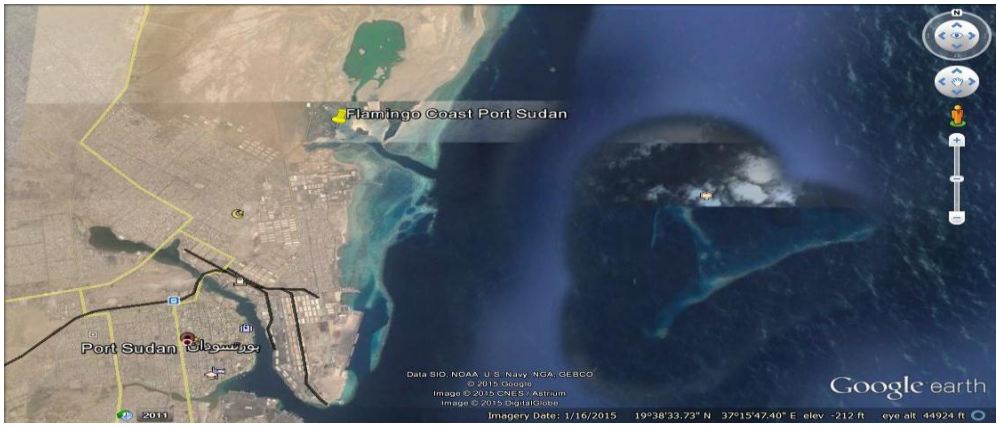

Fig. 2: Site of samples collection (Falamingo area) coast, Port Sudan

Table 1: Sample codes and its depths

No.	Sample Code	Depth in Meters (M)
1.	PS 1	6
2.	PS 2	7
3.	PS 3	8
4.	PS 4	2
5.	PS 5	7
6.	PS 6	9
7.	PS 7	6
8.	PS 8	5
9.	PS 9	4
10.	PS 10	5
11.	PS 11	9
12.	PS 12	4
13.	PS 13	10
14.	PS 14	12
15.	PS 15	11
16.	PS 16	10
17.	PS 17	11
18.	PS 18	15
19.	PS 19	15
20.	PS 20	5
21.	PS 21	8
22.	PS 22	8
23.	PS 23	0.5
24.	PS 24	2
25.	PS 25	2
26.	PS 26	1
27.	PS 27	1
28.	PS 28	4
29.	PS 29	1.5
30.	PS 30	0.5
31.	PS 31	1.5
32.	PS 32	2
33.	PS 33	3
34.	PS 34	3
35.	PS 35	4
36.	PS 36	5
37.	PS 37	5
38.	PS 38	2
39.	PS 39	1
40.	PS 40	1
41.	PS 41	18
42.	PS 42	18
43.	PS 43	20
44.	PS 44	20
45.	Ps 45	20
46.	PS 46	8
47.	PS 47	8
48.	PS 48	12
49.	PS 49	12
50.	PS 50	5

Table 2: Primers sequences used for detection of 16s rDNA gene from *Streptomyces* isolates

Target Gene	Primer Name	Sequence $5' \rightarrow 3'$	Position	Reference
16s rDNA	StrepB (F)	ACAAGCCCTGGAAACGGGGT	139–158	(**Malinovaa*et al*., 2014**)
16s rDNA	StrepE (R)	CACCAGGAATTCCGATCT	657–640	
16s rDNA	StrepF (R)	ACGTGTGCAGCCCAAGACA	1212–1194	

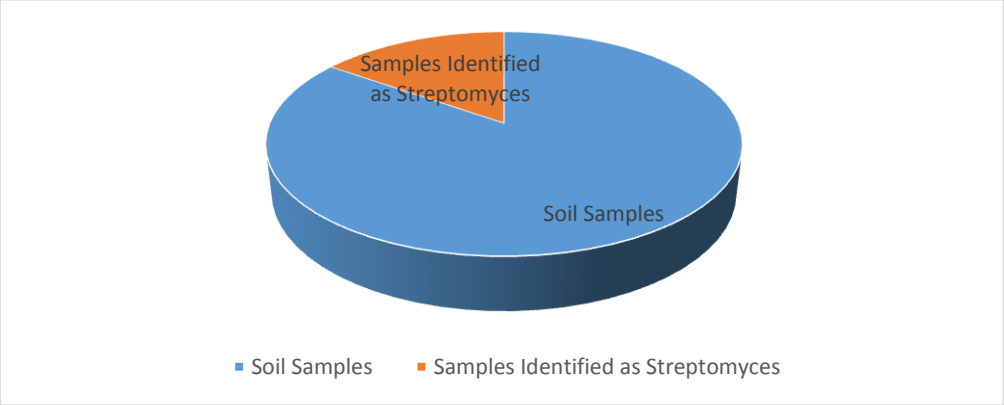

Fig. 3: The percentage of samples identified as genus *Streptomyces*

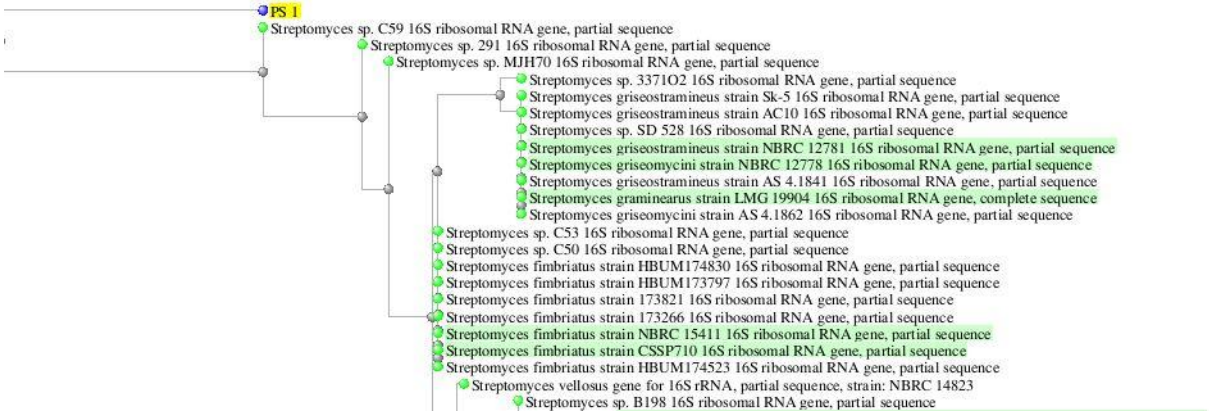

Fig. 4: Phylogenetic tree based on 16S rRNA gene showing the position of the isolate PS1

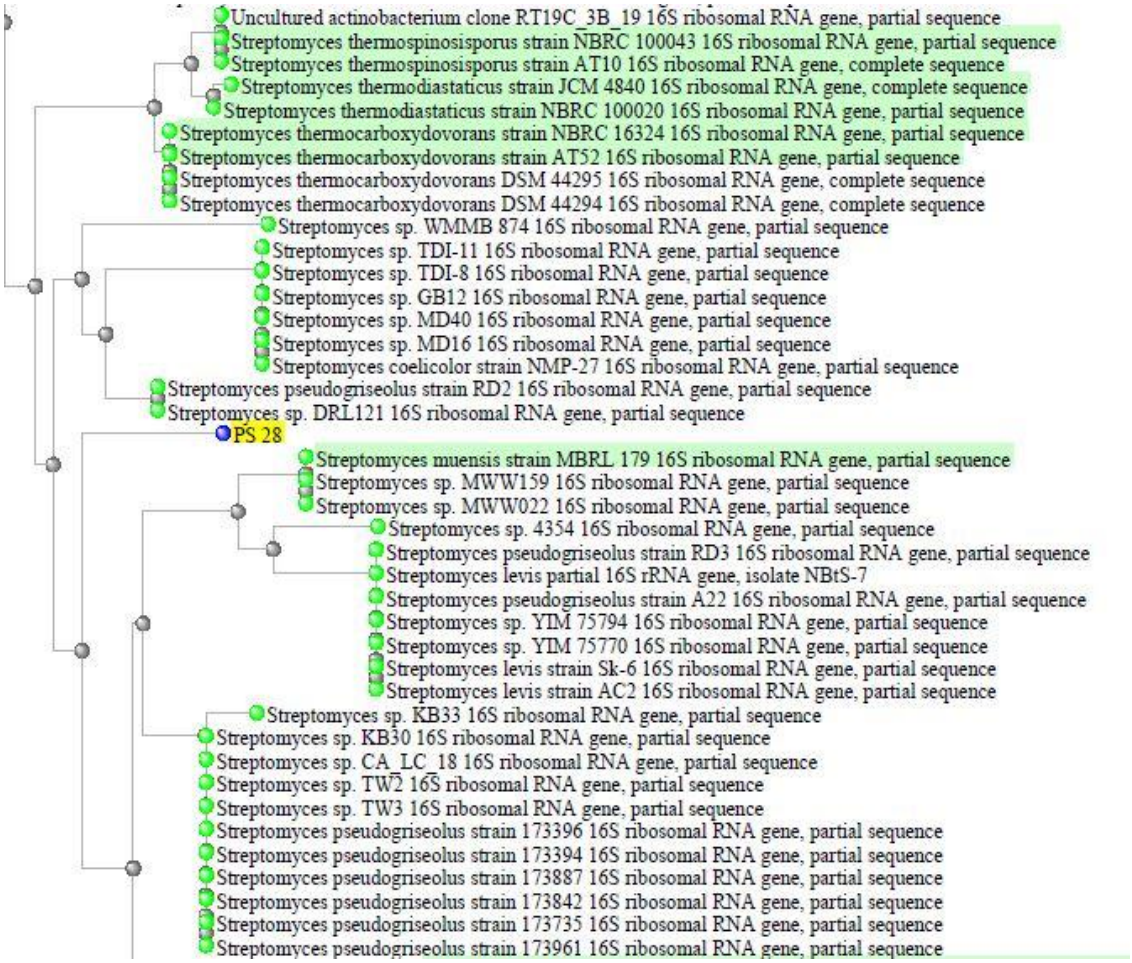

Fig. 5: Phylogenetic tree based on 16S rRNA gene showing the position of the isolate PS28

Table 3: Culture characteristics of the *Streptomyces* species

Isolate Code	Medium	Growth	Aerial Mycelium Color
PS 1	ISP No.7	Well	Dark Grey
	CSA	Well	Yellow
PS 5	ISP No.7	Well	Green
	CSA	Well	Creamy
PS 10	ISP No.7	Well	Grey
	CSA	Well	White
PS 13	ISP No.7	Well	White
	CSA	Well	Yellow
PS 20	ISP No.7	Well	Creamy
	CSA	Well	White
PS 21	ISP No.7	Well	White
	CSA	Well	White
PS 23	ISP No.7	Well	Brown
	CSA	Well	Grey
PS 24	ISP No.7	Well	Yellow
	CSA	Well	Yellow
PS 28	ISP No.7	Well	Yellow
	CSA	Well	Yellow

Table 4: The isolates activity against pathogenic bacteria

Isolate Code	Inhibition zone (mm) against	
	***S. aureus* ATCC 25923**	***E. coli* ATCC 25922**
PS 1	18	14
PS 5	13	6
PS10	10	-
PS 13	12	-
PS 20	11	7
PS 21	14	9
PS 23	14	-
PS 24	15	8
PS 28	20	14

Table 5: The five closest relativity of isolates to the genus *Streptomyces* by BLAST

Isolate Code	Closest relative	% Identity	Accession
PS 1	*Streptomyces afghaniensis*	95%	[GenBank:HG941978.1]
	Streptomyces fimbriatus	95%	[GenBank:EU841630.1]
	Streptomyces thermocarboxydovorans	95%	[GenBank:NR_112588.1]
	Streptomyces sp. C59 partial 16S rRNA gene, isolate SG 38	97%	[GenBank:EU551707.1]
	Streptomyces sp. 291 16S ribosomal RNA gene, partial sequence	97%	[GenBank:FJ754303.1]
PS 5	*Streptomyces griseostramineus*	99%	[GenBank:JQ955745.1]
	Streptomyces sp. SD 528 16S ribosomal RNA gene, partial sequence	99%	[GenBank:JN585736.1]
	Streptomyces vellosus	99%	[GenBank:AB184623.1]
	Streptomyces fimbriatus	98%	[GenBank:FJ486366.1]
	Streptomyces pseudogriseolus	98%	[GenBank:KT588652.1]
PS 10	*Streptomyces* sp. 4354 16S ribosomal RNA gene, partial sequence	98%	[GenBank:KC111835.1]
	Streptomyces afghaniensis	98%	[GenBank:HG941938.1]
	Streptomyces thermocarboxydovorans	98%	[GenBank:NR_112588.1]
	Streptomyces viridochromogenes	98%	[GenBank:AB184514.1]
	Streptomyces muensis	98%	[GenBank:JN560155.1]
PS 13	*Streptomyces capillispiralis*	99%	[GenBank:NR_041158.1]
	Streptomyces sp. SCAU5034 16S ribosomal RNA gene, partial sequence	99%	[GenBank:KR348890.1]
	Streptomyces gancidicus	99%	[GenBank:KP797911.1]
	Streptomyces sp. SXY37 16S ribosomal RNA gene, partial sequence	99%	[GenBank:GU045528.1]
	Streptomyces werraensis	99%	[GenBank:KT021807.1]
PS 20	*Streptomyces* sp. TDI-10 16S ribosomal RNA gene, partial sequence	99%	[GenBank:KT021816.1]
	Streptomyces rubiginosus	99%	[GenBank:LC034307.1]
	Streptomyces stramineus	99%	[GenBank:AY999907.1]
	Streptomyces caelestis	99%	[GenBank:KM081627.1]
	Streptomyces sp. 3482 16S ribosomal RNA gene, partial sequence	99%	[GenBank:DQ663172.1]
PS 21	*Streptomyces coelicolor*	95%	[GenBank:JN798179.1]
	Streptomyces caelestis	95%	[GenBank:KM081627.1]
	Streptomyces sp. MD16 16S ribosomal RNA gene, partial sequence	95%	[GenBank:JN896608.1]
	Streptomyces werraensis	95%	[GenBank:KT021807.1]
	Streptomyces sp. MJN18 16S ribosomal RNA gene, partial sequence	95%	[GenBank:HM026269.1]
PS 23	*Streptomyces* sp. 13658E 16S ribosomal RNA gene, partial sequence	92%	[GenBank:EU741184.1]
	Streptomyces fimbriatus	92%	[GenBank:EU841630.1]
	Streptomyces pseudogriseolus	92%	[GenBank:KT588652.1]
	Streptomyces thermocarboxydovorans strain NBRC 16324 16S ribosomal RNA gene, partial sequence	92%	[GenBank:NR_112588.1]
	Streptomyces sp. 4354 16S ribosomal RNA gene, partial sequence	92%	[GenBank:KC111835.1]
PS 24	*Streptomyces* sp. 1A14 16S ribosomal RNA gene, partial sequence	96%	[GenBank:KT153618.1]
	Streptomyces radiopugnans	96%	[GenBank:KC570323.1]
	Streptomyces nanhaiensis	96%	[GenBank:KJ947850.1]
	Streptomyces fenghuangensis	96%	[GenBank:KJ947857.1]
	Streptomyces sp. HA12301 16S ribosomal RNA gene, partial sequence	96%	[GenBank:KJ419956.1]
PS 28	*Streptomyces muensis*strain MBRL 179 16S ribosomal RNA gene, partial sequence	98%	[GenBank:JN560155.1]
	Streptomyces afghaniensis	99%	[GenBank:HG941938.1]
	Streptomyces sp. DRL121 16S ribosomal RNA gene, partial sequence	98%	[GenBank:FJ853201.1]
	Streptomyces fimbriatus	99%	[GenBank:EU841630.1]
	Streptomyces sp. MWW159 16S ribosomal RNA gene, partial sequence	98%	[GenBank:HM588208.1]

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