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Antimicrobial activity and molecular identification of *Streptomyces* strains isolated from Saudi Arabia

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Abstract

Actinomycetes are group of Gram-positive bacteria with high GC-content in their DNA. They are extremely useful for the pharmaceutical industry due to their seemingly unlimited capacity to produce secondary metabolites with diverse biological activities and chemical structure. The genus *Streptomyces* constitutes 50% of the total population of soil actinomycetes and about 75% of commercially and medicinally useful antibiotics that have been derived from this genus. The present study aimed in isolation of bioactive compound showing antimicrobial activities from soil *Streptomyces,* previously isolated and morphologically characterized from Jazan in Saudi Arabia. Six potent *Streptomyces* strains: JS3, JS4, JS6, JD7, JA8 and JA10 were chosen for antimicrobial activity screening against 5 human pathogenic bacteria and 5 phytopathogenic fungi before molecular identification was done. For antibacterial activity, the results showed that inhibition zones were found to range between 3.25-26.875 mm diameters, while for antifungal activity, it ranged between 13.3-40 mm diameters. The entire sequence of the 16S rDNA was determined for the strains JS6, JD7, JA8 and JA10 and deposited in the GenBank. Future studies of actinomycetes isolated from the Kingdom of Saudi Arabia's soils will assist in the discovery of new compounds that would be of industrial, pharmaceutical and agricultural importance.

Key words

Antibacterial activity, Antifungal activity, 16S rDNA sequence analysis, *Streptomyces*

Introduction

In the past few decades, repeated isolation of known natural products have limited the development of new and effective drugs for treating ever increasing human diseases (Debbab *et al.*, 2010). On the other hand, the arising antibiotic-resistant pathogens present an urgent requirement for new bioactive compounds discovery. Hence, more and more researchers have switched over to new or extreme environments for novel pharmaceutical compounds such as deep oceans (Abdel-Mageed *et al.*, 2010; Thornburg *et al.*, 2010), deserts (Bull and Asenjo, 2013), polar areas (Liu *et al.*, 2013) and mangroves (Amrita *et al.*, 2012). Microbial natural products are an important source and play a significant role in discovery and understanding of cellular pathways that are essential in the drug discovery process (Vincent *et al.*, 2006). The discovery of novel bioactive compounds through microbial secondary metabolite screening is becoming more important.

Gram positive bacteria today pose a challenge in anti-infection therapeutics, causing great concerns among medical doctors, microbiologists and the scientific community. This is due to resistance that microorganisms present against antimicrobial drugs used in clinical treatment protocols. Moreover, the paucity of new antibiotics introduced in markets makes it urgent to conceive measures to prevent health crisis at global level (Antunes *et al.*, 2014). Actinomycetes are diverse group of Gram positive bacteria that usually grow by filament formation. They belong to the

order Actinomycetales. They have high G+C (>55%) content in their DNA. Numerous researches have indicated that actinomycetes secondary metabolites have potential as new antibiotics, antitumor agents, immunosuppressive agents and enzyme inhibitors (Dharmaraj, 2010). Up till now, about 23,000 bioactive compounds produced by microorganisms have been reported and more than 10,000 among these compounds were isolated from actinomycetes (Berdy, 2005). It is worth mentioning that of 10,000 compounds, about 80% have been obtained from *Streptomyces* which is the most productive genus in the microbial world (Xu *et al.*, 2014).

Need of new antimicrobial agents is greater than ever because of emergence of multidrug resistance in common pathogens (Spellberg *et al.*, 2004). Resistance of bacteria to the effect of antibiotics has been a major problem in the treatment of diseases (Baltz, 2007). Choice of natural materials like soil in research is based on the assumption that samples from widely diverse locations are more likely to yield novel microorganisms and hopefully, novel metabolites as a result of the geographical variation (Gurung *et al.*, 2009). In this regard, Jazan region in Saudi Arabia is of significant interest. As not much studies have been carried out novel microorganisms. Keeping these points in view, the present study was undertaken to characterize antimicrobial effects and molecular identification of previously isolated *Streptomyces* strains from soil samples of this area.

Materials and Methods

Isolation of *Streptomyces* strains from soil : Soil samples were collected from 5 areas of Jazan (Sabya, Baish, Samtah, Alddarb and Abuarish) in Saudi Arabia and a total of 270 actinomycetes were isolated by modified standard dilution technique on water agar media. Identification and characterization were performed using cultural, physiological, biochemical and morphological analysis (Mujamammi *et al.*, 2014). Six strains of *Streptomyces* were chosen for this study based on their colour: JS3, JS4, JS6, JD7, JA8 and JA10.

Extraction of antimicrobial metabolites : The selective strains JS3, JS4, JS6, JD7, JA8 and JA10 chosen for producing antimicrobial compound were each inoculated into flasks containing starch glucose yeast (SGY) broth (10 g starch soluble,10 g glucose,10 g glycerol, 2.5 g corn flower, 5 g peptone, 2 g yeast extract, 3 g $CaCO_3$ and 1l distilled water) as described previously by Ara *et al.* (2012). The flasks were incubated at 37°C in a rotary shaker (200-250 rpm) for seven days. Antimicrobial compounds were recovered from the broth by solvent extraction method. Methanol was added to the flasks containing mixture of strains and broth in the ratio of 1:1 (v/v). The flasks were then returned to the shaker for 24 hrs and the contents were filtered to separate the mycelium from the liquid through filter papers such as blotting paper, Whatman No.1, and then through membrane filter to get cell free extract. From the liquid part, methanol was evaporated to dryness in hot air oven (40°C) and two drops of distilled water was added to the residue obtained. The crude extract obtained was used to determine the antimicrobial activity.

Screening of antibacterial activity of *Streptomyces* : The crude extract obtained from JS3, JS4, JS6, JD7, JA8 and JA10 was tested for its antibacterial activity by agar well diffusion method assay over Muller-Hinton agar (MHA) as a medium in triplicate (Shomurat *et al.*, 1979) against test human pathogenic gram-positive bacteria: *Bacillus subtilis* ATCC6633 and *Staphylococcus aureus* ATCC29213 and Gram-negative bacteria: *Pseudomonas aeruginosa ATCC27853*, *Escherichia coli* ATCC25966 and *Shigella sonnei* (clinical isolate). Subsequently, wells of 6 mm diameter were made over the cultures of bacteria and then 50 µl of crude extract from the isolates were added in the wells. The solution of 20 % ethanol was used as control. Discs containing antibiotic rifampicin (5 µg $disc^{-1}$) were used as positive control. The plates were incubated at 37°C. After 24-48 hrs of incubation, the diameter of the zone of inhibition was measured to evaluate the antibacterial activity of *Streptomyces* isolates.

Screening of antifungal activity of actinomycetes: Antifungal activity of JS3, JS4, JS6, JD7, JA8 and JA10 was evaluated against 5 phytopathogenic fungi, isolated from soil of Cairo (Egypt): *Fusarium moniliforme*, *Helminthosporium oryzae*, *Aspergilllus flavus*, *Aspergilllus japonicas* and *Fusarium verticillioides* by agar well diffusion method assay over Potato dextrose agar (PDA) as a medium (Shomurat *et al.*, 1979). Standard antifungal discs containing 50 µg of cycloheximide/ml were used as positive control, while methanol was used as negative control. After 7 days of incubation, the diameter of zone of inhibition was measured to evaluate the antimicrobial activity of *Streptomyces* isolates.

Molecular analysis:

DNA extraction: For DNA extraction, six *Streptomyces* strains were grown as mycelium in liquid culture of SGY broth from an inoculum of spores. Actinomycetes are highly aerobic and therefore, require shaking during incubation in vessels that allow good aeration at 30°C, 200 rpm for 7 days. After that, DNA was extracted using DNeasy Blood and Tissue Kit, Qiagen.

16S rRNA gene sequence analysis : The genomic DNA of JS3, JS4, JS6, JD7, JA8 and JA10 was used as template for PCR. The reaction was done using universal primers 24f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-

TACGGTTACCTTGTTACGACTT- 3') which amplify the totality of 16S rRNA gene (about 1500 bp). A further PCR was done for the same gene (16S rRNA gene), but with actinospecific primers: ACT235f (5'-CGCGGCCTATCAGCTTGTTG -3') and ACT878r (5'-CCGTACTCCCCAGGCGGGG -3'), which amplified an internal fragment of about 640 bp (Stach , 2003), in order to get the entire sequence of the gene 16S rRNA, which is relatively large. The PCR Reagent kit (Hot Star Taq Master Mix, Qiagen) was used to amplify the region of interest in all DNA samples. The reactions were performed in a final volume of 25µl. The DNA underwent a polymerization reaction in the chain in a Veriti 96 Well Thermal Cycler (Applied Biosystems®) using the program: initial denaturation at 95°C for 15 min and 35 cycles at 94°C for 1min; annealing at 63°C for 1 min, and primer extension at 72°C for 2 min followed by a final extension at 72°C for 10 min. With respect to ACT235f/ ACT878r, the program was same, except for annealing temperature which was 67°C for 1min and the extension was at 72°C for 1min.

Nucleotide sequencing: The 16S rDNA gene sequences were obtained using "Thermo sequenase big dye terminator cycle sequencing kit" (Applied biosystems ABI). The sequencing products were analyzed in an automated sequencer with ABI genetic analyzer model 3130xl (Applied Biosystems®). Phylogenetic analyses were performed by MEGA (version 5.2.2) software. The Neighbor-joining method (Saitou and Nei, 1987) was used for construction of phylogenetic trees and (Kimura, 1980) to calculate nucleotide distances. Reproducibility of tree was estimated with a bootstrapping 1000 times, only the values superior to 70% were considered. The nucleotide sequences were submitted to GenBank, under accession numbers KF725839–KF725842.

Statistical analysis : Experiments were done in triplicate. LSD ALPHA (0.05) was used for statistical analysis using one-way analysis of variance. $P<0.05$ was considered statistically significant. ANOVA was done with IBM SPSS Statistics 21 software.

Results and Discussion

Streptomyces flora isolated from soil samples, collected from Jazan in Saudi Arabia, were screened for their potential as a source of antibiotics active against pathogenic bacteria. The formation of inhibition zone around the pathogenic strains was indicative of production of secondary metabolites by the *Streptomyces* isolates. Strains JS3, JS4, JS6, JD7, JA8 and JA10 were tested for their antibacterial activity against *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli* and *Shigella sonnei*. Six strains showed antibacterial activity against all the five pathogenic bacteria with inhibition zones ranging between 3.25-26.875 mm in diameter (Table 1). Gram positive bacteria, *Staphylococcus aureus* was highly inhibited by almost all the six isolates. Isolates JS4, JS6, JD7 and JA8 showed strong activity against the tested bacteria *B. subtilis*, while the other two strains JS3 and JA10 appeared to have moderate effect on the same pathogenic bacterial strain. Isolates JA8, JD7 and JA10 inhibited *E. coli* growth moderately, JS3, JS4 and JS6 were most active against this bacteria strain. On the other hand, JS4, JS6, JD7 and JA10 strains had moderate effect against *P. aeruginosa*, but JS3 and JA8 exhibited higher antimicrobial activity. *Streptomyces* strains JS3, JD7, JA8 and JA10 exhibited moderate activity against *S. sonnei*, whereas JS4 and JS6 were highly active (Table 1). The obtained results are in confimation with the previous studies reported (Tamilarasi *et al.*, 2008 and Zaki et al., 2013) that high frequency of antibacterial activities among the *Streptomyces* species

Table 1 : Inhibition zone showing the antagonistic abilities of the bioproducts derived from the six strains of *Streptomyces* chosen for the study (diameter inhibition zone mm)

Isolate	Pathogenic bacteria				
	Gram positive bacteria		Gram negative bacteria		
	S. aureus	*B. subtilis*	*E. coli*	*P. aeruginosa*	*S. sonnei*
Control	20	20	15	15	15
Ethanol	1.5	0	0	0.5	0
JS3	19.75	14.5	17.625	17	11.625
JS4	18.875	18.875	21.5	12.5	15.375
JS6	19.75	22	19.625	14.625	19
JD7	12.25	15.125	12.125	10.125	13.25
JA8	26.875	26	4	21.25	10.75
JA10	15.375	14	3.25	13	10.875
LSD at 5%	14.89	15.77	9.808	15.33	16.18
LSD at 1%	10.87	11.51	7.16	11.19	11.818

Table 2 : Inhibition zone showing the antifungal activities of the bioproducts derived from the six strains of *Streptomyces* chosen for the study

Isolate	Phytopathogenic fungi				
	H. oryae	***A. flavus***	***F. moniliforme***	***A. japonicas***	***F. verticillioides***
Control	40	40	40	40	40
Methanol	6	0	0	1.75	0
JS3	22.23	26.67	13.3	40	33.3
JS4	21.1	40	18.3	40	35
JS6	21.1	40	15	29.76	40
JD7	35	35	40	36.67	35
JA8	40	40	31.67	28.3	36
JA10	31.67	30	16.67	31.67	30
LSD at 5%	26.14	25.41	35.84	22.78	21.21
LSD at 1%	18.97	18.44	26.01	16.53	15.39

Table 3 : Identification of *Streptomyces* strains by using 16S rDNA analysis.

Isolate	Accession number	Actinomycete species	Similarity
JS6	KF725839	*Streptomyces djakartensis*	99%
JD7	KF725840	*Streptomyces variabilis*	98%
JA8	KF725841	*Streptomyces minutiscleroticus*	99%
JA10	KF725842	*Streptomyces thermocarboxydus*	97%
JS3	na	*Streptomyces parvulus*	88%
JS4	na	*Streptomyces bullii*	96%

existing in other soil and aquatic isolates. The genus Streptomyces was dominant in soil samples and was found to have excellent microbial potential against several drug resistant bacterial pathogens.

Actinomycetes strains JS3, JS4, JS6, JD7, JA8 and JA10 were tested for their antifungal activity against five phytopathogenic fungi; *Fusarium moniliforme, Helminthosporium oryzae, Aspergilllus flavus, Aspergilllus japonicas and Fusarium verticillioides*. In the present study, it was found that most of the strains showed antifungal activity against these phytopathogenic fungi with an inhibition zone ranging between 13.3-40 mm diameter. Strain JA10 was able to inhibit growth of all the tested fungi. Of all the tested fungi, *F. moniliforme*, *H. oryzae* and *F. verticillioides* were highly sensitive to the most of the strains, (Table 2). The obtained results are in line with the studies of Abdel-Megeed *et al.* (2015) and Mangamuri *et al*. (2014) who reported a strong antagonistic activity of actinomycetes strains against phytopathogenic fungi.

After studying the morphology and pigmentation properties of colonies, all the isolates were presumptively assigned to the genus *Streptomyces* (Mujamammi *et al.*, 2014). Identification of six strains of actinobacteria selected in the present study was carried out by the 16S rDNA gene sequence analysis using universal primers 24f/1492r. BLAST searches within GenBank revealed high similarity with bacterial sequences from only one actinomycete genera: *Streptomyces*. These results support that *Streptomyces* strains are the most abundant in terrestrial soils (Hozzein *et al.*, 2011). Binary similarity values of each strain with other species of the genus *Streptomyces* ranged between 88 and 99% as shown in Table 3. In order to sequence the complete 16S rDNA (~1500 bp) sequence of the strains, internal primers ACT235f/ACT878r which were actinobacteria specific primers. Complete 16S rDNA sequences of JS6, JD7, JA8 and JA10 were successfully sequenced. Strains JS3 and JS4 were not sequenced completely. The sequences in the GenBank data library were deposited under the following accessing numbers: KF725839, KF725840, KF725841 and KF725842 for JS6, JD7, JA8 and JA10, respectively.

The taxonomical position of JS6, JD7, JA8 and JA10 strains were obtained after a stepwise phylogenetic analysis of 16S rRNA gene sequence with the closely related similar sequences. It is evident from the tree that each strain formed a distinct cluster in the *Streptomyces* 16S rRNA gene tree and could represent a new species of the genus *Streptomyces* (Fig. 1). However, sequence similarity values of ≥ 97% was reported to be of limited use in species differentiation (Hozzein *et al.*, 2004) and DNA_DNA hybridization and accurate polyphasic taxonomy, as well as, phenotypic descriptions need to be performed to clarify the taxonomic position of JS6, JD7, JA8 and JA10 strains.

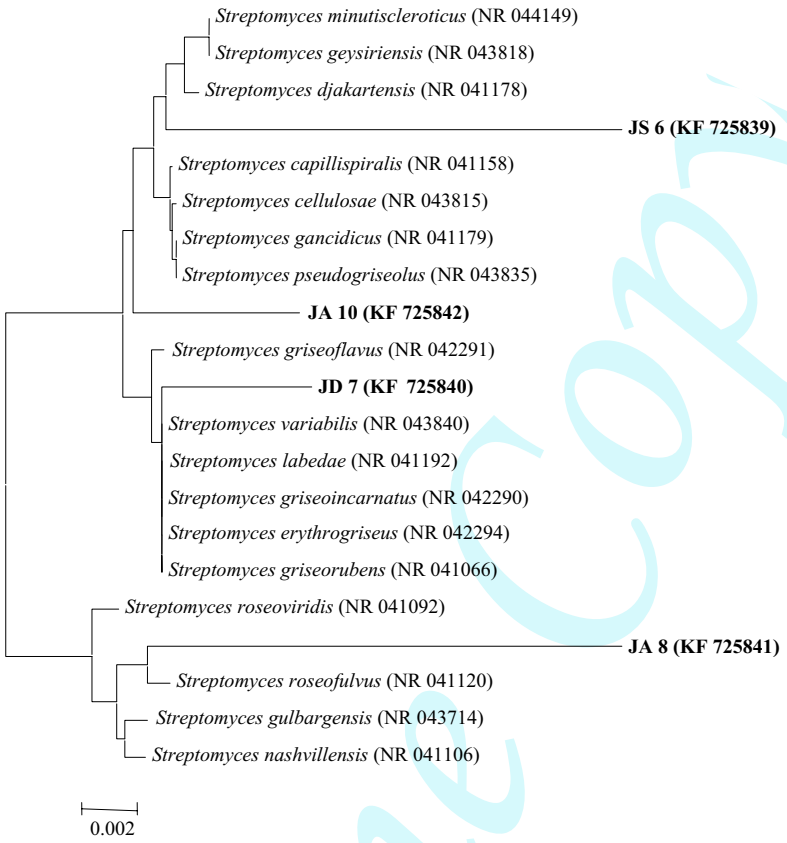

Fig. 1: Neighbour-joining tree based on nearly-complete 16S rDNA gene sequences showing relationships between JS6, JD7, JA8 and JA10 isolates and related *Streptomyces* type strain species. The tree was constructed using the neighbor-joining method

The results of the present study support the idea that actinomycetes possess a significant capacity to produce compounds having unique antibacterial and antifungal activity. The isolates JS6, JD7, JA8 and JA10 were found to have good antimicrobial activities. Further studies concerning purification, characterization and identification of secondary metabolites needs to be evaluated.

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