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Molecular Identification and Characterization of Antimicrobial Active *Actinomycetes* Strains from Some Egyptian Soils

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Abstract: Over classification problem of genus *Streptomyces* led the scientists to carry out many attempts to eliminate such problems. The current study aims at the molecular identification of antimicrobial activity of *Actinomycetes* from Egyptian soil. Among the 75 *Actinomycetes* collected isolates, 35 isolates by morphological and physiological studies were belonging to genus *Streptomyces* which were 64.3% exhibited activity against Gram positive bacteria, 48.5% showed activity towards Gram negative bacteria, 38.8% exhibited both Gram positive and negative bacteria and 80.85% isolates revealed significant antifungal activity from the total *Streptomyces* isolates. Analysis of the cell wall hydrolysates of the most potent two isolates showed the presence of LL-diaminopimelic acid and glycine. Molecular characterization of the two potent isolates was carried out based on PCR and sequencing. Sequence analysis indicated that two isolates shared 99-100% sequence identity to the 16S rRNA gene sequences with other *Streptomyces* taxons. Phylogenetic analysis revealed that the two isolates were belonging to genus *Streptomyces* within the same cluster. The obtained sequences were submitted to NCBI GenBank with accession numbers KM507575 and KM507576.

Key words: *Streptomyces* · Antimicrobial Activity · Egyptian Soil · Sequencing · Phylogenetic

INTRODUCTION

Actinomycetes constitute a diverse group of microorganisms that are widely distributed in terrestrial, freshwater and marine environments [1]. They play a vital role in decomposition of organic matter and thereby replenish the supply of nutrients in the soil. Among the various genera of *Actinomycetes* identified so far, the genus *Streptomyces* is represented in nature and differs greatly in their morphology, physiology and biochemical activities. It is well known as secondary metabolite producers and hence they are of high pharmacological and commercial interest [2, 3]. Around 80% of the total antibiotic products are obtained from *Streptomyces* spp. [4, 5]. The ability of *Streptomyces* spp. to synthesize several broad-spectrum antibiotics has made them interesting subjects of research due to the worldwide emergence of bacterial and fungal multi-drug resistance pathogens [6].

The evaluation of the data supplied by participants in the International *Streptomyces* Project (ISP) underlines the problem of the over classification. The data of the ISP comprising about 450 species of *Streptomyces* and Streptoverticillium have been used as the basis for several different classi?cation keys [7]. However, these approaches did not eliminate the over classification of genus *Streptomyces* and did not detect the numerous synonyms that still exist. The use of chemical procedures to correlate the composition of the cell wall of the *Actinomycetes* with their systematic position was discussed by Avery and Blank [8]. Later, in the past three decades, several approaches to solve this problem have been conducted by means of chemical taxonomy [9, 10]. Recently, new approaches involved the molecular methods as comparative sequencing and re-association of DNA [11, 12].

The most useful method for establishing the relatedness of higher taxa is the comparative analysis of the ribonuclease-resistant oligonucleotides of the 16S ribosomal RNA (rRNA) [13, 15]. The search for new molecules having unique therapeutic properties continues to be an active field of research and many studies are oriented towards the isolation of *Actinomycetes* species from new habitats.

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In the present study, morphological and physiological identification of 35 *Streptomyces* isolates were carried out and their potential antimicrobial activities were investigated. Molecular characterization of two isolates was conducted based on PCR, sequencing and phylogenetic analysis followed by submission to GenBank.

MATERIALS AND METHODS

Collection and Preparation of Soil Sample: Soil samples were collected from four locations: Giza, Cairo, Fayoum and Dakhlia Governorates. Each soil sample was air dried at room temperature, then mixed thoroughly and sieved through a 2 mm pore size sieve to get rid of large debris. The sieved soil was used for the isolation purpose.

Isolation and Purification of *Actinomycetes*: Sieved soil samples of 1 g were suspended in 100 mL sterile distilled water and incubated in an orbital shaker (Orbitek) at 28°C with shaking at 180 rpm for 1 h [16]. Mixtures were allowed to settle and then serial dilutions of the soil suspensions were prepared up to 10^{-4}. From each dilution, 0.1 mL was taken and spread evenly over the surface of starch nitrate agar (SNA) plates (supplemented with cycloheximide 50 µg mL^{-1}) (in triplicate), then incubated at 28°C for 10 days [17, 18]. *Actinomycetes* isolates were purified by streak-plate technique and the pure cultures were maintained on SNA slants at 4°C for further use.

Primary Screening Activity: Primary screening for the antimicrobial activity of the pure isolates were determined by perpendicular streak method on nutrient agar (NA) [19, 20]. The target reference strains were used as following *Bacillus subtilis* ATCC 5262, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 6821, *Pseudomonas aeruginosa* ATCC 10145, *Escherichia coli* ATCC 7624, *Salmonella* Typhimurium ATCC 14028, *Klebsiella pneumoniae* ATCC 8308 and clinical bacterial pathogens used were multi-drug resistant bacteria (Gram negative and Gram positive), fungi and yeast were collected from Microbiology Laboratory in Abo-Elresh for Children and El-Demerdash hospitals. The *Streptomyces* isolates were inoculated in SN broth and incubated on an orbital shaker for 7 days at 180 rpm. The broth cultures were filtered using Whatman No. 1 filter paper and centrifuged at 5000 rpm for 30 min at 4°C (Hettich Universal 32R, Germany).

Secondary screening was performed by well diffusion method on Mueller Hinton agar (Himedia) plates swabbed with the test bacteria. Following incubation at 37°C for 24 h, the diameter of the zones of complete inhibition was measured.

Effect of Different Media on Antimicrobial Activity: The effect of different media on antimicrobial activity was determined for the most potent isolates. Pre-cultures of *Streptomyces* isolates were carried out in 250 mL Erlenmeyer flasks containing 50 mL of SN broth, fishmeal extract broth, oatmeal extract broth, glycerol nitrate broth, glycerol asparagin broth and inorganic nitrate broth medium. After incubation for 24 h at 30°C under constant agitation at 180 rpm, culture filtrate used for antimicrobial activity testing by the agar well diffusion method [21].

Taxonomic Identification of the *Actinomycetes* Isolates: Cultural, Morphological and Physiological Identification of the Isolates: Only *Actinomycetes* isolates that gave a positive result for antimicrobial production were characterized morphologically and physiologically as previously described [7]. Morphological characters for isolates were observed by smears from colonies up to 10 days, stained by Gram's staining method as described by Hucker and Conn [22]. The spore chain morphology was determined by direct microscopic examination using the 10 days old cultures under a compound light microscope (Nikon, Japan) using 1000X magnification power. The organisms were identified also by color of aerial mycelia and substrate mycelia (reverse of the plate) along with diffusible pigments [23, 24]. Various physiological tests were performed for the identification of the potential isolates such as sugar utilization, NaCl tolerance and streptomycin sensitivity [25]. The observed structures were compared with those described by Bergey and Holt [26].

Chemotaxonomy: Identification of the most potent *Streptomyces* isolates was confirmed by chemotaxonomic analysis. Standard analytical procedures were used to extract and analyze the isomeric forms of diaminopimelic acid [27], whole-organism sugars [28] and polar lipids [29].

Molecular Identification of the Most Potent *Streptomyces* Isolates: In order to identify the isolates by molecular methods, PCR and 16S rRNA sequencing were carried out. Genomic DNA of pure subcultures of two antagonistic isolates was extracted using InstaGene Matrix (Bio-Rad,

USA) according to the manufacturer's specifications. PCR amplification reactions were performed using 1µL of genomic DNA in 20µL of PCR reaction solution by using 27F/1492R primers (27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-TACGGYTACCTTGTTACGACTT-3'). The cycling profile was as follows: 35 amplification cycles at 94°C for 45 sec, 55°C for 1 min and 72°C for 1 min. The PCR amplified product was purified by using Montage PCR Cleanup kit (Millipore) for sequencing. PCR product sends to Macrogen Company, South Korea for 16S rRNA sequencing by using the following primers (518F 5'-CCAGCAGCCGCGGTAATACG-3' and 800R 5'-TACCAGGGTATCTAATCC-3'). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing data were resolved on an Applied BioSystems model 330XL automated DNA sequencing system (Applied BioSystems, USA). Sequence data was analyzed with Chromas software version 2.33. The 16S rRNA gene sequence were compared to sequence in the public database using basic local alignment search tool (BLAST) on the national center for biotechnology information (NCBI) website(www.ncbi.nlm.nih.gov). Homology of the 16s rRNA sequence of isolate was analyzed by using BLAST program [30, 31].

RESULTS AND DISCUSSION

In the present study, 75 *Actinomycetes* strains were isolated from the collected soil samples. All of these isolates were selected based on their coloneal morphology on SNA medium producing dry, compact, chalky-like colonies slow growing, aerobic with aerial and substrate mycelia of different colors with an earthy odor. It has been observed that the environment of the soil such as the humidity, pH and diversity of plants species grown on that particular soil influence the growth rate of microorganisms [31]. Majority of the isolates in our study were recovered from the fertile soil that may be attributed to the presence of thick vegetation partly saline soil rich in humus content.

Out of the 75 *Actinomycete*s isolates, only 35 isolates demonstrated cultural characteristics similar to that of genus *Streptomyces* which grouped as shown in Figure 1. Microscopic examination revealed the typical long highly branched and non-fragmented aerial filamentous structures of the isolates bearing chains of conidia in spiral, coils arrangements and long chain

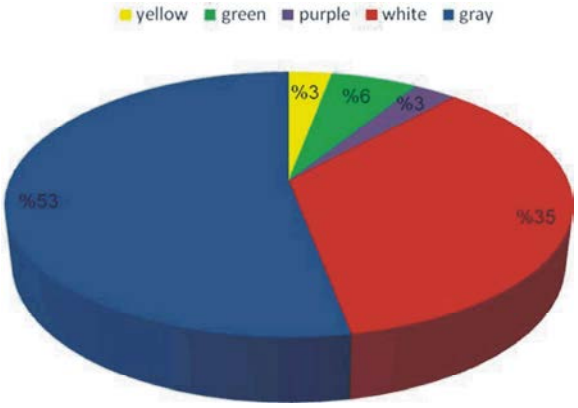

Fig. 1: Number of *Streptomyces* isolates according to the color of aerial mycelia.

spores. In accordance with the aerial mycelium color series established in the Bergey's manual of determinative bacteriology [32] and in the category IV of the Bergey's manual of systematic bacteriology [33].

The result of preliminary screening by perpendicular streak method revealed that all of 35 isolates have antimicrobial activity against indicator microorganisms and among them 10 *Streptomyces* isolates (No. 6, 14-16, 20, 21, 23, 30, 50, 69) were found to produce the most potent antimicrobial activity while two isolates (FS-16 and FS-20) were recorded highly antimicrobial activity (Table 1). Therefore, those ten isolates of *Streptomyces* were subjected to further morphological and physiological characterization.

Based on the results presented in Table 1, it seems that *S. aureus* and *B. cereus* were much more inhibited which supports the previous findings [34], who stated that Gram positive bacteria are more sensitive to metabolites produced by *Streptomyces*. This can be attributed to the cell wall structure of the Gram negative organisms that have an outer polysaccharide membrane carrying the structural lipopolysaccharide components that makes the cell wall impermeable to lipophilic solutes; while Gram positive organisms having only an outer peptidoglycan layer which is not an effective permeability barrier [35]. The fact that the bacterial isolates exhibited broad spectrum of antimicrobial activity, this signify possible production of several antimicrobial compounds and/or production of compounds with multiple microbial targets.

Several researchers have already reported that *Streptomyces* have biocontrol activity against pathogenic organisms [36, 37]. This has been shown that the principle mechanism of this biological activity involved the

Table 1: Antimicrobial activity of selected *Streptomyces* sp. against different sensitive and resistant test microorganisms:

	Streptomyces isolates									
Test Microorganism	FS-6	FS-14	FS-15	FS-16	FS-20	FS-21	FS-23	FS-30	FS-38	FS-50
Staphylococcus aureus	32	24.5	19	32.5	35	24	20	23	25.5	30
Staphylococcus epidermidis	30	14	30	32	35	31	22	18	20	25
Bacillus subtilis	24	27.5	12	25	26	0	17	16	20	28
Pseudomonas aeruginosa	15	15	25	29.5	28	20	22.5	15.5	18	20
Escerichia coli	20	21	18	22	24.5	25	22	23	24	18
Klebsiella pneumoniae	20	20.5	24	25	28	21	25	20	18	19
Salmonella Typhimurium	20	20	29	25	30	25	22	20	22	18
MDR Salmonella Typhimurium	20	0	26	20	16	18	20	19	18	20
MDR Salmonella Typhi	0	0	15.5	19	0	18	0	20	20	0
MDR *Klebsiella pneumoniae*	0	18	22	15.5	22	22	22	12	20	0
MDR *Pseudomonas aeruginosa*	0	0	18	18	18	18	18.5	0	0	0
MDR *Staphylococcus aureus,*	18	18	20	12	0	20	20	18	20	0
MDR *Escherichia coli*	14	18	15	18	15	0	28	20	20	0
Asperagillus flavus	18	0	20	20	22	0	24	18.5	0	22
Aspragillus niger	22	20	20	27.5	28	0	2	19	0	20
Rhizoctonia solani	22.5	20	21	30	26.5	0	27	0	0	20
Fusarium oxysporum	20	22	18.5	16.5	20	24	25	0	0	0
Candida albicans	31	20	25	30	25	30	20	0	0	22.5

Table 2: Selection the best media for inhibitory effect by tested *Streptomyces* isolate against *S. aureus*

	Diameter of inhibition zone (mm)										
Media	FS-6	FS-14	FS-15	FS-16	FS-20	FS-21	FS-23	FS-30	FS-38	FS-50	FS-59
Sstarch nitrate B.	15	21.5	32	20	25	22.5	23.5	27	25	29.5	30
Fishmeal extract B.	25	24	41	35	33	25	27.5	22.5	30	23	25
Oatmeal extract	17	17	0.0	18	22	20	18.5	0.0	20	0.0	22
Glycerol nitrate B.	19	20	30	12	18.5	30	22.5	26	28	30	21.5
Glycerol aspragine B.	18	15.5	17	0.0	22	25	30	20	00.0	23.5	0.0
Inorganic nitrate B.	18	0	19	19	0.0	22	0.0	0.0	20	19.5	0.0

Table 3: Cultural, morphological and physiological characteristics of isolates:

Characteristics																						
Physiological Characteristics													Cultural and morphological Characteristics									*Streptomyces* isolates
salicin	Sucrose	Cellulose	L-Inositol	D-Mannitol	Raffinose	D-Galactose	D-fructose	L-rhamnose	L-arabinose	D-xylose	D-Glucose	No carbon source	Growth on Czapek's agar medium	streptomycin sensitivity	Sodium chloride tolerance	Melanin pigment	Spore surface	Shape of spore chain	Soluble pigment	Substrate mycelium	Aerial mycelium	Growth of vegetative mycelium	
+	-	-	+	+	+	+	+	+	+	+	+	-	+++	+	6%	brown	smooth	spiral	Pale brown	Pale yellow	White	+++	6
-	+	-	+	+	+	+	+	+	+	+	+	-	++	+	6%	colorless	hairy	RF	-	colorless	white	++	14
	+	-	+	+	+	+	+	+	+	++	++	-	++++	Resistant	4%	brown	smooth	spiral	brown	Brown	gray	++	15
+	+	-	+	++	-	+	++	+	+	+	++	-	+++	+	6%	brown	smooth	spiral	colorless	Grayish white	gray	+++	16
+	±	-	-	++	-	++	-	+++	-	ND	++	-	++	+	7%	brown	smooth	spiral	Brown	Pale brown	gray	++	20
	-	ND	+	+	+	+	+	+	+	ND	+	-	++	+	5%	colorless	smooth	loose spiral	colorless	colorless	gray	+	21
	-	-	-	+	+	+	+	++	++	++	++	-	+	+	7% ≤ 10%	green	spiral	spiral	Green	Grayed - yellow	Gray	Good	23
	+	+	+	+	±	-	+	±	++	++	++	-	+	Resistant	10%	colorless	smooth	loose spiral	Colorless	colorless	white	+++	30
	-	-	+	+	-	+	+	+	+	+	+	-	++	Resistant	10%	green	hairy	spiral	Green	Grayed – yellow	Green yellowish	Good	38
-	-	-	-	+	-	-	+	±	+	+	+	-	+	Resistant	7%	colorless	smooth	spiral	Colorless	colorless	White	Good	50

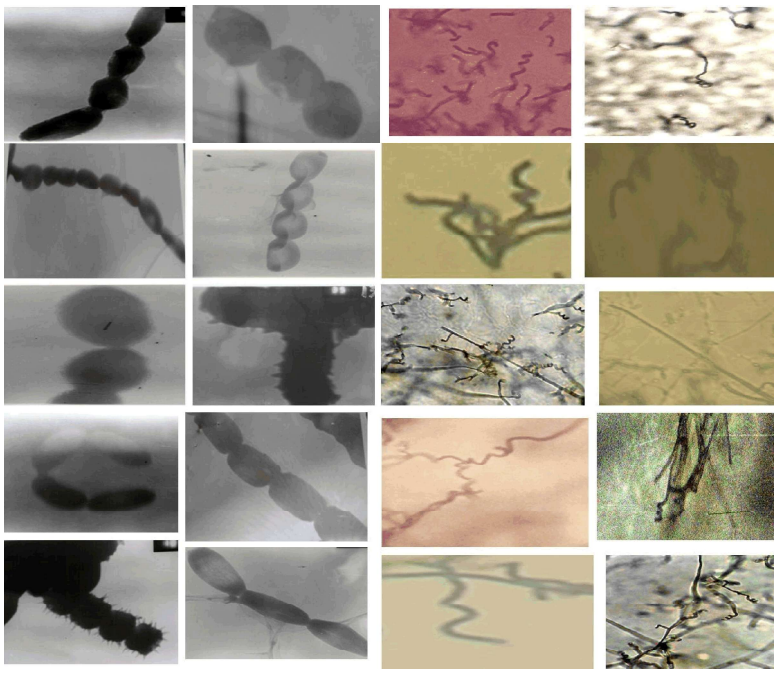

Fig. 2: Spore chain and spore surface image of isolates.

production of secondary metabolites [38]. According to selection of best media, fishmeal extract broth was the best media for the production of antimicrobial activity for the ten *Streptomyces* isolates followed by starch nitrate broth then glycerol nitrate broth but the unsuitable media was oatmeal extract broth and inorganic nitrate broth media (Table 2).

The identification of the *Streptomyces* isolates was further confirmed with reference to the Bergey's Manual of Determinative Bacteriology [26]. Besides the aerial mycelia, majority of them produced distinct pigment on the reverse of the colonies probably due to the diffusion of pigments and/or secondary metabolites into the media. The detailed results of the cultural, morphological and physiological tests have been summarized in Table 3.

Microscopically, it was observed that the morphology of the spore chains varied depending on the species where the majority spore chains were spiral belonging to the gray series and white series (Figure 2). It was also observed that some of the strains produced diffusible pigments in the surrounding medium, some of them melanoid. The production of melanoid pigments was variable in all the series, where only two white strains produced melanoid pigments and four gray strains. Con?rmatory identification to genus was based on degradation of carbohydrates where all *Streptomyces* strains have variable degradation ability of carbohydrates (Table 3).

Strains FS-16 and FS-20 showed abundant growth on all ISP media used with aerial mycelium color varied from grayish white to moderate grey. The substrate mycelium color varied from grayish white to pale brown. Brown soluble pigments were produced on any medium for FS-20 while colorless for FS-16. It formed aerial mycelium with short to long spore chains with spiral ends and smooth-surfaced spores (Figure 2). The most potent *Streptomyces* strain FS-16 showed good utilization of glucose, fructose, mannitol and starch, while moderate utilization of galactose, salicin, arabinose, rhaminose while no utilization for sucrose, cellulose and raffinose and FS-20 showed good utilization of galactose, mannitol, rhaminose, starch and glucose while moderate utilization of sucrose and no utilization of fructose, arabinose, raffinose inistol and cellulose. Both strains showed good growth at different NaCl concentration at 6 and 7% respectively (Table 3).

The taxonomic position of the isolated strain was confirmed by 16S rRNA gene sequencing. Considering all the complications facing with biochemical and morphological test which are laborious, time consuming and sometimes imprecise because of the subjectivity of interpreting the results, currently there exist molecular methods available for the isolation and characterization of new strain from different soil samples. Among them, application of 16S rRNA gene is more simple, yet efficient, in identification of new *Streptomyces* strains [39].

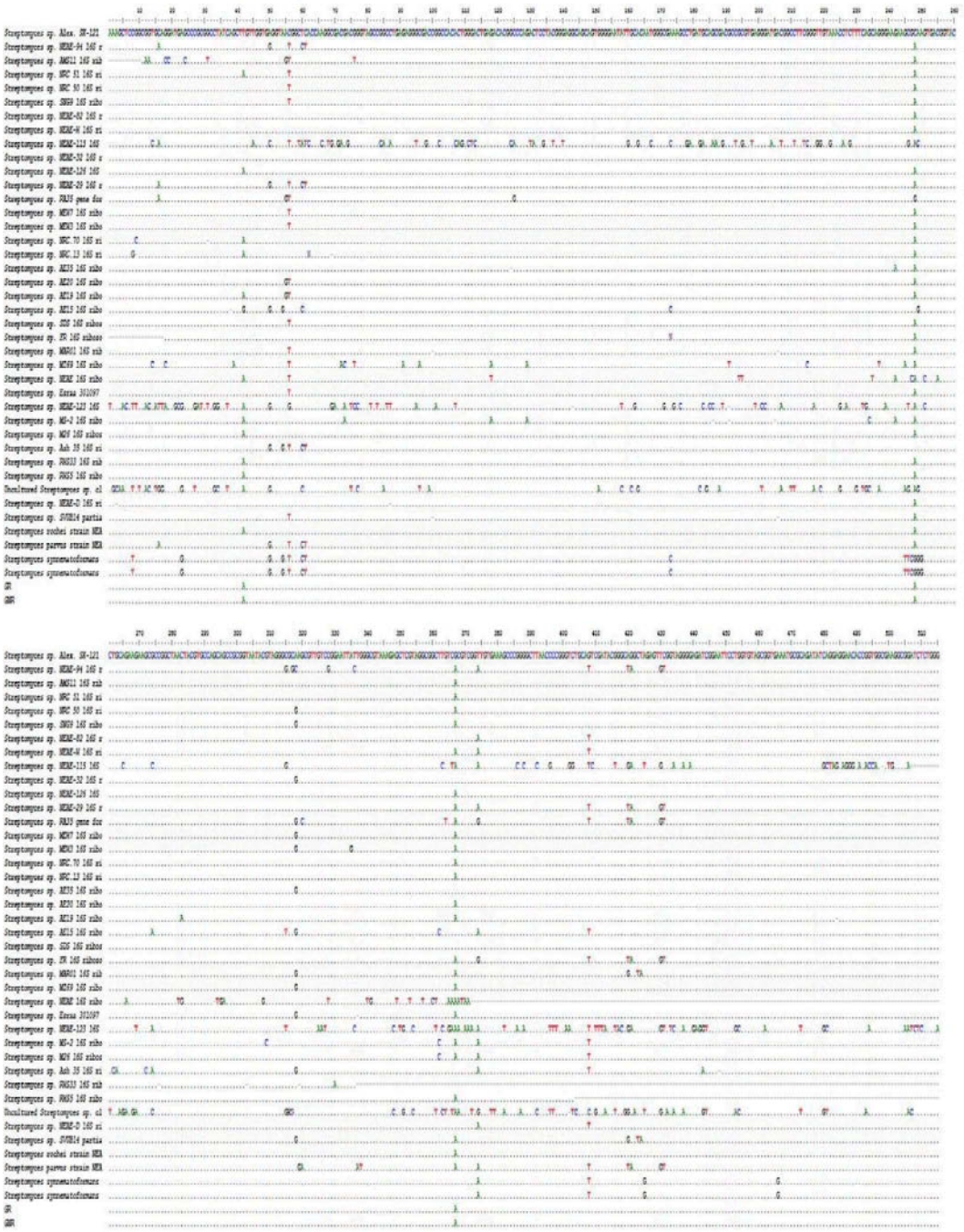

Fig. 3: Multiple nucleotide sequences alignment of 16S rRNA gene of our two Egyptian *Streptomyces* strains in comparison with other representative strains of *Streptomyces*. The Dot (.) represents identity whereas single alphabet represents difference in the nucleotide sequence.

Unlike conventional methods of identification of *Actinomycetes* which are long, difficult and very expensive, the molecular approach is often used for its speed and efficiency. Among molecular techniques, PCR remains the most widely used. The 16S rRNA gene is mainly used for molecular identification of bacteria. Indeed, some regions of this gene are highly conserved and common to all bacteria, while others are specific to species. Thus, 16S rRNA analysis offers a great advantage for the identification of *Actinomycetes* [40].

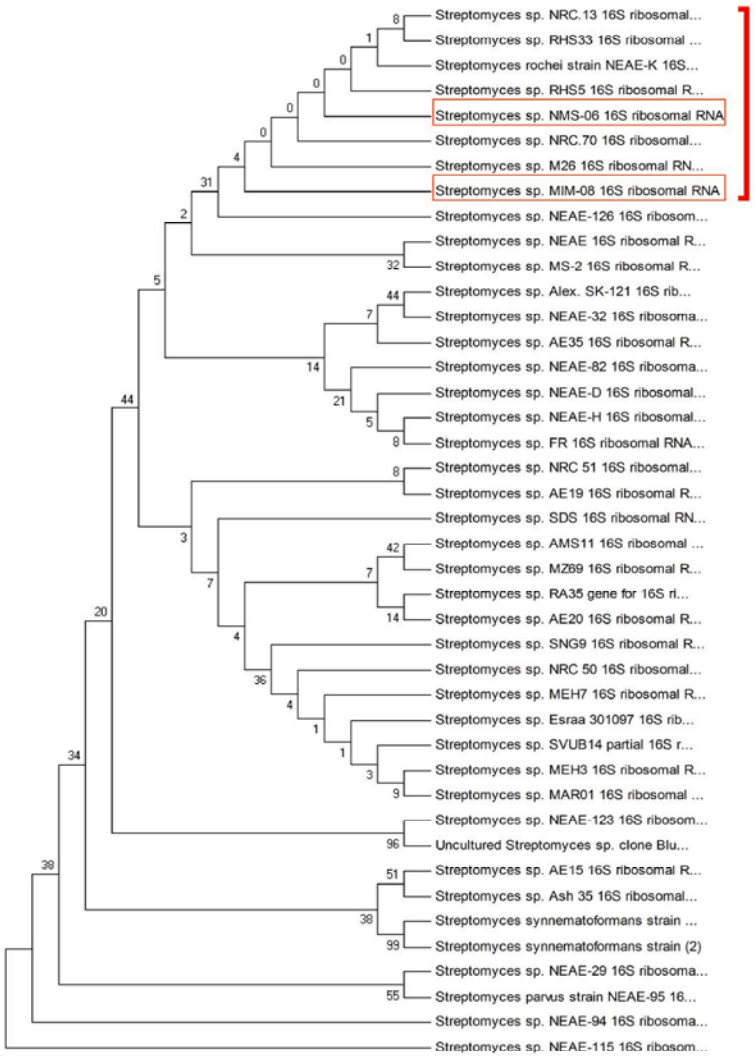

Fig. 4: Phylogenetic tree based on 16S rRNA gene sequence analysis, showing the relationship of active *Streptomyces* isolates A: FS-16 and B: FS-20 with reference strains.

In the present study, the results showed that 42.67% of the isolated *Actinomycetes* are biologically active. These results are very encouraging to continue screening more *Actinomycetes* strains from desert, fertile soil and other habitats and strong support the idea that species of *Actinomycetes* from underexploited environments could be a very fruitful source of novel bioactive secondary metabolites. The results showed also that two isolates FS-16 and FS-20 similar to *S. albus* and *S. vinaceusdrappus*, which was isolated from the Egyptian fertile soil produced biologically active compound with high broad spectrum activity against Gram-positive, Gram-negative bacteria, fungi and yeasts suggesting that this strain is a promising producer of an antimicrobial compound.

All the closest strains to the bacterial isolates have been linked to the production of one or more bioactive compounds. *S. albus* had been described as a soil dwelling Gram positive bacteria with antibacterial, antifungal and antitumor activities as previously described [41].

Previous studies reported that the antibiotic laspartomycins were originally discovered by Naganawa *et al*. in 1968 from the soil bacterium *Streptomyces viridochomogenes* var. *komabensis* (ATCC 29814) [42]. They were produced as a mixture of at least three peptide compounds which differ in their attached fatty acid side chains [43]. Laspartomycin C is the major component of this mixture and its structure was recently fully elucidated as a cyclic lipopeptide with a 2, 3-unsaturated C15-fatty acid side chain [44, 45].

Other reports recorded that avilamycin, an oligosaccharide antimicrobial belonging to the orthosomycin group of antibiotics, produced by *Streptomyces viridochomogenes* and it inhibits the growth of Gram positive bacteria effectively and is one of the antimicrobial agents approved for growth promotion in many countries [46, 47].

Comparison of the resulting nearly complete sequences of 16S rRNA genes of the two active *Streptomyces* isolates with those of public databases allowed the affiliation of two isolates to genus *Streptomyces*. As graphical examples, we report the dendrograms of the strain FS-16 and FS-20 with 99 and 100% similarity with the known species *Streptomyce* followed by submission of the obtained sequences to GenBank with accession numbers KM507575 and KM507576. Multiple nucleotide alignment shows the high similarity between our two isolates with similar sequences and prominent nucleotide substitution sites (Figure 3).

According to the taxonomical position of strain FS-16 and FS-20 was obtained after a stepwise phylogenetic analysis of the 16S rRNA gene sequence with the closely related similar sequences. It was found that they belong to genus *Streptomyces* as obvious from the phylogenetic tree (Figure 4). It is evident also from the tree that strain FS-16 and FS-20 formed a monophyletic line with the *Streptomyces* 16S rRNA gene. The affiliation of strain FS-16 and FS-20 to genus *Streptomyces* was supported by its chemotaxonomical characteristics. It contained LL-diaminopimelic acid as the characteristic diamino acid of the peptidoglycan in the whole-cell hydrolysate, glucose and g alactose as whole organism sugars (Wall chemotype I). The polar lipid pattern revealed the presence of phosphatidyl ethanolamine, phosphatidy inositol mannosides, diphosphatidyl glycerol (Phospholipid type II).

CONCLUSION

The present study aims at the molecular identification as a alternative confirmation technique instated of traditional identification method of active *Streptomyces* isolates in Egyptian fertile soil. *Streptomyces* are known as powerful antibiotic producer and show potent antimicrobial activity against different multi-drug resistant Gram positive and Gram negative bacteria.

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