Morphological, Biochemical and Sequence-Based Identification of Some Selenium Tolerant Actinomycetes

El-Meleigy, M . A.[1] ; Mohamadein, M. M.[2] ; Mohamed, H.F.[3] and Salem ,M. S.[3]

[1]Biology Dept., Faculty of Education, Shaqraa University ; K.S.A
[2]Medical Laboratory Sciences Dept., College of Applied Medical Sciences, Alkharj University K.S.A.
[3]Botany and Microbiology Dept., Faculty of Science Al-Azhar University, Cairo, Egypt
mereamsalah@yahoo.com

Abstract: Microbial tolerance to heavy metals is of great concern. Environmental pollution with different heavy metals came from many industrial applications. In this study, actinomycetes communities of heavy metal contaminated soil collected from Hellwan city were examined. Two actinomycetes were isolated from such soil using starch nitrate agar medium supplemented with different concentrations (50,100, 200,400,800 and 1000 ppm) of sodium selenite. The two isolates were subjected to a process of characterization and identification depending on their morphological , physiological and biochemical characterization , chemical analysis of the cell wall, cultural characteristics using the recommended media of the international *Streptomyces* project (for actinomycetes) and with help of 16S rRNA sequencing. The two isolates belonged to members of *Streptomyces* .Representative strains were identified *Streptomyces variabilis* strain NRRL B-3984T (GenBank accession number (DQ442551.1), Identities =99 % and *Streptomyces fradia* strain HBUM174185 GenBank accession number(FJ486352.1), Identities = 95 %. The study demonstrates the ability of partial 16S rRNA gene sequencing to identify members of the isolated actinomycetes.

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1. Introduction:

Actinomycetes are found worldwide, maintaining a saprophytic existence in soil and other natural habitats. Soil is a major reservoir for retention of bacteria and fungi and wind can transmit these agents in companion with dust and dirt into lung or skin and produce diseases like nocardiosis, sporotrichosis, chromomycosis, mycetoma etc (Department of Social Affairs and *Health*, 1997).

The aerobic actinomycetes are a large group of soil-inhabiting bacteria that occur worldwide. Some of them are the main cause of two important diseases, nocardiosis and actinomycetoma (Aghamirian and Ghiasian, 2009). *Streptomyces* are a group of aerobic high %G+C Gram positive bacteria that undergo complex differentiation to form filamentous mycelium, aerial hyphae and spores. In addition, they produce a broad range of secondary metabolites including antibiotics, antiparasitic agents, herbicides, anti-cancer drugs and various enzymes of industrial importance. Two *Streptomyces* species had their complete genome sequences published, namely the model organism *Streptomyces coelicolor* (%G+C = 72.1) and avermictin producer *Streptomyces avermitilis* (%G+C = 70.7) (Bentley *et al*., 2002 and Ikeda *et al*., 2003). Heavy metals are relatively abundant in the earth's crust. They are elements having atomic weights between 36.5 and 200.6, and a specific gravity greater than 5.0. Living organisms require trace amounts of some essential heavy metals including cobalt, copper, iron manganese, molybdenum, vanadium, strontium and zinc. Excessive levels of essential metals, however, can be detrimental to the organism. Non essential heavy metals are cadmium, chromium, mercury, lead, arsenic and antimony (Srivastava and Majumder, 2008).

Identification of aerobic actinomycetes by conventional biochemical assays requires expertise and time, and newer species such as *Nocardia nova* can be difficult to separate with accuracy from other related species (Wallace *et al* ., 1991). These organisms are isolated infrequently enough that it is difficult to develop or maintain technical expertise among laboratory personnel. Since these bacteria are slowly growing, 2 to 4 weeks is required for genus level identification and an additional 4 weeks or more is required for species-level identification. Alternative methods of identification, including high-performance liquid chromatography (HPLC) and molecular techniques have been applied to this group of bacteria (Butler *et al*., 1987). HPLC is limited by the inability to determine species-level identification. DNA amplification followed by PCR-restriction endonuclease analysis (PRA) of the 65 *hsp* gene has proven to be a more effective method of rapid

identification (Steingrube *et al*., 1995). Although the 16S rRNA gene is generally used as a framework for modern bacterial classification, it often shows limited variation for the discrimination of closely related taxa (Fox *et al*., 1992). Protein-coding genes exhibit higher genetic variation, which can be used for the classification and identification of closely related taxa (Yamada *et al*., 1999; Chun and Bae**,** 2000). 16S rRNA gene sequence analysis lacks widespread use beyond the large and reference laboratories because of technical and cost considerations. Thus, a future challenge is to translate information from 16S rRNA gene sequencing into convenient biochemical testing schemes, making the accuracy of the genotypic identification available to the smaller and routine clinical microbiology laboratories (Clarridge, 2004). Aghamirian and Ghiasian, 2009 studied to identify the prevalence and geographic distribution of aerobic actinomycetes in soil of Qazvin province, a study was carried out during 2006-2007. The incidence and diversity of medically important aerobic actinomycetes was determined in 300 soil samples of different parts of Qazvin. The isolated microorganisms were examined by Gram and acid-fast stains and were identified biochemically and morphologically. We investigated partial 16S rRNA gene sequence analysis for the identification of aerobic actinomycete isolates. Since the accuracy of sequence identification is directly dependent upon the sequence database that is queried, we evaluated GenBank database, the GenBank database is a public database that contains a large number of sequences, including 16S rRNA sequences (Benson *et al*., 2004).

This study aims at isolation and identification of some selenium tolerant actinomycetes using morphological, physiological, biochemical and 16S rRNA sequences properties.

2. Material and Methods

Samples origin and isolation procedure

Samples were taken from Egyptian localities during the autumn of 2009. (i) The first one was collected from Hellwan Governorate at Hellwan City beside cement factory (ii) the other one was collected from Wady El-Natron City, El-Beheraa Governorate. After removing the surface loose litter layer (approximately top 4 cm), the soil samples were taken from a depth of 5 to 10 centimeters of the superficial layers in each location. Soil samples were numbered and put in a sterilized paper sack, transferred to the microbiology laboratory of the Faculty of Science , Al-Azhar University (girls branch).Sulpher free medium (starch-nitrate agar) supplemented with different concentrations(50,100, 200,400,800 and 1000 ppm) of sodium selenite was used. Direct spreading of soil technique was used in the isolation of heavy metal-tolerant actinomycetes .0.5 gm of each soil was added per each plate, and then incubated at 30 ^{0}C for 5 days. Representative colonies were selected and streaked on new plates of the same culture media. Actinomycetes on the plates were identified as colored, dried, with regular margin, generally raised colony. Pink and gray colors of actinomycetes were considered, in order to purification of actinomycetes, the streak plate method were used. After isolation of the pure colonies based on their colonial morphology, color of substrate and aerial mycelia, they were individually plated on the same agar media was determined.

Morphological examination of the actinomycetes was done by cover slip technique according to Kawato and Shinobu (1959). The mycelium structure, and arrangement of spores on the mycelium were examined using light microscopy under oil immersion (1,00 x). Since a single method could not identify all actinomycete isolates to the species level; therefore, a combination of methods was necessary. Biochemical tests and physiological criteria such as the capability to degrade the organic compounds such as casein, xanthine, pectin and starch as substrates, the utilization of different carbon and nitrogen sources , growth at 45°C at 3 days as well as growth in (%w/v): 7 NaCl, 0.1 Phenol , 0.01 Sodium Azid and 0.001 Potassium tellurite were studied in order to reach a possible classification to the species level .In addition to Cell Hydrolysis for Diaminopimelic Acid (DAP) ,detection and hydrolysis of cells for sugar analysis which carried out according to Becker *et al*. (1964) and Lechevalier *et al*. (1968) .

Melanoid pigments were observed on i-Peptone yeast extract-iron agar medium (ISP, International Streptomyces Project-6) and ii-Tyrosine agar medium (ISP-7):

Medium used for isolation and identification of actinomycetes

Starch-nitrate agar (Tadashi, 1975) :It contains (g): soluble starch 20.0 ; KNO_3 2.0 ; K_2HPO_4 1.0 ; KCl 0.5 ; $MgSO_4.7H_2O$ 0.5 ; $CaCO_3$ 2.0 ; agar 20 and tap water up to 1000 ml . The pH of the medium was adjusted at pH 7 – 7.4 before sterilization.

Peptone yeast extract-iron agar medium contains (g/l) : bacto-peptone 15.0; protease peptone (Difco) 5.0 ; ferric ammonium citrate 0.5 ; K_2HPO_4 1.0 ; sodium thiosulphate 0.08 ; bacto-yeast extract 1.0 ; agar 20 and distilled water up to 1000 ml . The pH value was adjusted at 7-7.2 before autoclaving, Tyrosine agar medium contains (g/l) :glycerol 15 ; L-tyrosine (Difco) 0.5 ; L-asparagine (Difco) 1.0; K_2HPO_4 (anhydrous basis) 0.5 ; $MgSO_4.7H_2O$ 0.5 ;

NaCl 0.5 ; $FeSO_4.7H_2O$ 0.01 ; trace salt solution 1.0 ml ; agar 20.0 and distilled water up to 1000 ml . The pH value was adjusted at 7-7.4.

16S rDNA extraction (Sambrook *et al.*, 1989), amplification, and sequencing

The genomic DNA of the isolates under study was isolated according to Sambrook *et al*., 1989. Cells were collected from overnight starch-nitrate broth cultures by centrifugation and resuspended in MLTEN buffer. Twenty five µl of 10 mg/ml of lysozyme was added and the tubes were incubated at 37^0C for 30 min , followed by the addition of 75 µl of 10 % stock DNA solution (SDS) and the tubes were inverted gently several times till complete lysis. Three of 20 mg/ml of protienase k was added and the tubes were incubated at 37^0C for one hour. After incubation 100 µl of 5 M NaCl was added and 800 µl of phenol/ chloroform: isoamyl alchol(24:1) was added and the tubes were inverted several times and centrifuged for 10 min. The upper phase was transferred to afresh tube and extracted once with chloroform. The upper phase was again transferred to a fresh tube and 0.7 volume of isopropanol was added and mixed gently and centrifuged for 10 min. The supernatant was removed carefully and the pellet was washed with 1ml of 70% ethanol. The pellet was collected by centrifugation for 5 min. The DNA was dried and dissolved in 100 µl TE buffer and stored at -20 ^{0}C. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using eubacterial universal primers. That were F27 with the sequence 5-AGAGTTTGATCMTGGCTCAG-3 and R1492 with the sequence 5-TACGGGYTACCTTGTTACGACTT-3.The PCR mixture consisted of 30 picomoles of each primer, 10µg of chromosomal DNA, 200 µl dNTPs and 2.5 units of Taq polymerase in 50 µl of polymerase buffer. The PCR was carried out for 30 cycles of 94 ^{0}C for 1 min, 55^0C for 1 min and 27^0C for 2 min. After completion, PCR product was purified using PCR purification kit (Qiagen, Germany). DNA sequences were obtained using an ABI PRISM 3700 DNA sequencer and ABI PRISM Big Dye Terminator Cycle Sequencing.

DNA sequence similarities and phylogenetic analysis.

Sequences data were analyzed in the GenBank database by using the BLAST program available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The unknown sequences were compared to all of the sequences in the database to assess the DNA similarities (Altschul *et al*., 1990).The GenBank entry with the highest score from the search with the BLAST program was downloaded.

3. Results

Two actinomycetes tolerated selenite were obtained from contaminated Hellwan soil, that tolerate selenium as sodium selenite up to 300×10^{-4} ppm for the two isolates designated A1 and A2 .No isolates were obtained from the other soil sample. Microscopic analysis revealed that isolates were spiral forming aerial mycelium (Plates, 1 and 2) , and therefore these isolates were expected to belong to *Streptomyces* or related genera in addition to cell wall analysis that contains LL-diaminopimelic acid (LL-DAP) and sugar pattern not detected. Based on morphological, physiological and biochemical characters , and according to the recommended international Key's viz. (Williams, 1989; and Hensyl, 1994), the first isolate, A1 was identified *Streptomyces variabilis* , it had spiral spore chains with smooth spore surface ,color of spore mass belong to red series, there is no diffusible pigment, melanin pigment is not produced , hydrolysis of pectin and xanthin ,nitrate reduction and H_2S production were positive, grow at 45^0C, grow in the presence of % (w/v) : 0.01 sodium azide, 0.001 potassium tellurite and not in presence of 7 NaCl, it utilizes L- Cysteine, L- Valine, L- phenylalanine, *meso*-Inositol, Mannitol and Rhamnose, table (1) shows the comparative study between the isolate A1, to reference strains *Streptomyces variabilis* , 16S rDNA sequencing was performed for this isolate as a confirmed identification tool .

The extracted DNA sample from this isolate was amplified by PCR using 16S rRNA eubacterial universal primer. The PCR product was sequenced directly and 469 bp sequence of the isolate was successfully determined using an ABI PRISM 3700 DNA sequencer. The obtained sequence was compared to all of the sequences in Gene bank by using Basic Local Alignment Search Tool (BLAST). The isolate A1 was found to be similar to *Streptomyces variabilis* strain NRRL B-3984T (GenBank accession number(DQ442551.1), Identities =99 %, Gaps = 0 % , where isolate, A2 was identified as strain of *Streptomyces fradia* and this was confirmed by using BLST tool of 16S rDNA sequence of this isolate which have high similarity with reference *Streptomyces fradiae* strain HBUM174185 GenBank accession number (FJ486352.1), Identities = 95 % , Gaps = 2 % .

The isolate A2 are characterized by , the color of sore mass is gray with spiral spore chain, positive production of diffusible and melanin pigments, degradation of xanthin and growth at 7% NaCl (w/v) were negative, grow at 45^0C, grow in

presences of %(x/v): 0.001 potassium tellurite with utilization of L- phenylalanine and Rhamnose.

Table (2) shows the comparative study between the isolate A2, to reference strains *Streptomyces fradiae*.

Table (1): A comparative study of the identification properties of the isolate A1, in relation to the reference strains *Streptomyces variabilis*

Characteristic	Isolate A1	*Streptomyces variabilis*
Morphological Characteristics		
Motility	-	-
Spore mass white	-	-
Spore mass red series	+	±
Spore mass gray	-	±
Spore chain Spirales	+	±
Spore chain Rectiflexibiles	-	-
Spore surface smooth	+	+
Diffusible pigment produced	-	-
Mycelial pigment red –orang	-	-
Diffusible pigment yellow-brown	-	-
Melanin pigment on 1-Peptone yeast extract-iron agar medium (ISP-6)	-	-
2- Tyrosin agar medium (ISP-7)	-	-
Pectin hydrolysis	+	±
Nitrate reduction	+	±
H_2S production	+	+
Degradation of Xanthin	+	+
Growth at 45°C	+	±
Growth at NaCl 7% (w/v)	-	±
Growth inhibitor (% w/v)		
Phenol (0.1)	-	+
Sodium Azid (0.01)	+	±
Potassium tellurite (0.001)	+	±
Utilization of		
L- Cysteine	+	±
L- Valine	+	±
L- phenylalanine	+	±
Sucrose	-	±
meso-Inositol	+	+
Mannitol	+	+
Rhamnose	+	+

Symbols: ±, 11-89% of strains are positive. +, 90 % or more of strains are positive. - , 10 % or less of strains are positive

Table (2) : A comparative study of the identification properties of the isolate A2, in relation to the reference strains *Streptomyces fradiae*

Characteristic	Isolate A2	*Streptomyces fradiae*
Morphological Characteristics		
Motility	-	-
Spore mass white	-	-
Spore mass gray	+	±
Spore chain Spirales	+	±
Spore chain Rectiflexibiles	-	-
Diffusible pigment produced	+	-
Melanin pigment on 1-Peptone yeast extract-iron agar medium (ISP-6)	+	+
2- Tyrosin agar medium (ISP-7)	+	+
Degradation of Xanthin	-	-
Growth at 45°C	+	+
Growth at NaCl 7% (w/v)	-	-
Growth inhibitor (% w/v)		
Phenol (0.1)	-	-
Sodium Azid (0.01)	+	-
Potassium tellurite (0.001)	+	+
Utilization of		
L- phenylalanine	+	±
Sucrose	-	-
Meso-Inositol	-	-
Mannitol	-	-
Rhamnose	+	+

Symbols: ±, 11-89% of strains are positive. +, 90 % or more of strains are positive. - , 10 % or less of strains are positive

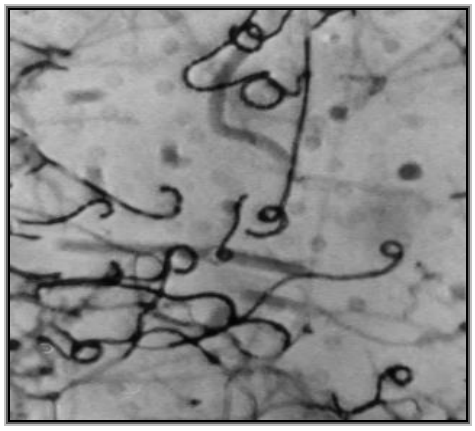

Plate (1). A photograph of the actinomycete isolate A1 grown on starch-nitrate agar medium showing spore chain spiral and coil shape(X 100).

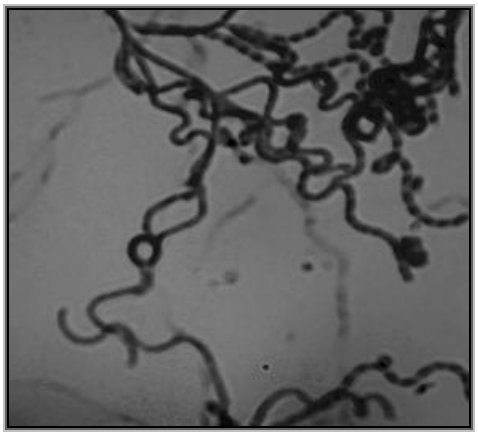

Plate (2). A photograph of the actinomycete isolate A2 grown on starch-nitrate agar medium showing spore chain spiral and coil shape (X 100).

4. Discussion

The aerobic actinomycetes are a various group of Gram positive, branching, filamentous, obligate aerobic and relatively slow-growing bacteria. Actinomycetes are considered as Spore-forming bacteria with indication of health problems (Department of Social Affairs and *Health*, 1997)

In this study, isolation, characterization, and identification of selenium tolerant actinomycetes obtained from contaminated Hellwan soil was done. Two actinomycetes are resist to 300×10^{-4} ppm selenium were isolated. Such resistance to selenium was higher than that obtained by different organisms as , the MICs of sodium selenite determined for pyridine-2,6-bis (thiocarboxylic acid) pdtc-producing strain KC and pdtc-negative mutant CTN1 in iron-limited medium were 3 and 0.5 mM, respectively (Zawadzka *et al* ., 2006).

The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique (Clarridge, 2004), Although the 16S rRNA gene is generally used as a framework for modern bacterial classification, it often shows limited variation for the discrimination of closely related taxa (Fox *et al*., 1992 ; Stackebrandt and Swiderski, 2002). For these reasons we evaluated partial 16S rRNA gene sequences and compared the result of identification to the profiles obtained by morphological, physiological and biochemical analysis. In accordance , evaluated partial 16S rRNA gene sequences and compared them to the profiles obtained by biochemical analysis, HPLC, fatty acid analysis, drug susceptibility testing, and PRA of the 65 *hsp* Telenti sequence for identification of isolates of the aerobic actinomycetes were carried out (Patel *et al*., 2004). PCR fingerprinting was used to identify our isolates at the subspecies level. PCR is a relatively rapid DNA fingerprinting technique that is known to discriminate bacterial isolates at the intraspecific level and potentially up to the strain level **(**Versalovic *et al*., 1994).As expected from morphological studies and cell hydrolysis for diaminopimelic acid (DAP), the two isolate were identified as members of S*treptomyces* . Depending on morphological, physiological and biochemical analysis, the two isolates were identified as *Streptomyces variabilis* and *Streptomyces fradiae* respectively. 16S rDNA sequence analysis of these representative isolates allowed a confirmed species identification. Sequences of the 16S rRNA gene are generally used as a framework for bacterial classification. Therefore, sequencing of this gene was used as a first identification tool According to Stackebrandt and Goebel, 1994; De Clerck *et al*., 2004 .The two isolates had 469 and 435 bp 16S rRNA sequences and these sequences match with *Streptomyces variabilis* strain NRRL B-3984T (GenBank accession number(DQ442551.1), Identities =99 %, and *Streptomyces fradiae* strain HBUM174185 GenBank accession number (FJ486352.1), Identities = 95 % in Gene bank database respectively and therefore , the 16S rRNA gene confirmed the phenotypic . Similarly, Patel *et al*., 2004 investigated the utility of 500-bp 16S rRNA gene sequencing for identifying clinically significant species of aerobic actinomycetes. The partial sequence of the 16S rRNA of *Streptomyces albus* isolate was aligned and compared with all eubacterial 16S rRNA gene sequences available in the GenBank and EMBL databases by multisequence analysis (Altschul *et al*., 1997), giving the maximum identity (99%) with 16S rDNA sequences from *Streptomyces albus* subsp. a*lbus* type strain DSM (AJ621602). Organisms showing less than 97% 16S rDNA sequence similarity will have less than 70% DNA-DNA relatedness, and, according to the recommendations for species delineation(Stackebrandt *et al*., 2002), these strains should be considered to belong to different species. Sequence-based identification is becoming an increasingly important identification tool. Although the present cost of performing such tests limits its application in most microbiological laboratories, it is a useful alternative for the identification of bacterial isolates that are slowly growing or for which specialized identification techniques are required. Our results indicate that sequencing of the 469 and 436 bp, 16S rRNA gene provides enough information for the sub species-level identification of isolated actinomycetes.

The isolates are promising so much work will be done to understand their resistance to heavy metal and their mode of tolerance.

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