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16S rRNA Characterization of a *Bacillus* Isolates From Egyptian Soil and its Plasmid Profile.

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ABSTRACT

Bacillus species constitute a diverse group of bacteria widely distributed in soil. In this study, sixteen *Bacillus* local strains isolated from different Egyptian soils and three identified strains; (*Bacillus thuringiensis* subsp. *Kurstaki* (*B.t.*40), *Bacillus subtilis* subsp. subtilis strain ATCC 168(*B.s*) and *Bacillus licheniforms* strain ATCC 14580**)** (*B.l*); were identified by detailed conventional biochemical methods, plasmid pattern and partial 16S rRNA sequencing. All isolates were Gram-positive, aerobic, and motile, Oxidase-positive, rod shaped, endospore-forming bacteria. These isolate was phenotypically identified as *Bacillus*. The crude polysaccharides were separated from the supernatant and examined for sulphate content. In this study, five different isolates were selected according to their plasmids patterns and polysaccharide production, the primary aim was to characterize these isolates using 16S rRNA partial gene amplification and sequencing. The five isolates were assigned as *Bacillus cereus* strain and *Bacillus sp* BAB3450 16S rRNA gene. All *Bacillus* strains were characterized by whole-cell protein profiles using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analysis of dendrogram using UN weighted pair group method with arithmetic averages algorithm (UPGMA).

Keywords: 16S rRNA, *Bacillus*, SDS

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INTRODUCTION

Soil is an excellent source for unknown microorganisms; the most frequently isolated genus has been *Bacillus.* The use of 16S rRNA gene sequences to study bacterial evolution has been the most common house-keeping genetic marker used for a number of reasons. They include (i) existence in almost all bacteria, often as a multigene family, or operon; (ii) the function has not changed over time, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for bioinformatics purposes [1].

The cumulative results from a limited number of studies to date suggest that 16S rRNA gene sequencing provides identification on the genus level (90%) but less so with regard to species level (65 to 83%), with from 1 to 14% of the isolates remaining unidentified after testing [2-3-4]. 16S rRNA and its gene have proven to be useful and powerful markers for the presence of bacteria in biological samples [5]. Several *Bacillus* species were reclassified based on 16S rRNA and separated into different phylogenetically distinct clusters [6]. Partial 16S rDNA sequence [7] and rRNA gene restriction pattern [8] have been used for the rapid identification or classification of *Bacillus* species and related genera [6]. *Bacillus* species is ubiquitous and broadly adapted to grow in diverse settings within the biosphere. *B*. *subtilis* can be isolated in greater numbers than most other spore-forming bacteria from the rhizosphere of a variety of plants like Rhazyastricta. There is evidence that through these associations, *B. subtilis* can promote plant growth [9-10-11]. The traditional methods for isolating *B. subtilis* require that the organism be in its spore form. Besides, there is no guarantee that the isolated strain from a particular environment actually grow at that location [12]. It is important to realize that libraries of PCR-amplified 16S rRNA and 16S rRNA genes may not represent a complete or accurate picture of the bacterial community in a given biological sample. Firstly, the species diversity is so great; the available libraries of 16S rRNA genes permit an initial survey of the global soil bacterial community structure. The majority (79 to 89%) of 16S rRNA gene sequences are from bacteria that are not affiliated with known genera [13]. The gene encoding the small subunit rRNA serves as a prominent tool for the phylogenetic analysis and classification of bacteria and archaea owing to its high degree of conservation and its fundamental function in living organisms [14]. Plasmid profiles of *B. thuringiensis* strains from different environmental zones. Totally 35 *B. thuringiensis* strains were subjected to plasmid profiling. Different sizes of plasmids ranging from 108 kb to 2 kb in 97.22% strains were isolated [15]. Exopolysaccharides are naturally formed macromolecules during growth of many organisms. Among a group of bacilli, *Bacillus* species strain-QS5 producing exopolysaccharides, locally isolated from Eastern Province in Saudi Arabia, was characterized and identified based on 16S rRNA sequencing [16].

In the present study, we performed an investigation for *Bacillus* strains by isolating and identifying sixteen- local strains grown in Egyptian soil rhizosphere from different area and three identified strains (*B.t*. *B.s* and *B.l*). The objectives of the present study are to screen *Bacillus* species isolated from Egyptians soil rhizosphere in order to evaluate the usefulness of the technique as a taxonomic tool in this genus and to study their suitability with regard to exopolysaccharides and sulphate production. Also SDS-PAGE method combined with computerized analysis of cellular protein profiles provide an effective

approach to investigate of taxonomic relationships within *Bacillus* species. In this study, the taxonomic position of *Bacillus sp* is determined using 16S rRNA partial gene amplification and sequencing also, through 16S rRNA phylogenetic identification.

MATERIALS AND METHODS

Microorganism sources

Sixteen isolates were collected from Egyptian soils at different locals and three identified strains (*Bacillus thuringiensis* subsp. *kurstaki* strain Bt40, *Bacillus subtilis* subsp. *subtilis* strain ATCC 168 and *Bacillus licheniforms* strain ATCC 14580**)** obtained from National Research Center, Microbial Genetics Dept. Dokki, Cairo, Egypt used in this study are listed in Table (1).

Isolation of *Bacillus* isolates from field

One gm. of soil sample from each collected soil sample transferred into 10 ml tube containing saline solution (NaCl 0.1 & w/v) and mixed vigorously with vortexes for 5 min. until soil particles settled and left for 30 min at room temperature, sample solutions were diluted ten- fold with 0.1% NaCl, spread onto Luria-Bertani (LB) agar plates [17], and incubated overnight at 37^{o}C. Bacilli-like colonies were isolated according to their morphological characters.

Culture media and growth conditions

Bacillus isolates were cultivated at 37^{o}C in Luria-Bertani (LB) medium composed of 1% NaCl, 1% tryptone, and 0.5% yeast extract. A single colony of an isolate was three times streaked over LB agar and incubated at 37^{o}C for 24 h. Purified colonies cultured under various pH (between pH5 and pH9). Salt tolerance experiments were performed on LB broth with NaCl, in various concentrations (between 0.5 and 10%, w/v) and temperature (between 25^{o}C and 50^{o}C) at 37^{o}C. Purified *Bacillus* growth was numbered as indicated in Table (1).

Identification of selected bacterial isolates

Identification of the bacterial isolates was performed based on their morphological, physiological, and biochemical characteristics, as described in Bergey's Manual of Systematic Bacteriology [18] then confirmed by PCR amplification of 16S rDNA. As well as electrophoretic separation of total protein (SDS-PAGE) according to the method described [19].

Isolation of exopolysaccharide and sulphate content from liquid culture.

Exopolysaccharide and sulphate content of all the local isolates and three identified strains were done using the method described [16-20].

Plasmid DNA pattern of *Bacillus* isolates

Plasmid profiles of the Egyptian *Bacillus* isolates and three identified strains (*Bacillus thuringiensis* subsp. ***kurstaki*** strain Bt40, *Bacillus subtilis* subsp. *subtilis* strain ATCC 168 and *Bacillus licheniforms* strain ATCC 14580) were done by using (Rouch) plasmid isolation kit.

Agarose gel electrophoresis of plasmid DNA

Ten micro liters of each sample were applied to a 0.5% agarose gel and run for approximately 4h at 100 V. TAE buffer was the running buffer (0.001M EDTA pH 8.0, 0.04 M TRIS pH 8.0, 0.02 M acetic acid), and the gel was stained in a solution of ethidium bromide (1 µg/ml) for approximately 15 min, and washed in water for about 30 min. Gel images were recorded in Gel Doc XR+ Bio RAD with Image Lab Software.

DNA Preparation and PCR Amplification

Genomic DNA was extracted from all the isolates using Qiagen DNeasy Tissues isolation Kit. Each genomic DNA used as template was amplified by PCR with the aid of 16S rRNA 8F (5-AGTTGATCCTGGCTCAG-3), and reverse primer 1492R (5-ACCTTGTTACGACTT-3) with the programme consisted of denaturation at 94°C for 5 min and subsequent 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min followed by final extension at 72°C for 5 min. The presence of PCR products was determined by electrophoresis of 10 µl the reaction product in a 1% agarose gel.

16S rRNA Sequencing and Data Analysis

Sequencing analysis was performed on a 1500 bp PCR product. The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit. The five 16S rRNA sequences were aligned and compared with other 16SrRNA genes in the GenBank by using the NCBI Basic Local alignment search tools BLAST-n program (http://www.ncbi.nlm.nih.gov/BLAST). A distance matrix was generated using the Jukes-cantor corrected distance model. The phylogenetic trees created using (Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction) with alphabet size 4 and length size 1000. The 16SrRNA gene sequences have been deposited to Genbank using BankIt submission tool and has been assigned with NCBI accession numbers.

SDS-PAGE protein analysis

Solubilized proteins were subjected to SDS-PAGE in gel slabs of 1 mm thickness (3.5cm, 4% stacking and 16.5cm, 12.5% resolving gels) as described by [21] Electrophoresis was performed with a discontinuous buffer system in a BRL gel apparatus model V16-2BRL Gaithersburg MD, USA. The gel was run at 30 mA until the bromophenol blue marker had reached to the bottom of the gel. Gels were then stained with Coomassie Brillant Blue R-250.

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Statistical method

Data from SDS-PAGE were pooled and transferred into 1 and 0. The data were then fed into the statistical data analysis software package SPSS system (SPSS for windows, Ver. 18.0- Lz0-up.for.vdown, 2002, Chicago, SPSS Inc.). The statistical analysis of the data was carried out using the SPSS software package and utilizing the method described [22] with the least significant difference values calculated at the 5% probability level.

RESULTS AND DISCUSSION

Collection and isolation of bacteria

Morphological, physiological and biochemical characters of *Bacillus* isolates.

In the course of a screening program for new bioactive polysaccharides, the present study was carried out on sixteen local isolates, which were isolated from different areas in Egypt and three identified strains (*Bacillus thuringiensis* subsp. *kurstaki* strain Bt40, *Bacillus subtilis* subsp. *subtilis* strain ATCC 168 and *Bacillus licheniforms* strain ATCC 14580**)**. The samples were collected from 16 different areas and the isolates were obtained using standard serial dilution technique from the original samples.

The genus *Bacillus* is generally defined according to classical phenotypic characterization based primarily on morphology, nutrition, growth characteristics; and various substrate utilization and physiological assessments. Physiological reactions are generally used to determine the species of the genus [18-23].

Table 1: Characterization of the sixteen local *Bacillus* bacterial isolates and the three identified strains (*B.t*. *B.s* and *B.l*).

latelso	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	*B.t*	*B.s*	*B.l*
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	-	-	+	-	-	-	-	+	+	+	+	-	+	+	+	-	+
Catalase	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aerobic	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	+
Indole	-	-	-	-	-	-	-	-	-	-	-	--	-	-	-	-	-	-	-
Production of acids gas from carbohydrates																			
Glucose	-	-	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+
Sucrose	-	+	+	+	-	-	-	-	+	+	+	+	-	-	+	-	+	-	+
Xylose	+	+	+	-	-	-	-	-	+	+	-	-	+	+	+	+	+	-	-
Arabinose	-	+	-	-	+	+	+	-	+	+	+	+	+	-	+	+	+	-	+
Utilization	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Hydrolysis	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+
Reduction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6.5% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Data in Tables (1) showed the morphological and the physiological parameters, identified bacteria isolates, of the sixteen local bacteria isolates and three identified strains

(*B.t*. *B.s* and *B.l*). Data in Table (1) represent the sixteen bacteria isolates and three identified strains (*Bacillus thuringiensis* subsp. *kurstaki* strain Bt40, *Bacillus subtilis* subsp. *subtilis* strain ATCC 168 and *Bacillus licheniforms* strain ATCC 14580**)**. According to the obtained parameters which compared with those mentioned [18] found to be short rods, spore form, gram positive, capable of growing on different sugars such as: Glucose, Sucrose, Xylose and Arabinose. In addition, the isolates showed aerobic growth, catalase positive, tolerance to 6.5 NaCl fermented glucose and could hydrolysis starch or gelatin. These strains were negative for indole production, Reduction of nitrate to nitrite and utilized citrate.

Exopolysaccharide (EPS) and sulfate groups production from *Bacillus sp.*

The sixteen purified local strains of *Bacillus sp* and three identified strains (*Bacillus thuringiensis* subsp. *kurstaki* strain Bt40, *Bacillus subtilis* subsp. *subtilis* strain ATCC 168 and *Bacillus licheniforms* strain ATCC 14580**)** were tested for production of sulfated exopolysaccharides. Data in Table (2) clearly show the production of strains greatly in sulfated polysaccharide. The maximum value of polysaccharide obtained by the strain No.(3) was 5.7 g/L EPS which isolate from Ismailia; while the minimum value; 3.8 g/L EPS; obtained by the strain No.(11) which isolate from Damietta. On the other side the results in Table (2) gives the sulfate production by the strains. The results indicate that the highest productivity was on the strain No.(2) at a rate of 0.102 mg/ml these isolate from Monofeya; while the lowest isolate productivity was the strain No. (12) 0.034 mg/ml these isolate from Dakahlia. These results are in agreement with results obtained from previous studies [24-16] in *Bacillus subtilis* and *Bacillus licheniformis*.

Table 2: Sulfated and polysaccharide production from the sixteen local *Bacillus* isolates and three identified strains (*B.t*. *B.s* and *B.l*).

Code	Strains	Source	Mean of polysaccharide g/L	Mean of sulfate mg/ml
1	*Bacillus sp*	Gharba	4.4	0.060
2	*Bacillus sp*	Monofeya	5.2	0.102
3	*Bacillus sp*	Ismailia	5.7	0.095
4	*Bacillus sp*	Behara	5.1	0.062
5	*Bacillus sp*	Kafr Al-Shaikh	4.6	0.101
6	*Bacillus sp*	Alexandria	4.7	0.064
7	*Bacillus sp*	Cairo	5.2	0.054
8	*Bacillus sp*	Giza	4.6	0.058
9	*Bacillus sp*	Fayoum	5.5	0.061
10	*Bacillus sp*	Kaliobeya	5.1	0.053
11	*Bacillus sp*	Damietta	3.8	0.044
12	*Bacillus sp*	Dakahlia	4.2	0.034
13	*Bacillus sp*	Porsaid	4.5	0.072
14	*Bacillus sp*	Shrkia	4.1	0.040
15	*Bacillus sp*	Suez	5.2	0.097
16	*Bacillus sp*	BeniSuef	4.7	0.079
17	*Bacillus thuringiensis B.t40*	NRC*	4.4	0.064
18	*Bacillus subtilis ATCC168*	ATCC	4.7	0.076
19	*Bacillus licheniformsATCC14580*	ATCC	5.0	0.075

*NRC. National Research Center ,Microbial Genetics Dept.Dokki,Cairo,Egypt

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Plasmid DNA profiles of *Bacillus* isolates

Summary of results of plasmid profiling of the various isolates has shown in Fig.(1) 16 isolates and three identified strains (*Bacillus thuringiensis* subsp. *kurstaki* strain Bt40, *Bacillus subtilis* subsp. *subtilis* strain ATCC 168 and *Bacillus licheniforms* strain ATCC 14580**)**were ascertained for the presence of plasmids. The isolates from the various samples were determined for the presence of DNA plasmids using agarose gel electrophoresis. The results obtained revealed that the samples actually contained plasmids. The molecular weight of the plasmids was determined using lambda DNA digested with Hind III. The bands obtained from the plasmids are presented in Fig. (1). It was observed that the isolates contained plasmids with molecular weights ranging between 3958bp to 27344bp. A total of five different molecular weights were obtained for all the 19 strains**.** Fig. (1) Show the plasmid patterns of all the *Bacillus* type strains included in this report. All the strains contain at least one plasmid, and some strains have a maximum of seven plasmids. In general, all plasmid patterns are unique to each strain. Comparative resolution of megaplasmid bands (plasmids above the chromosomal DNA band) in the agarose gel was difficult and was an unreliable way to differentiate plasmid patterns. This is the reason why the comparison of plasmid patterns focused on the plasmids below the chromosomal band. However, this decision made it impossible to compare the plasmid patterns of those strains that contained only megaplasmids (Fig. 1). The strains (lane 2 to lane 10), showed similar migration of plasmids, and two monomorphic band at molecular weight 18125bp and 8411bp, and polymorphic percentage 77.7% also isolates lane 11-16 and lane 17, 18 and 19 identified strains (Fig.1) plasmids have the same size, and showed polymorphic percentage about 100%, the DNA sequence may be different, and should be considered for future work. The isolate (lanes 2, Fig. 1) showed unique plasmid patterns at molecular weight 9748 and lane 7 showed 2 unique bands at 6691 and 5085 bp. Also, the isolates 12, 16 and strain 19 (*Bacillus licheniforms*) showed unique patterns with 9 total numbers of bands.

All of nineteen strains, contained megaplasmids except strain 18 (*Bacillus subtilis*) that leads to the hypothesis that genes responsible for their efficiency are located on the chromosome [25], such genes encoding the Cry proteins are found in chromosomes or plasmids of different sizes.

Among the isolates and strains collected (lanes 1-19 Fig. 1) ten of them (strains 1, 4, 5, 6, 9, 10, 11, 16 ,17; *B.t*.40 and 18; *B.s* ATCC 168) showed a similar plasmid profile, have a five bands, with one small plasmids, except the isolate 7 presented three small plasmids (Fig. 1). All isolates collected had small plasmids varying in number from one to seven. Isolate 7 showed only seven plasmids (Fig.1), the similarity between the plasmid profile of all isolates reports and these results agreement with the results obtained previously [26]. Most *Bacillus* species, regardless of their source, harbor at least one indigenous plasmid [27]. Many of the functional differences between *B. cereus*, *B. thuringiensis* and *B. anthracis* are due to presence of plasmid that varies in number and size [28]. These results are in good agreement with previous investigations [29-30].

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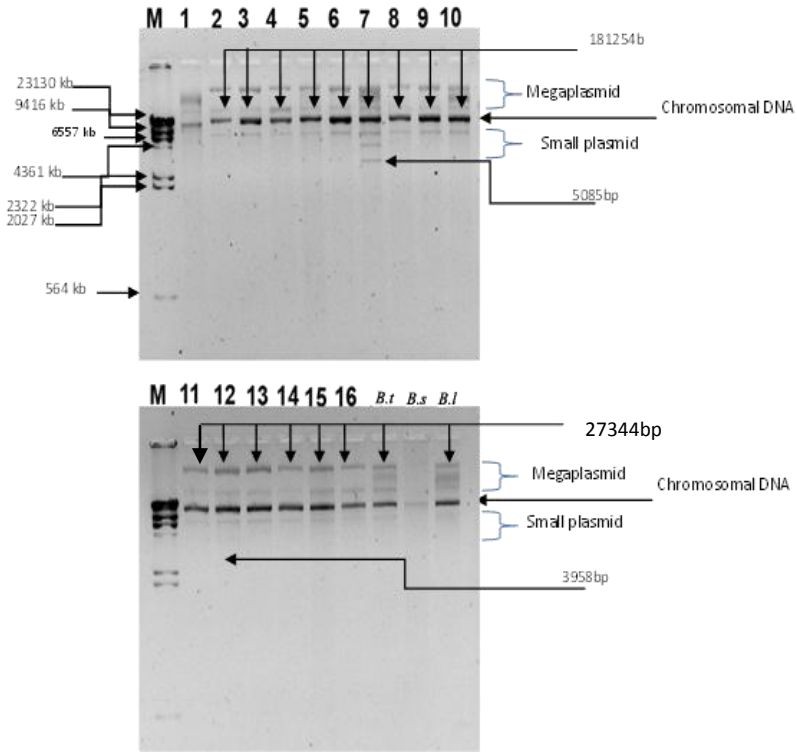

Figure 1: Electrophoretic pattern for plasmid profile of the 16 local *Bacillus* isolates (lane 2-16) and three identified strains (*B.t. B.s* and *B.l*). M, Lambda DNA digested with HindIII.

Cluster analysis with SPSS: Hierarchical Cluster Analysis.

Cluster analysis works upwards to place every variable (strain) into a single cluster. The main part of the output from SPSS is the Dendrogram that represents the phylogenetic relationships among the variable (strains) under study. The dendrogram for plasmid profiles banding patterns of the all strains using average linkage (between groups) is presented in (Fig.2). The horizontal dotted line of the dendrogram indicates the rescaled distance, in which the clusters are formed. Two main clusters were apparent in the dendrogram made by the Jaccard analysis method using similarity matrices based on hybridization information (Fig.2).

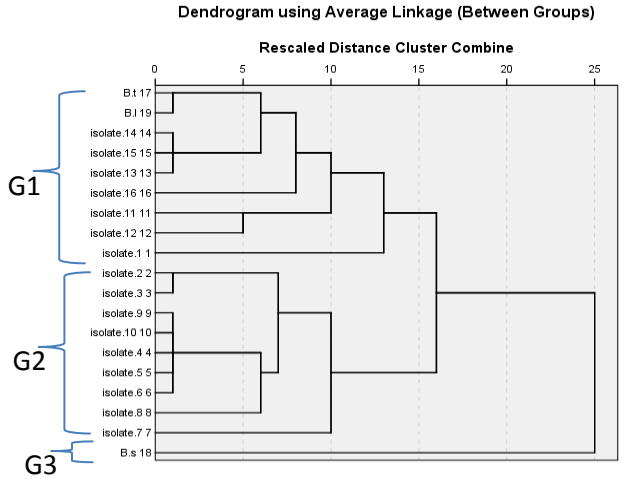

Figure 2: Dendrogram showing the genetic relationship among the 16 local *Bacillus* isolates and three identified strains (*B.t., B.s.* and *B.l.*).

PCR amplification of the 16S rDNA gene

The 16S rRNA gene sequence is about 1,550 bp long and is composed of both variable and conserved regions. The gene is large enough, with sufficient interspecific polymorphisms of 16S rRNA gene, to provide distinguishing and statistically valid measurements. Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene and at either the 540-bp region or at the end of the whole sequence (about the 1,550-bp region), and the sequence of the variable region in between is used for the comparative taxonomy [31-32].

PCR targeting the 16S rRNA has been used extensively to study prokaryote diversity and allows identification of prokaryotes as well as the prediction of Phylogenetic relationships [33]. Nucleic acids extracted from the formed bacterial colonies were subjected to primer pair designation to amplify a region of the 16S rRNA gene and all produced an expected size (1550 bp), as shown in Figure (3). This result does not indicate that the tested bacteria belong to a specific genus or a particular species, because the primer pair used was a universal eubacterial primer designed to classify related bacteria according to sequencing results [34].

16S rRNA sequencing and PCR.

The 16S rRNA gene sequence is about 1,550 bp long and is composed of both variable and conserved regions. The gene is large enough, with sufficient interspecific polymorphisms of 16S rRNA gene, to provide distinguishing and statistically valid measurements. Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene and at either the 540-bp region or at the end of the whole sequence (about the 1,550-bp region), and the sequence of the variable region in between is used for the comparative taxonomy [30-31]. Although 500 and 1,500 bp is common lengths to sequence and compare, sequences in databases can be of various lengths. The 16S rRNA gene sequence has been determined for a large number of strains. GenBank, the largest databank of nucleotide sequences, has over 20 million deposited sequences, of which over 90,000 are of 16S rRNA gene. This means that there are many previously deposited sequences against which to compare the sequence of an unknown strain. Lastly, the 16S rRNA gene is universal in bacteria, and so relationships can be measured among all bacteria [35-36] (Fig. 3). In general, the comparison of the 16S rRNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels, including what we now call the species and subspecies level. The occasional exceptions to the usefulness of 16S rRNA gene sequencing usually relate to more than one well-known species having the same or very similar sequences.

In the present study we determined the 16S rRNA gene sequence of 16 isolates of *Bacillus* form Egyptian soil and three identified strains *B.t*. *B.s* and *B.l* (Table 2). All strains divided into five groups depending on their plasmids pattern and polysaccharide production Fig.2 and Table 2. The phylogenetic tree of the five groups based on 16S rRNA sequences was generated in comparison with 100 Genbank 16S *Bacillus* partial sequences. In silico analysis;(computer simulation); the generated showed that the 16S sequence size for the

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five groups ranged between 641 to 1193 nt, while their counterparts in the Genbank ranged between 1486-1487 nt. The percentage for GC content for all five 16S rRNA gene sequences ranged between 52-54%.

Using BLAST search, we found that all strains belonged to species *Bacillus.* The identities of the 16 soil *Bacillus* isolates were determined by comparing them to the available 16S rRNA sequences found in Genbank and with high-scored rRNA sequences in BLAST searches. BLAST similarity scores ranged between 97% to 95%, where 100 matches of 16S rRNA gene sequence were included in the analysis.

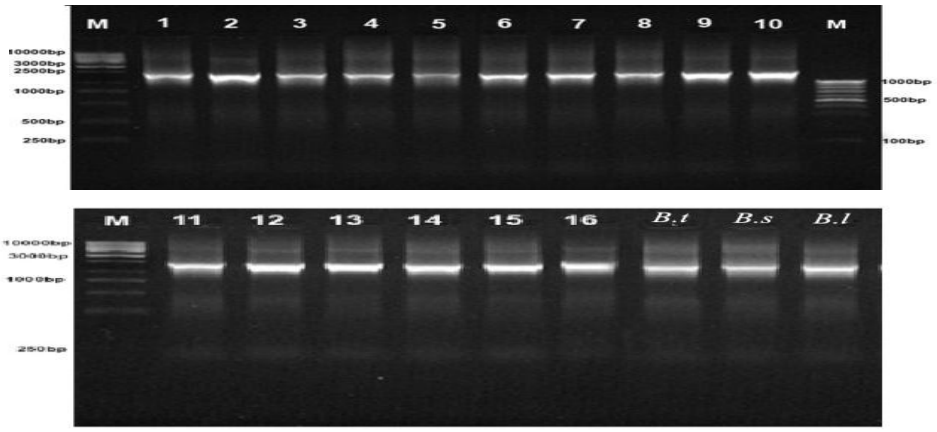

Figure 3: Full-length 16S rRNA gene (1500 bp) of the 16 *Bacillus* isolates and three identified strains (*B.t*. *B.s* and *B.l*). amplified with universal primers. The amplicon was electrophoretically resolved on a 1.2% agarose gel in 1X TBE buffer Lane1: DNA ladder, Lane 1-20: amplified product of full length 16S rRNA gene.

16S rRNA gene sequence similarity and Phylogenetic analysis:

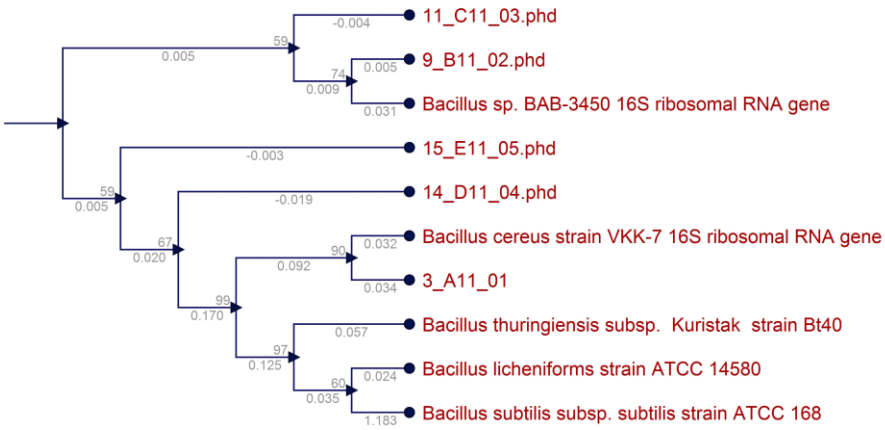

Figure 4: Phylogenetic tree based on partial 16S rDNA sequences, showing the relationship between isolates 3_A11, 9_B11, 11_C11, 14_D11 and 15 _E11 and other species belong to the genus *Bacillus*. The tree was constructed using the CLUSTAL-X and neighbour-joining method.

Molecular identification of the isolated strains was carried out based on 16S rRNA sequence analysis. The partial sequence of 16SrRNA obtained from *Bacillus* strains were aligned with all the presently available 16S rRNA sequences in the GenBank data base**.** Species identity of the five strains was further confirmation by molecular study of 16S rRNA gene. The query sequence 3_A11 showed closely similarity with the strain *Bacillus cereus* Vkk-7 also, strains 9_B11 and 11_C11 are placed in the same group. The rest strains 14_E11 and 15_E11 are related to the group of *Bacillus sp*. strain BAB 3450 .the NCBI BLAST suggests the relatedness of the isolates with same and identity within the genus *Bacillus*.

As a result, a Phylogenetic tree was mapped using the neighbor joining method, and is shown in Fig.(4). Phylogenetic analysis using the 16S rRNA sequences indicated that isolate (3_A11,9_B11, 11_C11 , 14_D11 and 15 _E11) belonged to the genus *Bacillus*, according to blast result shown in Fig.(4) isolate (3_A11,14_D11 and 15 _E11)) were identified as *Bacillus cereus* VKK-7 and isolates (9_B11, 11_C11) were identified as *Bacillus sp***.** BAB3450 .

Protein Profiles of *Bacillus* Isolates

Sodium Dedocyle Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis is generally used to compare protein profiles of *Bacillus* isolates. For example, [37] used SDS-PAGE analysis in order to determine the differences among the *Bacillus* isolates collected from different aria in Egypt and three identified strains (*B.t*. *B.s* and *B.l*). SDS-PAGE of whole-cell protein extract of sixteen bacterial isolates and three identified strains (*B.t*. *B.s* and *B.l*) showed a high heterogeneous profile (Fig.5). The main difference in protein pattern was related to some high-accumulated polypeptides with different molecular weight present in almost strains. Protein profile allowed the comparison of the nineteen strains which were distributed into five groups of bacteria; two main group and 3 individuals.

The high level of protein polymorphism observed in bacteria isolates, indicates that protein profile is an effective method for bacterial fingerprinting when a high number of isolates are necessary to be identified. Furthermore, SDS-PAGE could be an inexpensive and fast procedure allowing the rational use of microorganism collections.

The Second level information for a cell, other than sequencing of bacterial genome, can be obtained from cellular protein profiles. Different types of electrophoresis were used to explore the profiles. The protein profiles produced y SDS-PAGE of whole cell extract have been found correlates closely with DNA-DNA hybridization on results suggests it could be appropriate to use SDS-PAGE for rapid bacterial identification [38-39-40]. Combination of polyacrylamide gel electrophoresis (PAGE) of proteins with computerized analysis of profiles provided an effective approach to investigate the taxonomic relationships among many bacterial species [41]. In addition, the results of 16S rRNA sequence analysis reconfirm the insufficient defined genera on the basis of phenotypic criteria [36]. Figure (4) shows the whole -cell protein profiles of selected *Bacillus* strains obtained by sodium dodecyl sulphate polyacrylamide gel electrophoresis. A dendrogram produced after numerical analysis of the whole- cell protein profiles using the Neiand Li, coefficient and UN weighted pair group method with arithmetic averages algorithm (UPGMA) is shown in Figure (4) Numerical analysis revealed clearly two main clusters and three separate isolates in individual; in

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individual; B13, B1, B11 and B18 as shown in the dendrogram (Fig. 4) Cluster 1 divided into four subgroup. Strain B15 and B16 was closely related together with 100% similarity this strain; B15; belongs to *B. cereus*. in 16S rRNA. All strains in this cluster are belongs to *B. cereus.* The cluster 2 divided into 3 subgroup; the strains of the subgroup1; B7,B8 is closely related also B6 and B9 in subgroup 2 also closed together and belongs to *B. cereus* ,B3, is nearst to strain 4, 5 and 2. The strain B3 (3A_11_01) which gave similarity with *Bacillus cereus* in 16S rRNA(Fig.4). The same result B11(11_c11_03) and B9(9_B11_02) were belongs to *B. cereus.* Our results have showed that electrophoretic methods can vide valuable information which may be used identification of Bacillus strains. These results are in good agreement with previous researches [40-42]. It is known that protein profiles of whole-cell and extracellular protein are good enough to distinguish most of bacterial genera at species level [40]. Some researchers [19-43] also differentiated the strains of *Bacillus subtilis* and strains of *Bacillus pumilus* species by whole-cell proteins using SDS PAGE at the subspecies level. Numerical analysis of SDS-PAGE of whole-cell proteins is an extremely useful in taxonomic assessment in studying *Bacillus* species [19].

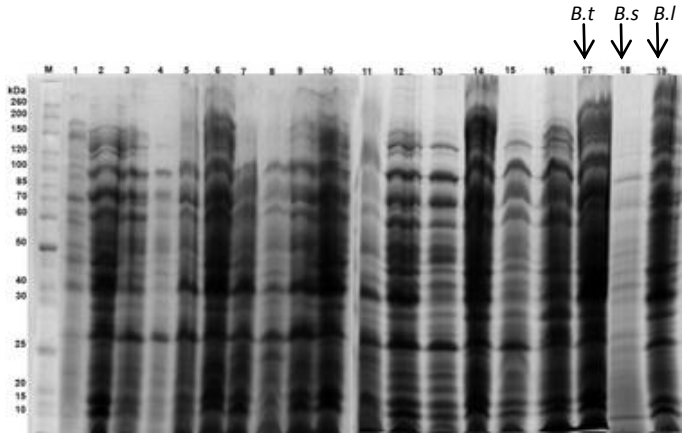

Figure 4: Electrophoretic profile (SDS-PAGE) of whole protein extract of 16 bacteria isolated from Egyptian soil and three identified strains (*B.t.*, *B.s* and *B.l*). Numbers 1 to 20 indicate bacterial isolates 1 to 20, respectively. M = Protein Molecular weight markers, PageRuler™ Unstained Protein Ladder (Fermentas).

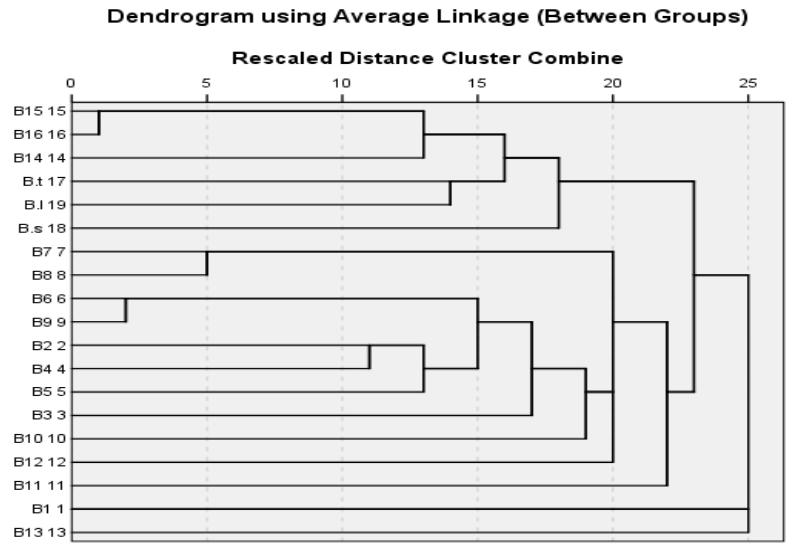

Figure 5: Dendrogram based on u nweighted pair group method with arithmetic averages algorithm (UPGMA) of the protein patterns of whole-cell of of the 16 *Bacillus* isolates and three identified strains (*B.t. B.s* and *B.l*).

CONCLUSION

The characterization of the isolates was performed using various morphological, biochemical and physiological parameters. The results indicate the identity of the isolates under the genus *Bacillus*. Molecular characterization of based on 16S rRNA and NCBI BLAST search confirms the identity of these isolates close to the *Bacillus cereus* and *Bacillus sp*. The results obtained in this study showed that a combined identification method using SDS-PAGE patterns of whole-cell proteins and subsequent 16S rRNA gene sequence analysis could successfully identify *Bacillus* species isolated from soils. The identification of *Bacillus* species based on whole-cell protein patterns by SDS-PAGE has not been previously reported. This result suggests that such an approach will be useful for grouping and identifying *Bacillus* species in soils, as a complementary identification method.

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