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16s rDNA sequence characterization and homogeneity of halophilic *Bacillus* sp. isolated from athalosaline lake (Al Jouf, Saudi Arabia)

Maher M. Al-Enazi[1,2], A. Z. El-Bahrawy[3], Bilal Ahmad Tantry[4] and Rahiman Shaik[5]*

[1]Vice Rector of Academic Affairs, Aljouf Univeristy, Saudi Arabia.
[2]Department of Clinical Laboratory Sciences, College of Applied Medical sciences, Salman Bin Abdulaziz University, Saudi Arabia.
[3]Faculty of Science, biology department , Aljouf University, Saudi Arabia.
[4]Department of Microbiology, College of Medicine, Aljouf University, Saudi Arabia.
[5]Department of Biochemistry, College of Medicine, Aljouf University, Saudi Arabia.

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Moderate halophilic bacteria from Dowma al Jandal in extreme north Al Jouf were isolated using cultural-dependent methods. Halophiles from hypersaline environments possess a biotechnological potential. Polymerase chain reaction (PCR) amplification of the 16s rRNA gene and phylogentic analysis were used to identify strains. Culturing was done aerobically in chemically defined media (CDM). Salt concentration (15%) was used at pH 7.2. Universal bacterial primers were used to amplify 16SrDNA from chromosomal DNA isolated from the four distinct colonies. Four moderate halophilic bacterial isolates were analyzed and identified wih 16srDNA sequencing as *Virgibacillus salarius, Bacillus subtilis, Bacillus* sp., and *Virgibacillus marismortui*. Comparison of the 16srDNA sequence alignment to reference sequence data bases showed samples M1, M2, M3 and M4 have 95-99% homology. All of the four isolates had at least 95% similarity to the published sequences implying that they could be species within the described genera.

Key words: Moderate halophiles, hypersaline lake, *Bacillus* sp., 16srDNA sequencing.

INTRODUCTION

Halophiles employ different morphological, physiological, and genetic mechanisms to withstand the environmental conditions in which they live. In recent decades many halophilic microorganisms have been isolated from many athalosaline environments, such as the Dead lake (Arahal et al., 1999), the Great Salt Lake (Waino et al., 2000), the Solar Lake (Cytryn et al., 2000) and the Wadi lake (Weisser and Truper, 1985). Earlier studies reported the significance of halophilic microorganisms in the biogeochemistry of carbon and phosphorus in saline environments (Sánchez-Román et al., 2007) and degradation of organic compounds for potential use in bioremediation studies (Zhao et al., 2009,

*Corresponding author. Email: rahimhi@gmail.com. Tel: +966502450484.

Al-Mailem et al., 2010) and also used as biocontrol agents against certain pathogenic fungi (Sadfi-Zouaoui et al., 2008, Chen et al., 2010). The diversity amongst halophiles among the domains of bacteria, archaea, and eukarya depends on the salinity, temperature, pH and redox conditions of the environments that the organisms are adapted (Oren, 2008). Most of the halophilic and halotolerant micro-organisms represent the domain bacteria that contain moderate rather than extreme halophiles in its phylo-genetic subgroups (Oren, 2002). Kushner (1978) defined the moderate halophiles as organisms growing optimally between 0.5 and 2.5 M salt. Recent studies showed that an extensive research work has been done on isolation and characterization of large number of moderately halo-philic Gram-positive, endospore-forming aquatic isolates in the genus. *Bacillus* have been reported but most of that belongs to marine environments (Yoon et al, 2003; Noguchi et al., 2004; Lee et al., 2006). However, limited studies have been done regarding the microbial species inhabiting athalosaline environments (Lim et al., 2006; Souza et al., 2006). In this study, we characterized the moderate microorganisms using 16S rDNA sequencing techniques and compared the homogeneity with the relevant microorganism by NCBI BLAST analysis.

MATERIALS AND METHODS

Sample collection

Four saline soil samples were collected from Dowma Al Jandal athalosaline lake from random locations along the lake shore at 29° 48′ 41.1″ N, 39° 52′ 5.9″ E. Sample M1, M2 was obtained from loose saline soil. The third and fourth (M3, M4 respectively) was obtained from the lake sediment at 10 cm depth. Soil samples were collected in sterile bags and appropriately labeled and dated. The samples were transported in a cool box with ice packs and transported to the laboratory and stored at 4°C in College of Medicine Aljouf University. Samples were processed within 24 h of collection. Salinity of the saline soil samples was measured using a conductivity meter model KL-1385 (Kelilong Electron Co. Ltd, China).

Sample culturing

Soil sample (5 g) was inoculated into 45 ml on chemically defined medium (CDM). The contents of the CDM per liter were as follows: NaCl (81.0 g), $MgCl_2{\cdot}6H_2O$ (7.0 g), $MgSO_4{\cdot}7H_2O$ (9.6 g),$CaCl_2{\cdot}2H_2O$ (0.36 g), KCl (2.0 g), $NaHCO_3$ (60.0 mg), NaBr (26.0 mg), Ferric chloride (trace), Peptone (5.0 g), yeast extract (10.0 g), glucose (1.0 g), pH 7.2 ± 0.1 at 25°C (Kushner DJ, 1993). All components were added to distilled water and volume was brought up to 1.0 L. The medium was mixed thoroughly and gently heated until dissolved and then autoclaved. After 5 days of incubation, serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) of soil sample were made in sterile saline (0.85%). A 100 µL aliquot of each diluted sample was spread on chemically defined medium (CDM) agar (15-20% NaCl w/v) plates. Dilution of each soil sample was analyzed in triplicate. The plates were inverted and incubated for 3 to 7 days at 38°C. Results were recorded as colony forming unit (CFU). Colonies growing on the plates were counted and the density of microorganisms in the original sample was estimated by multiplying the colony count times the dilution.

Light microscopy

On day 7, selected isolated colony samples were characterized by gram-stain and by observed cell morphology (shape, size and color) using a light microscope (Leica DMD108 digital microscope).

Genomic DNA extraction

Genomic DNA was extracted from 5 ml late exponential Phase of culture growth, using Invitrogen Kit (USA) as per manufacture instructions.

PCR amplification and 16s ribosomal RNA sequencing

PCR amplification of the 16S rRNA gene from the purified genomic DNAs, was performed using ABI 9700 thermocycler (Applied Biosystems) using the primer sets, 16S forward primer AGAGTTTGATCHYGGYTYAG and the 16S reverse primer ACGGCTACCTTGTTACGACTT.

Purification of PCR products by gel elution protocol

The purification of the PCR products was done by using inhouse Gel elution kit.

DNA sequencing and analysis

Sequencing was done by using ABI 3730XLS sequencer. The unincorporated dye terminators were removed by using ABI Big dye Terminator kit. The chromatograms were analyzed by Genetool version 1.0. Assembly program is also carried out using the same program. The assembled sequences were analyzed by NCBI BLAST program (hhttp://ncbi.nlm.nih.gov/BLAST) to identify the microorganisms and compared with the published sequences.

RESULTS AND DISCUSSION

The four distinct moderate halophilic bacteria obtained from the saline soil of the Dowma al Jandal Lake designnated as M1, M2, M3 and M4 were analyzed. The isolates were identified as *Virgibacillus salarius, Bacillus subtilis, Bacillus* sp*. and Virgibacillus marismortui.* Mentioned isolates belong to the phylum of *Firmicutes* and *Bacillaceae* family. The salinity of the Dowma al Jandal athalosaline lake samples varied from a low of 1.3% to a high of 21%. Significant growth was observed after 5-7 days of the incubation, and occurred at 3-15% (W/V), NaCl (optimum 15%), pH 5-9 (optimum 7.2) and temperature 35-40°C (optimum 38°C). All the isolates were Gram positive, strictly aerobic rods, occurring in pairs or small chains, cells were motile, colonies were white to pale yellow and mucoid. And were strongly catalase and oxidase positive.

Alignment and sequencing comparison of samples M1 and M4 show high homology (95-98%) to*Virgibacillus* sp. SK31 and *V. marismortui* TPA3-3 respectively, as shown

Table 1. BLAST Similarity Search Results for M1: Similarity to *Virgibacillus salarius* strain SA-Vb1 by 16S ribosomal RNA gene partial sequence.

Accession	Description	Max score	Total score	Max Identity (%)
NR_041270.1	*Virgibacillus salarius* strain SA-Vb1 16S ribosomal RNA, complete sequence	2372	2372	96
GU397387.1	*Virgibacillus marismortui* strain B33 16S ribosomal RNA gene, partial sequence	2368	2368	96
JN998437.1	*Virgibacillus* sp. SK2 16S ribosomal RNA gene, partial sequence	2366	2366	96
GQ181204.1	*Virgibacillus* sp. Ez223 16S ribosomal RNA gene, partial sequence	2366	2366	96

Table 2. BLAST Similarity Search Results for M2 : Similarity to *Bacillus subtilis* strain H12 by 16S ribosomal RNA gene, partial sequence.

Accession	Description	Max score	Total score	Max Identity (%)
KC441785.1	*Bacillus subtilis* strain H12 16S ribosomal RNA gene, partial sequence	2590	2590	99
JN641292.1	*Geobacillus stearothermophilus* strain DDKRC4 16S ribosomal RNA gene, partial sequence	2588	2588	99
JF411313.1	*Bacillus tequilensis* strain KM34 16S ribosomal RNA gene, partial sequence	2588	2588	99
JF411297.1	*Bacillus tequilensis* strain M60 16S ribosomal RNA gene, partial sequence	2588	2588	99

Table 3. BLAST Similarity Search Results for M3: Similarity *to Bacillus* sp. 2BSG-10NA-12 gene by 16S ribosomal RNA gene, partial sequence.

Accession	Description	Max score	Total score	Max Identity (%)
AB533728.1	*Bacillus* sp. 2BSG-10NA-12 gene for 16S ribosomal RNA, partial sequence	2440	2440	97
AB533758.1	*Bacillus* sp. 2BSG-PDA-4 gene for 16S ribosomal RNA, partial sequence	2435	2435	97
AB533743.1	*Bacillus* sp. 2BSG-10TSA-3 gene for 16S ribosomal RNA, partial sequence	2435	2435	97
GQ407241.1	*Bacillus* sp. DV2-37 16S ribosomal RNA gene, partial sequence	2435	2435	97
JF411333.1	Uncultured *Bacillus* sp. clone GT1-7 16S ribosomal RNA gene, partial sequence	2431	2431	97

in Tables 1 and 4. Samples M2 and M3 demonstrated close relation (97-99%) to *B. subtilis* H12 and Bacillus *sp*. 2BSG-10NA, respectively (Tables 2 and 3). All the isolates had at least 95% and up to 9% similarity to the described sequences within the phylum of *Firmicutes* and which in turn means that the isolates are closely related to genus level and belong to different species. Phylogenetic analysis gives an idea of relationship between the isolates which was made on the basis of 16S rDNA data. Phylogentic interferences made on the basis of 16S rDNA data indicated that the four isolates may be previously undescribed halophilic bacteria. It was the first molecular study conducted by us on virgin athalosaline lake in Dowma al Jandal. These four isolates may be previously undescribed moderate halophilic bacteria which belong to domain bacteria (Oren, 2002). The growth of halophiles mainly depends upon the salt concentration and other factors, like pH and media composition (Oren, 2002). Till date, the MIDI Sherlock microbial identification system does not contain the library for halophilic bacteria. The data and isolates obtained by us can be used to develop the database in MIDI system library for future identification of moderate halophilic bacteria. Furthermore, library can be constructed if the diversity of halophiles are extensively studied throughout the world. Building up a library for halophiles can assist the accurate identification of novel moderate halophiles. Molecular method is the most accurate way to identify the microorganisms than by the

Table 4. BLAST Similarity Search Results for M4: Similarity to *Virgibacillus marismortui* strain TPA3-3 by 16S ribosomal RNA gene, partial sequence.

Accession	Description	Max score	Total score	Max Identity (%)
GU172145.1	*Virgibacillus marismortui* strain TPA3-3 16S ribosomal RNA gene, partial sequence	1877	2440	95
EU435360.1	*Virgibacillus sp*. B1-21 16S ribosomal RNA gene, partial sequence	1871	2435	95
AY505533.1	*Virgibacillus marismortui* strain GSP17 16S ribosomal RNA gene, partial sequence	1871	2435	95
JN624920.1	*Virgibacillus* sp. NBSL35 16S ribosomal RNA gene, partial sequence	1869	2435	95
JF680941.1	*Virgibacillus sp*. IEGM 795 16S ribosomal RNA gene, partial sequence	1868	2431	95

identification of conventional methods, as a matter of fact the later can lead to identification problems.

Conclusion

The extreme environment of the Dowma al Jandal Lake still remains a virgin in exploring of halophiles. The results of this study will be useful to produce novel enzymes that will have potential biotechnological applications in biogeochemistry, bioremediation and can be applied as biocontrol agents. Such a unique environment in this lake provides a scope for further study of prokaryotic diversity.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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