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Full Length Research Paper

Isolation and characterization of Gram negative obligate and facultative alkalophilic *Bacillus* sp. from desert soil of Saudi Arabia

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Five new alkalophilic strains were isolated from soil samples collected from the western region of Saudi Arabia. They were identified by partial sequencing of 16s rRNA polymerase chain reaction (PCR) amplicons and morphological and biochemical characterization. Two of the isolates (number 2 and 3) were found to be facultative alkalophiles, while the other three (numbers 1, 4 and 5) were found to be obligate alkalophiles. Total protein profile analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed no significant differences between isolates No. 2 and No. 3. On the other hand, there were major differences recognized in the banding pattern of the protein for isolate numbers 1, 4 and 5. All the isolates were 98% identical to *Bacillus cohnii* at the 16s rRNA gene.

Key words: *Bacillus cohnii*, 16SrRNA, Alkalophilic bacteria.

INTRODUCTION

The adaption of microorganisms to alkaline environments has been studied over the last three decades in many laboratories all over the world (Muntyan et al., 2005). The genus *Bacillus* consists of an extremely diverse collection of aerobic or facultatively anaerobic, rod shaped endospore forming bacteria that are ubiquitous in many environments (Xu and Cote 2003; Claus & Barkeley 1986). In the last decade, characterization of *Bacillus* species has led to the formation of several new genera. Members of the *Bacillus* genus exhibit a wide range of nutritional requirements, growth conditions and metabolic diversity (Joung and Cote, 2002). Furthermore, *Bacillus* species have fast growth rates and can secret proteins into their growth medium, and they often produce antibiotics, which make them ideal candidates for industrial applications, such as, degradation of starches and production of bioinsecticides, vitamins, antibiotics, flavor enhancers and biodegradation of plastics. More specifically, alkalophilic *Bacillus* species often produce industrially important alkaline stable enzymes, such as, protease, amylases and cellulases(Marie et al.,2005). Recently, studies on the classification and characterization of alkalophilic *Bacillus* strains have been increasing because these strains possess commercially valuable enzymes (Schallmey et al., 2004). To date, several researchers have identified and characterized alkalophilic *Bacillus* strains using phenotypic characteristics, DNA-DNA relatedness data, and analysis of the 16SrRNA sequence (Felske et al., 2003). Although, these methods have been used for the classification of alkalophilic *Bacillus* species, but the characterization of these microorganisms is considered complicated due to their slow growth and their extreme pH which interfere with the results of phenotypic tests. Alkalophilic bacteria that grow well at pH range of 10 to 11 are widely distributed throughout the world and have been isolated from a variety of ecosystems including soil (Guffanti et al., 1986). There are only a few reports on alkalophilic bacteria isolated from Saudi Arabia (Salama et al., 1993). The objectives of this study were to isolate, characterize and identify alkalophilic bacteria from the western region of Kingdom of Saudi Arabia. Strains were characterized using phenotypic characteristics, 16SrRNA gene sequencing and SDS-PAGE protein profiles.

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Table 1. Showing the primers used in amplification of specific 16S rRNA and for DNA sequencing.

No	Primer	Sequence	Product size
F	F63	'5- gcctaacacatgcaagtc-3'	1387bp
R	R1387	5'- gggcggwgtgtacaaggc-3'	Primer A
F	F16 S1	5'- ccggaggaaggtggggatg-3'	290bp
R	R16S2 (270)	5'-CCCACTGCTGCCTCCCGTAGGAGT-3'	Primer B

MATERIALS AND METHODS

Collection of soil samples

A total of 50 soil samples were collected from different locations surrounding Al-Qunfudhah city (beach sand) (41.5E and 19.8N) located in the western region of Saudi Arabia. Samples were collected from 2 to 5 cm below the surface with a shovel. Each soil sample was placed in a plastic bag at ambient temperature following the method described by Horikoshi and Akiba (1982). Samples were stored in ice until (at about +4°C) they were transported to the laboratory.

Growth medium and isolation

The samples were first incubated for 10 min at 80°C in a water bath to kill most of the vegetative cells (Mora et al., 1998). Different dilutions of samples were then plated on M1 agar medium as described by Horikoshi and Akiba (1982). The contents of the medium were 1% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4$ $7H_2O$, 2% Agar, pH 10.5. The solution of Na_2CO_3 was autoclaved separately and added to the medium. Plates were incubated at 30°C for 48 h. Single colonies showing different morphologies were picked and re-streaked for 2 to 3 times on agar medium until single uniform colonies were obtained. Isolates were then stored in 20% glycerol at -80°C. The recipe for liquid media was same as the composition of M1 medium but without addition of agar.

PCR of 16S rRNA genes sequencing

The isolates were identified using phenotypic characterization. Isolates were characterized by studying their biochemical and phenotypic properties (Cappuccino and Sharmen, 1999), in addition to 16S rRNA gene sequencing using primers as shown in Table 1. These primers were also used for PCR aplification of the 16S rRNA genes. Genomic DNA was isolated as described by Wang et al. (2001). Cells from 5 ml overnight culture for each isolate were harvested. Cell pellets were rinsed with 200 µl of NET buffer (0.1 M NaCl, 50 mM EDTA, 10 mM Tris-Cl, pH 8.0) and re-suspended in 200 µl of GET buffer (50 mM glucose,10 mM EDTA, 25 mM Tris-Cl, pH 8.0). 0.001 ug of lysozyme was added and the mixture was incubated at 37°C for 3 h. Twenty microliter of 10 mg/ml proteinase K was then added and the mixture was incubated at 37°C for 1 h. One hundred microliter 10% SDS was then added, and the mixture was incubated at 37°C for 1 h. The mixture was extracted several times with phenol: chloroform: isoamyl alcohol (24 : 24 : 1, v/v) until the interface was clear. DNA was precipitated by adding 1/25 volume of 5 M NaCl and 2.5 volumes of 95% chilled ethanol. The precipitated DNA was rinsed with 1 ml of ice cold 70% ethanol, air dried, and re-suspended in 30 µl of sterilized distilled water. Selection of primers (Invitrogen, Paisley, UK) for PCR was according to Marchesi et al. (1998); primers sequences are shown in Table 1. The PCR reaction mixture (50 µl total volume) contained 200 µM of each dNTP, 0.5 for each µM primer, 10 mM tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 2.5 U Taq polymerase (ABgene, Surry, UK) and 100 ng of template DNA. DNA amplification using primers (B)F-16S-1 and R-16S-2 was performed at the following temperature cycle: denaturation at 94°C for 5 min, 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, final extension at 72°C for 7 min, respectively. For primers (A) F63 and R1378, the cycling program was 95°C for 3 min, 30 cycles at 95°C for 30 s, 55°C for 15 s and 72°C for 1.5 min, with the final extension at 72°C for 5 min. A total of 10 µl of PCR products were analyzed by 1.5% agarose gel (Bioline, London, UK) electrophoresis and made visible by ethidium bromide (0.5 mg/ml) staining and ultraviolet (UV) transillumination. Sequencing of PCR products was performed by the research team of the biotechnology lab company, Cairo, Egypt; following the procedure described by Sanger et al. (1977).

Total protein analysis by SDS-PAGE

Total protein from the five isolates was determined using SDS-PAGE. Isolates were grown in M1 broth for 48 h at 30°C and samples were taken when optimal dietary (OD) reaches 0.6. Protein analysis was performed on 10% SDS-PAGE. Protein samples were boiled for 5 min in loading buffer containing 5% β-mercaptoethanol then centrifuged at 10,000 g for 3 min and directly loaded the gel. Protein electrophoresis was performed in vertical sub-cells (Bio-RAD, England). Slab gels containing 10% (w/v) resolving gel and 5% stacking gel concentrations of acrylamide were run at a constant current of 80V for 2 h. The proteins on PAGE gels were fixed in 45% methanol and 10% acetic acid in distilled water stained with 0.25% Coomassie brilliant blue R-250 previously dissolved in 10% acetic acid, 50% methanol and water. The gels were destined for 3 h in 5% methanol and 7% acetic acid in distilled water (Laemmli, 1970).

RESULTS AND DISCUSSION

Morphological and phenotypic characterization

An extreme pH environment interferes with ion transport across cell membranes, and can also damage cellular components, such as, DNA and RNA. Because our understanding of the adaption of extreme alkalophiles is very limited, identifying the unique properties of alkalophiles may contribute new insights on this field. A total of 50 samples were collected from different areas surrounding Al-Qunfudhah city, western Saudi Arabia. Strains were examined for their alkalophilic properties; of the 50 isolates, only 5 were culturable in M1 medium agar at pH 7 and 10. Two of the strains were able to grow at both pH (facultative alkalophilic, numbers 2 and 3),

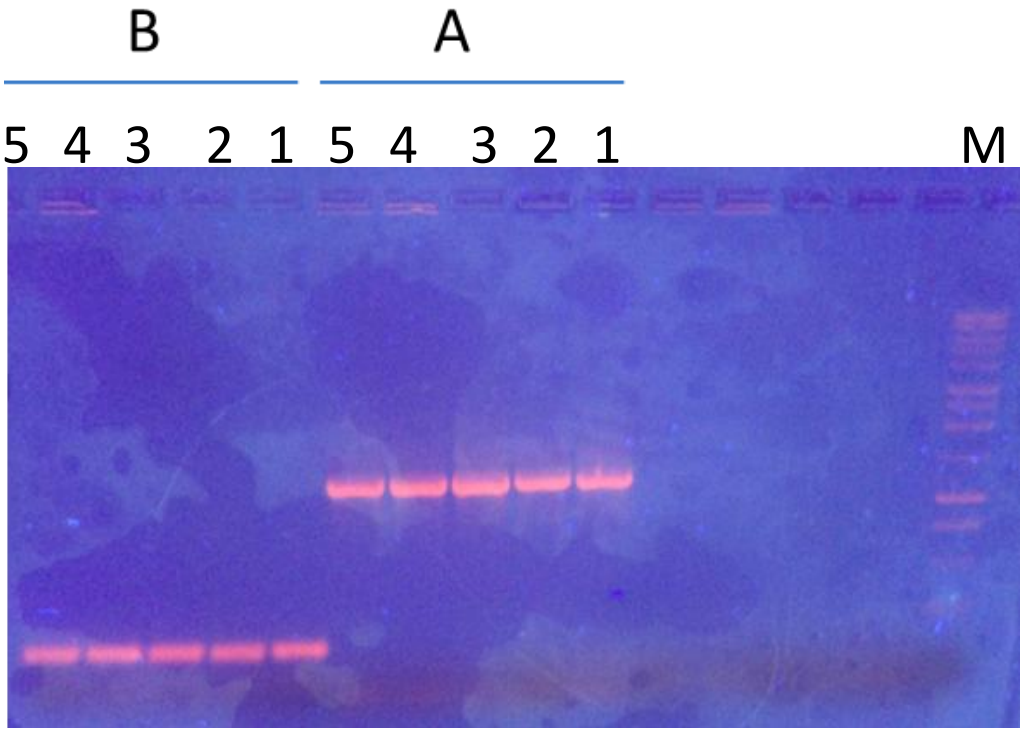

Figure 1. Agarose gel electrophoresis of PCR products of the 16S rRNA fragments Panel A with primer (A) or isolates number 1, 2, 3, 4 and 5. Panel B: with primer (B) for isolates 1, 2, 3, 4 and 5 lane M: 1Kb DNA ladder.

whereas the other three isolates (numbers 1, 4 and 5) were only able to grow at pH 10 (obligate alkalophilic). Gram staining of microorganisms is the first step towards identification and taxonomic characterization (Gram, 1884). All isolates were aerobic, motile, spore forming, Gram-negative staining and straight rods. This phenomenon which is spore-formers would stain Gram-negative, similar to that shown by Gram negative *Bacillus subterraneus* sp. nov. (Kanso et al., 2002; Ashis and Sudhir, 2010). During Gram-staining, the crystal violet iodine dye was extracted easily from the cells when samples were stained within 12 h of culture. After treatment with ethanol, the rate of extraction was not as rapid as shown by Gram-negative bacteria. This indicates that the peptidoglycan layers of the isolates are thicker than those of Gram negative bacteria, but thinner than that of ordinary Gram positive bacteria (Yumoto et al., 1998). These strains therefore represent unique members of the genus *Bacillus* as there are few reports available on Gram-negative *Bacillus* species (Yumoto et al., 1998; Kanso et al., 2002). It is to be noted that certain organisms with a defective cell wall structure may also stain Gram-negative (Wiege, 1981).

PCR of 16S rRNA genes sequencing

There is a diverse group of *Bacillus* species living in highly alkaline terrestrial and aquatic environments. These species are difficult to identify by traditional methods based on phenotypic characteristics (Woese, 1987). In past decades, there was a full revision of alkalophilic *Bacillus* classification according to their phenotypic characteristics (Fritze et al., 1990). Sequence analysis of a 16S rRNA hyper-variant region has been a widely accepted technique (Saitou, 1987), and was reported to be a useful tool in the discrimination between the species in the *Bacillus* group (Jill, 2004). To identify the taxonomy of our alkalophilic isolates, DNA was isolated and PCR amplification of the 16S rRNA was performed using two primer sets (Table 1). Primer set (A) was able to amplify a 1387 bp fragment and primer set (B) was able to amplify a 290bp fragment (Figure 1). Homologs of the deduced sequence were identified using BLAST and Gene Bank from the National Centre of Biotechnology, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov). The partial 16S rRNA gene sequence was aligned with reference homologous DNA sequences from Gene Bank using the multiple sequence alignment program in MEGA4. Alignment of the top 100 homologs identified by BLAST showed that the primers only targeted *Bacillus* genera. The results reveal that our 5 alkalophilic isolates each, shared 98% sequence similarity with *B. cohnii*, suggesting that our 5 isolates belong to this species. The 16s rRNA sequence data showed that isolates number 1, 2 and 3 had high

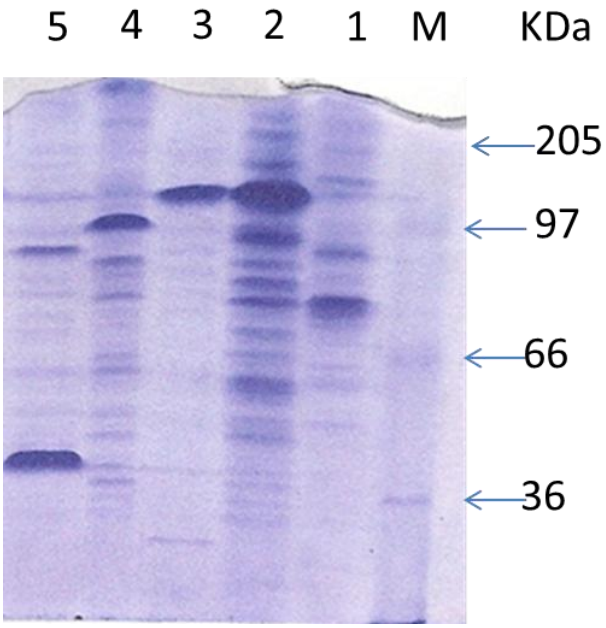

Figure 2. SDS-PAGE analysis of total cell protein profiles after 48 h growth of B. cohnii isolates. Lane M: High range proteins; marker: lanes 1 to 5 represent the isolates from 1 to 5 respectively.

similarity with *B. cohnii* strain D7023, while isolates number 4 and 5 had high similarity with *B. cohnii* strain T-46. All sequence data were deposited into Gene Bank with accession numbers: JN035905, JN035906, JN035907, JN035908 and JF957615.

Protein banding patterns

Protein profiles of whole cells are sufficient for distinguishing species of most bacterial genera, but not subspecies (Berber et al., 2003). However, the protein profiles produced by SDS-PAGE of whole cells and extracellular cells of *Bacillus* strains are useful for characterizing these microorganisms at species and subspecies level (Berber, 2004). The protein profiles of the alkalophilic isolates are shown in Figure 2. The protein profiles were visually inspected and compared. Isolates 2 and 3 had similar protein profiles and were distinguished from the other isolates by the presence of a highly expressed protein band at 130 KDa (Figure 2). Isolate number 1 was characterized by 2 bands at 75 and 85 KDa, isolate number 4 was characterized by a specific protein at 97KDa and isolate numbers 2 and 5 had proteins at 90 and 40 KDa respectively (Figure 2). The SDS-PAGE revealed that we have 4 different protein profiles for the 5 strains, suggesting we have isolated 4 subspecies of *B. cohnii.*

In conclusion the results obtained in this study suggest that variety of alkali tolerant *B. cohnii* strains inhibit the western region of KSA. Interestingly the SDS-PAGE revealed 4 unique protein profiles for the 5 strains, indicating that we have 4 subspecies of *B. cohnii*. Our results suggest that further study for these subspecies is warranted, because this may result in finding extracellular enzymes with novel proteins that could be useful for diverse industrial applications.

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