Isolation and Phylogenetic Identification of Halotolerant/Halophilic Bacteria from the Salt Mines of Karak, Pakistan

Aneela Roohi1,2, Iftikhar Ahmed1, Nauman Khalid2, Muhammad Iqbal1 and Muhammad Jamil1,2

1National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre (NARC), Park Road, Islamabad-45500, Pakistan
2Department of Biotechnology and Genetic Engineering, Kohat University of Science and Technology, Pakistan

Abstract

Extreme environments like salt mines are inhabited by a variety of bacteria that are well-adapted to such environments. The bacterial populations provide economic benefits in terms of enzymes synthesis. The salt mines of Karak region in Pakistan are extremely saline and the microbial communities found here have not yet been explored. In the present study, 57 halotolerant/halophilic bacterial strains were isolated from the salt mines of Karak. These strains were grown in media with 0-35% NaCl concentration. The morphological and physiological characteristics of the isolated strains were studied to optimize the growth conditions and to classify the isolated bacterial strains into slightly halotolerant/halophilic, moderately halophilic and extreme halophilic. The phylogenetic analyses inferred from 16S rRNA gene sequence of the isolated strains demonstrated that the major population were closely related to species belonging to Planococcus, Jeotgalicoccus, Staphylococcus, Halobacillus, Halomonas, Brevibacterium, Gracilibacillus, Kocuria, Salinivibrio, Salinicoccus, Oceanobacillus and Bacillus genera. Results showed that the salt mines of Karak region are rich in halotolerant/halophilic bacterial population with diverse bacterial communities, which may be utilized in various industrial applications after proper screening and identification. © 2014 Friends Science Publishers

Keywords: Extremophile; Halotolerant; Halophilic; Karak region; 16S rRNA gene sequence

Introduction

Bacteria play an important role in the development of viable habitats. Saline environments harbor taxonomically diverse group of bacteria that show improved structural and physiological characteristics in extreme (saline) conditions. To survive in saline environments, bacteria developed adaptive mechanisms like surface layer modifications especially in cellular phospholipid composition (Ventosa et al., 2008). An increasing interest in microorganisms from hyper-saline environments has resulted in the discovery of several new species and genera in Bacteria and Archaea domains (Woese and Fox, 1977). Extremely halophilic Archaea belong to the order Halobacteriales, which contains one family, the Halobacteriaceae (Ozcan et al., 2007). The family Halobacteriaceae belongs to domain Archaea and presently comprised of 40 genera (Euzéby, 2013).

Among the halophilic microorganisms, Archaea are the most common (Antón et al., 2000; Oren, 2002). Microorganisms that form part of domain Archaea require high salt concentration for growth and are coined as halobacteria (Kamekura, 1998). These groups of microbes have been isolated from diverse environments like salterns, salt lakes, salt marshes and saline desert soils (Ventosa et al., 2008). Halobacteria are either Gram-negative or Gram-positive and have aerobic, facultative anaerobic or obligatory anaerobic metabolism (Johnson et al., 2007). Halophilic bacteria have the ability to grow in media containing 3 to 15% (w/v) NaCl (Oren and Rodríguez-Valera, 2001; Oren, 2002). Halophilic microorganisms have many biotechnological applications like β-carotene production from fermented foods, hydrolytic enzymes or exopolysaccharides, stabilizers and other valuable compounds (Quesada et al., 1982, 2004; Ventosa et al., 2008).

Bahadur Kheil and Jatta Ismail Kheil are two of the biggest and well protected salt mines in Karak District, Khyberpakhtun Khwa (KPK) Province of Pakistan, and it has an estimated salt reserve of about 52,563 tons (PMDC, 2013). Being of high quality, Karak rock salt is consumed as table salt after a simple treatment instead of complex refining. However, there are insufficient reports on exploration of bacterial diversity with respect to halophilic/halotolerant characteristics from Pakistan (Roohi et al., 2012). Exploration of such diversity might result in considerable economic benefits in food and leather

The objective of this research was to explore the novelty associated with extremely halotolerant/halophilic aerobic or facultative anaerobic bacteria and to assess the bacterial biodiversity of these extremophiles from Pakistan using molecular techniques that has not been previously reported.

Materials and Methods

Collection of Samples

The samples were collected from the Karak Salt Mines of KPK Province, Pakistan. The area is situated at 32° 47 to 33° 28 North and 70° 30 to 71° 30 East. The sampling sites were divided into two different areas according to the difference in location of salt quarries, which are located at Bahadur Khel and Jatta Ismail Khel. Different salt mines were selected for the sampling (Fig. 1). The salt beds are approximately 105 m thick in Bahadur Khel, while more than 30 m thick in Jatta Ismail Khel. Different rock salt was collected from 5 rock salt mines. The samples were randomly taken in sterile plastic bags and vials. These samples were transported to the lab for the isolation of bacteria and processing for further analysis.

Physicochemical Analysis of Brine Samples

Physico-chemical properties of the brine samples taken from the salt mines (Bahadur Khel and Jatta Ismail Khel) were determined including moisture contents, water insoluble impurities $SO_4^{2-}$, $Cl^-$, $HCO_3^-$, $CO_3^{2-}$ with standard procedures while $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, and some trace elements like $Zn^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Mn^{2+}$, $Pb^{2+}$, $Cr^{3+}$ and $Cd^{2+}$ were determined with atomic absorption spectrophotometer (Z-8000, Hitachi, Japan). The pH meter (Model, 3510 Jenway) was employed for pH measurement, while laboratory thermometer was used to determine temperature on site.

Isolation and Enrichment of Halophilic Bacteria

To recover moderately to extremely halotolerant/halophilic bacteria, enrichment cultures and isolation procedures were performed by dilution plate method on tryptic soy agar medium. 1-2 mL of diluted sample was streaked on agar medium containing various concentrations (5-25%, w/v) of NaCl and incubated at 28°C. The isolated strains were subcultured several times under same conditions to obtain pure cultures of morphologically different bacteria. The purified strains were stored at – 80°C for further characterization.

Characterization and Identification of Halotolerant Bacterial Strains

The purified bacterial colonies were morphologically characterized for colony color, form, elevation, margin etc.

Table 1: Chemical analysis of rock salt samples collected from different location from Bahadur Khel and Jatta Ismail Khel salt mines

<table>
<thead>
<tr>
<th>Element</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfur (SO$_4^{2-}$)</td>
<td>1.31</td>
</tr>
<tr>
<td>Calcium (CaO)</td>
<td>1.73</td>
</tr>
<tr>
<td>Potassium (K$^+$)</td>
<td>1.65</td>
</tr>
<tr>
<td>Magnesium (Mg$^{2+}$)</td>
<td>0.11</td>
</tr>
<tr>
<td>Chloride (Cl$^-$)</td>
<td>53.91</td>
</tr>
<tr>
<td>Sodium (Na$^+$)</td>
<td>34.70</td>
</tr>
<tr>
<td>Moisture</td>
<td>0.13</td>
</tr>
<tr>
<td>Water insoluble impurities</td>
<td>7.98</td>
</tr>
<tr>
<td>Trace elements</td>
<td>(mg/Kg)</td>
</tr>
<tr>
<td>Zinc (Zn)</td>
<td>0.15</td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>0.02</td>
</tr>
<tr>
<td>Iron (Fe)</td>
<td>0.57</td>
</tr>
<tr>
<td>Manganese (Mn)</td>
<td>0.00</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>0.02</td>
</tr>
<tr>
<td>Chromium (Cr)</td>
<td>0.37</td>
</tr>
<tr>
<td>Cadmium (Cd)</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Fig. 1: Location of sample collection sites in Karak Salt Mines, Khyber Pakhtunkhwa Province, Pakistan

Bacterial strains were characterized on the basis of Gram’s staining, cell morphology and motility using microscope (Olympus, CX31 equipped with Digital Camera 5A). The growth characteristics of bacterial strains were determined at various pH range (4-11), NaCl concentrations (0-40%) and temperatures (4, 10, 20, 28, 35, 37, 40, 45 and 50°C).

PCR Amplification and Sequencing of 16S rRNA Gene

Genomic DNA extraction for amplification of 16S rRNA gene was performed as described by Roohi et al. (2012) by suspending few isolated colonies in TE buffer in a micro-centrifuge tube. These cells were heated for 10 min at 95°C and were centrifuged at 6,000 rpm for 5 min.
The supernatant was used as template DNA for the amplification of 16S rRNA gene. The 16S rRNA gene of all the isolated strains was amplified by polymerase chain reaction using primers 9F (5'-GAGTTTGATCCTGGCTAG-3') and 1510R (5'-GGCTACCTTGTAGCTAG-3') using Premix ExTaq (Takara, Japan) following protocol described previously (Ahmed et al., 2007). The PCR was carried out in ABI Veriti PCR Machine (Applied Biosystems, USA) using optimized PCR Program: initial denaturation at 94ºC for 2 min; 30 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min and extension at 72ºC for 1:30 min. The final extension was performed at 72ºC for 5 min. The amplified PCR products of 16S rRNA gene of bacterial strains were purified and sequenced using the primers 27F (5'-AAGTGTGTATCCTGGCTAG-3') and 1492R (5'-ACCTTGTAGCTAG-3') using commercial service of Macrogen Inc. Korea (http://dna.macrogen.com/eng/).

**Phylogenetic Analysis of Bacterial Isolates**

BioEdit software (Hall, 1999) was used to assemble the fragment sequences of 16S rRNA gene. The 16S rRNA gene sequences were submitted to DDBJ (http://www.ddbj.nig.ac.jp). Using 16S rRNA gene sequences, the strains were identified by BLAST search on EzBiocloud Server). Closely related type species sequences were retrieved to assess the molecular evolutionary relations and to construct phylogenetic tree using MEGA version 5 (Tamura et al., 2011). A phylogenetic tree was constructed from unambiguously aligned nucleotides using the neighbour-joining (NJ) algorithm. The relationship stability was evaluated by boot strap analysis performed using 1000 re-samplings of the neighbour-joining data for the tree topology.

**Results**

**Physical and Chemical Properties of Samples**

The rock salt samples named M1, M2, M3, M4 and M5 were collected from 5 different sites. The results indicated that the mineral content, pH and temperature of samples were suitable for the growth of halophilic bacteria. The average temperature of all samples ranged from 23.8 to 34.7ºC. The above temperature varied according to sampling sites and changes in air temperature. The chemical analysis showed that major components in samples were Cl⁻ (53.91%) and Na⁺ (34.70%). Small amount of Mg²⁺ (0.11%) and trace elements were also detected from rock salt. Cd²⁺ was absent from observed salt samples (Table 1). The average pH of samples ranged between 7.8 to 7.95.

**Colony, Cell Morphology and Density of Halotolerant/Halophilic Bacteria**

Fifty seven strains were isolated and purified on the basis of morphology. Pigmentation of the strains included pale yellow, creamy and whitish colonies. Most of the colonies had entire margin, while some had undulate and wavy filamentous margins (Table 2). Few of them also had lobate margins. The growth conditions of all the isolated strains were optimized for pH, NaCl tolerance and temperature. Our results showed that the bacterial strains grew best at pH 7 to 9 and at a temperature range of 28-37ºC. The isolated strains tolerated NaCl up to 0-30% and based on optimization conditions the isolated strains were classified as slightly halotolerant (0-5% NaCl), moderately halophilic (5-15% NaCl) and extremely halophilic (15-35% NaCl).

**Phylogenetic Analysis**

Phylogenetic analysis of the halophilic isolated strains were performed by constructing a phylogenetic tree based on the 16S rRNA gene sequences (Fig. 2). Phylogenetic analysis indicated that the majority of isolated strains belonged to the genera, Planococcus, Jeotgalicoccus, Staphylococcus, Thalassobacillus, Halobacillus, Halomonas, Brevibacterium, Gracilibacillus, Kocuria, Salinivibrio, Salinicoccus, Oceanobacillus and Bacillus. All strains shared more than 97% identity with their closest phylogenetic relatives except the strain NCCP-176. The phylogenetic analyses showed that eight strains (NCCP-71, 91, 93, 168, 176, 701, 705, 710) can further be studied taxonomically to delineate as novel species. The phylogenetic analysis reflected the evolutionary relationships among halophilic bacteria (Table 3).

**Discussion**

In the present study, we isolated and characterized extremely halotolerant/halophilic aerobic or facultative anaerobic bacteria from Pakistan using molecular techniques that has not been previously reported. The above growth condition are in line with several other studies that concluded that most species show optimum growth at 3.5 to 4.5 M NaCl and pH 7.0 to 7.5 (Kushner, 1985; Oren and Rodriguez-Valera, 2001). Similarly, our results of chemical analysis of rock salt showed that these contain proper amounts of essential ions to support the growth of extremely halophilic microorganisms (Mancinelli and Hochstein, 1986; Oren, 1993; Birbir et al., 2007).

The colonial morphology of the isolated strains were described using standard microbiological criteria. These standards are based on colonial pigmentation, opacity, diameter, consistency and elevation (Oren et al., 1997). In these studies, we observed polymorphism with respect to colonial morphology of the isolated strains as reported by Castillo et al. (2007). Halobacterial members exhibited various tones pigmentation ranging from red to pink-orange due to high rate of carotenoid pigments in their cell membrane. In addition, appeared opaque, transparent or translucent, mucoid or non mucoid, with entire edges and convex (Oren et al., 1997; Castillo et al., 2006). The bacteria isolated from the various samples belonged to...
Diverse group of halotolerant and halophilic bacteria with different phenotypic characteristics. However, the phenotypic characteristics alone were not enough to differentiate the bacterial isolates and could lead to false identification. The main reason for this is that the phenotypic characteristics depend on the growth conditions, which were different in terms of NaCl concentration, temperature, pH and medium composition. In this regard, Fritz (2002) recommended that phenotypic characterization results cannot be directly compared without full background knowledge of the accurate conditions used for a particular test.

Several studies have assessed the taxonomic status, ecological characteristics, phylogenetic relationship and biotechnological applications of halobacteria (Litchfield and Gillevet, 2002). Halophilic bacteria were categorized on the
basis of tolerance to different NaCl concentrations into slightly, moderately, and extremely-halophilic bacteria. However, this approach is practically ineffective for this purpose as the optimum and range of halophilic bacteria for tolerating NaCl is critical. Previously, the halophilic bacteria were grouped by using the results of salt tolerance test proposed by Kushner (1993). Our results showed that the slightly- and moderately halophilic bacteria were more abundant than the extremely halophilic bacteria (Table 2).

Molecular phylogeny provides information regarding organism relationships that is the basis for the conventional identification techniques (Singh et al., 2007). 16S rRNA gene sequence analysis is a widely used tool for reconstructing microbial phylogeny (Dewhirst et al., 2005).
In a previous study, Rohban et al. (2009) also isolated bacterial strains belonging to the genera Salicola, Halovibrio, Halomonas, Bacillus, Oceanobacillus, Thalassobacillus, Virgibacillus, Gracilibacillus, Halobacillus, Piscibacillus and Salinicoccus from Saltan lake of Iran.

**Staphylococci** has ability to grow in a wide range of salt concentrations (Graham and Wilkinson, 1992; Garzoni and Kelley, 2009; Morikawa et al., 2010) and is in accordance with our study. *Staphylococcus* strains were isolated from various salt samples during these studies and the results agreed with previous reports. Highly diverse nature of *Bacillus* genus found in this study is in accordance with the observations made by Claus and Berkeley (1986). Due to the ubiquity and capability to survive under adverse conditions, heterotrophic *Bacillus* strains cannot be considered species of certain specific habitats (Claus and Berkeley, 1986). It is therefore not surprising that a large number of the isolates recovered in the present study belonged to the same bacterial group.

In conclusion, the strains from salt mines of Karak can be further characterized using polyphasic taxonomic approach and other molecular methods. This is the first study on investigating the bacterial diversity of the Karak salt mines of Pakistan and it provided a large number of strains which are candidate novel species. The rich diversity of genus *Bacillus* found in this study points out to the extensive distribution and ecological relationship with other microorganisms, e.g. halophilic bacteria demands further comprehensive study. The microbial diversity can prove to be a valuable future resource in various industrial and biotechnological processes. Such microbes can also be used as a source of gene(s) that can increase salt tolerance in different crop species through genetic transformation.

**Acknowledgements**

This work was supported partly by financial assistance from PSDP funded Project *Research for Agricultural Development Project* under a sub-project (Grant No. CS-55/RADP/PARC) entitled “Establishment of Microbial Bio-Resource Laboratories: National Culture Collection of Pakistan (NCCP)” from Pakistan Agricultural Research Council, Islamabad, Pakistan.

**References**


(Received 30 September 2013; Accepted 24 February 2014)