INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
13–1198/2014/16–3–564–570
http://www.fspublishers.org

Full Length Article

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

Aneela Roohi[1,2], Iftikhar Ahmed[1*], Nauman Khalid[3], Muhammad Iqbal[1] and Muhammad Jamil[2*]
[1]*National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre (NARC), Park Road, Islamabad-45500, Pakistan*
[2]*Department of Biotechnology and Genetic Engineering, Kohat University of Science and Technology, Pakistan*
[3]*Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku 113-8657, Tokyo, Japan*
*For correspondences: iftikharnarc@hotmail.com; jamilkhattak@yahoo.com

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.

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 adoptive 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 mines, salted food, saline 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

To cite this paper: Roohi, A., I. Ahmed, N. Khalid, M. Iqbal and M. Jamil, 2014. Isolation and phylogenetic identification of halotolerant/halophilic bacteria from the salt mines of Karak, Pakistan, *Int. J. Agric. Biol.,* 16: 564–570

industries. 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^{2+} 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 28ºC. The isolated strains were sub-cultured several times under same conditions to obtain pure cultures of morphologically different bacteria. The purified strains were stored at – 80ºC 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

Element	(%)
Sulfur (SO_3^{-2})	1.31
Calcium (CaO)	1.73
Potassium (K^+)	1.65
Magnesium (Mg^{+2})	0.11
Chloride (Cl^{-1})	53.91
Sodium (Na^+)	34.70
Moisture	0.13
Water insoluble impurities	7.98
Trace elements	(mg/Kg)
Zinc (Zn)	0.15
Copper (Cu)	0.02
Iron (Fe)	0.57
Manganese (Mn)	0.00
Lead (Pb)	0.02
Chromium (Cr)	0.37
Cadmium (Cd)	0.00

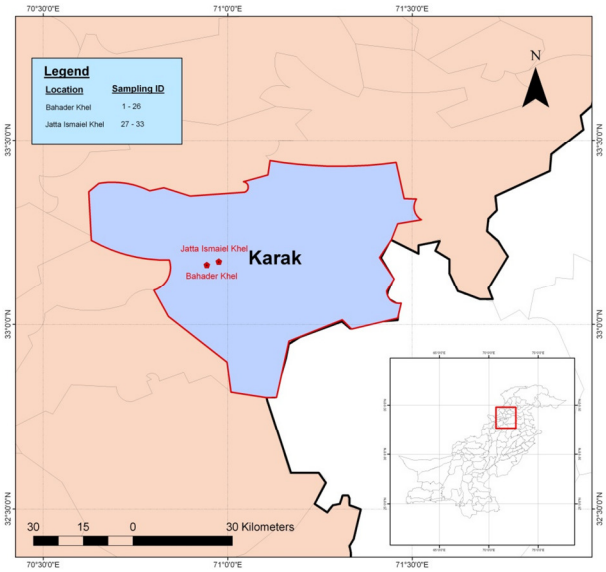

Fig. 1: Location of sample collection sites in Karak Salt Mines, Khyber Pakhtunkhwa Province, Pakistan

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 50ºC).

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 95ºC 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'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-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' AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACCTTGTTACGACTT-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 M_1, M_2, M_3, M_4 and M_5 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 34.7°C. 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 Mg^{2+} (0.11%) and trace elements were also detected from rock salt. Cd^{2+} 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 28-37^{o}C. The isolated strains toleratedNaCl 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 Rodríguez-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

Table 2: Phenotypic characteristics of isolated halotolerant/halophilic strains

Strain ID	Gram stain	Cell shape	Colony color	Form	Surface	Margin	Opacity	Motility	A/An	pH for range growth (Optimum)	NaCl Range (%) for growth	Temperature Range (°C) for growth (Optimum)
NCCP-59	+	R	C	Irregular	Rough	Lobate	Opaque	+	A	5-9 (7)	2-20	4-45 (28)
NCCP-60	+	C	O	Round	Smooth	Entire	Opaque	+	A	6-9 (8)	1-15	4-40 (30)
NCCP-61	+	R	PY	Round	Smooth	Entire	Opaque	+	A	5-9 (7)	2-25	10-45 (35)
NCCP-62	+	R	PY	Irregular	Smooth	Lobate	Opaque	+	An	6-9 (8)	3-20	4-50 (30)
NCCP-63	+	R	PY	Irregular	Smooth	Lobate	Opaque	+	A	6-8 (7)	2-20	10-40 (35)
NCCP-65	+	R	W	Irregular	Rough	Undulate	Opaque	+	A	5-9 (7)	1-20	10-50 (35)
NCCP-66	+	R	PY	Circular	Shiny	Entire	Opaque	-	An	6-9 (8)	1-30	4-40 (30)
NCCP-71	+	R	C	Irregular	Smooth	Filamentous	Opaque	+	A	5-10 (7)	1-25	4-50 (37)
NCCP-73	+	R	W	Irregular	Rough	Undulate	Opaque	+	A	5-9 (7)	1-20	10-50 (35)
NCCP-74	+	R	W	Irregular	Rough	Undulate	Opaque	+	A	5-9 (7)	1-20	10-50 (35)
NCCP-75	+	R	W	Irregular	Rough	Undulate	Opaque	+	A	5-9 (7)	1-20	10-50 (35)
NCCP-77	+	R	PY	Round	Smooth	Entire	Opaque	+	A	5-9 (7)	2-25	10-45 (35)
NCCP-79	+	R	C	Round	Smooth	Entire	Opaque	+	A	5-9 (8)	2-15	10-40 (28)
NCCP-80	+	R	PY	Round	Smooth	Entire	Opaque	+	A	5-9 (7)	2-25	10-45 (35)
NCCP-81	+	R	C	Round	Smooth	Entire	Opaque	+	A	5-9 (8)	0-15	10-40 (35)
NCCP-82	+	R	C	Irregular	Smooth	Filamentous	Opaque	+	A	5-10 (7)	1-25	4-50 (37)
NCCP-83	+	R	W	Irregular	Rough	Undulate	Opaque	+	A	5-9 (7)	1-20	10-50 (35)
NCCP-84	+	C	C	Round	Smooth	Entire	Opaque	-	A	5-9 (8)	2-30	10-40 (35)
NCCP-85	+	R	W	Irregular	Rough	Undulate	Opaque	+	A	5-9 (7)	1-20	10-50 (35)
NCCP-86	+	R	PY	Circular	Smooth	Entire	Opaque	+	A	6-9 (7)	2-15	4-50 (28)
NCCP-87	-	R	C	Circular	Smooth	Entire	Opaque	+	A	6-8 (7)	0-15	4-45 (35)
NCCP-88	+	R	W	Irregular	Rough	Undulate	Opaque	+	A	5-9 (7)	1-20	10-50 (35)
NCCP-91	+	R	PY	Irregular	Smooth	Lobate	Opaque	+	An	5-9 (7)	3-20	4-50 (30)
NCCP-92	+	R	PY	Round	Shiny	Entire	Opaque	-	A	6-9 (7)	2-20	10-45 (35)
NCCP-93	+	R	W	Round	Mucoid	Entire	Transparent	-	A	6-10 (7)	2-20	10-45 (30)
NCCP-166	+	R	C	Irregular	Smooth	Filamentous	Opaque	+	A	5-10 (7)	1-25	4-50 (37)
NCCP-168	+	R	PY	Circular	smooth	Entire	Opaque	-	A	5-9 (8)	0-15	10-40 (35)
NCCP-170	+	R	C	Circular	Smooth	Filamentous	Opaque	+	A	6-9 (7)	2-25	4-40 (37)
NCCP-171	-	R	W	Irregular	Smooth	Lobate	Opaque	+	A	5-9 (7)	1-19	10-45 (37)
NCCP-172	+	R	C	Circular	Rough	Irregular	Opaque	+	A	5-10 (7)	1-25	4-50 (37)
NCCP-173	+	R	C	Circular	Rough	Irregular	Opaque	+	A	6-8 (7)	2-20	10-40 (35)
NCCP-174	+	R	C	Round	Smooth	Entire	Opaque	+	A	5-9 (8)	0-15	10-40 (35)
NCCP-175	+	C	O	Round	Smooth	Entire	Opaque	-	A	6-9 (7)	2-20	4-40 (28)
NCCP-176	+	R	PY	Circular	Smooth	Entire	Opaque	-	A	6-9 (8)	1-35	4-45 (35)
NCCP-184	+	C	C	Round	Smooth	Entire	Opaque	-	A	5-9 (8)	2-30	10-40 (35)
NCCP-204	+	C	C	Round	Smooth	Entire	Opaque	-	A	5-9 (8)	2-30	10-40 (35)
NCCP-701	-	R	W	Circular	Smooth	Entire	Transparent	+	An	6-10 (8)	2-30	15-40 (30)
NCCP-704	+	C	C	Round	Smooth	Entire	Opaque	-	A	5-9 (8)	2-30	10-40 (35)
NCCP-705	+	C	Orange	Circular	Smooth	Entire	Opaque	-	A	6-10 (8)	1-30	4-50 (37)
NCCP-706	+	R	C	Irregular	Rough	Lobate	Opaque	+	A	5-9 (7)	2-18	4-45 (28)
NCCP-708	-	R	Cream	Circular	Smooth	Entire	Opaque	+	A	5-9(8)	0-30	4-45 (37)
NCCP-710	+	C	Orange	Circular	Smooth	Entire	Opaque	-	A	6-10 (8)	1-30	4-50 (37)
NCCP-713	-	R	Cream	Circular	Smooth	Entire	Opaque		A	6-10 (8)	0-25	4-45 (30)
NCCP-716	+	R	Cream	Circular	Rough	Irregular	Opaque	+	A	5-10 (7)	1-25	4-50(37)
NCCP-717	+	R	PY	Circular	Shiny	Entire	Opaque	-	An	6-9 (8)	1-30	4-40 (30)
NCCP-718	+	C	Orange	Circular	Smooth	Entire	Opaque	-	A	6-9 (7)	0-25	15-45 (35)
NCCP-721	+	R	PY	Circular	Smooth	Entire	Opaque	+	A	8-10 (9)	0-25	10-45(37)
NCCP-722	+	C	C	Round	Smooth	Entire	Opaque	-	A	5-9 (8)	2-30	10-40 (35)
NCCP-724	+	R	PY	Circular	Smooth	Entire	Opaque	+	A	6-9 (7)	2-35	4-45 (30)
NCCP-726			W	Irregular	Smooth	Irregular	Opaque		A	6-10 (7)	0-20	4-35 (28)
NCCP-727			W	Irregular	Smooth	Irregular	Opaque		A	6-10 (7)	0-20	4-35 (28)
NCCP-729	+	R	W	Round	Mucoid	Entire	Transparent	-	A	6-10 (7)	2-20	10-45 (30)
NCCP-730	+	C	C	Round	Smooth	Entire	Opaque	-	A	5-9 (8)	2-30	10-40 (35)
NCCP-731	+	R	Cream	Circular	Rough	Irregular	Opaque	+	A	5-10 (7)	1-25	4-50 (37)
NCCP-732	+	C	Orange	Circular	Smooth	Entire	Opaque	-	A	6-10 (8)	1-30	4-50 (37)
NCCP-734	+	C	Orange	Circular	Smooth	Entire	Opaque	-	A	6-10 (8)	1-30	4-50 (37)
NCCP-735	-	R	W	Circular	Smooth	Entire	Opaque	-	A	6-9 (8)	3-35	4-45 (37)

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, Fritze (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

Table 3: Molecular identification of halophilic/halotolerant bacterial strains

Strain ID	Strain Name / Genus	16S rRNA gene (nt)	Accession	Closely Related validly published taxa	Similarity of 16S rRNA gene sequence (%)	No. of Species having >97% similarity of 16S rRNA gene
NCCP-59	*Bacillus sp.*	1435	AB698777	*Bacillus sonorensis* (AF302118)	99.36	14
NCCP-60	*Planococcus sp.*	1441	AB698778	*Planococcus citreus* (X62172)	98.39	5
NCCP-61	*Bacillus sp.*	1452	AB715332	*Bacillus subtilissubsp. inaquosorum,* (EU138467)	99.74	15
NCCP-62	*Bacillus sp.*	1541	AB698779	*Bacillus aquimaris* (AF483625)	98.17	3
NCCP-63	*Bacillus sp.*	1499	AB698780	*Bacillus licheniformis* (AE017333)	99.60	10
NCCP-65	*Bacillus sp.*	1132	AB715333	*Bacillus safensis*(AF234854)	100.00	9
NCCP-66	*Jeotgalicoccus sp.*	1137	AB735682	*Jeotgalicoccus halotolerans* (AY028925)	99.82	6
NCCP-71	*Bacillus sp.*	1547	AB575949	*Bacillus clausii* (X76440)	99.18	2
NCCP-73	*Bacillus sp.*	1452	AB715334	*Bacillus safensis* (AF234854)	98.46	5
NCCP-74	*Bacillus sp.*	1096	AB715335	*Bacillus safensis* (AF234854)	99.90	5
NCCP-75	*Bacillus sp.*	1174	AB715336	*Bacillus safensis* (AF234854)	98.86	5
NCCP-77	*Bacillus sp.*	1410	AB698788	*Bacillus subtilissubsp. Inaquosorum* (EU138467)	99.91	12
NCCP-79	*Bacillus sp.*	1423	AB698789	*Bacillus oceanisediminis* (GQ292772)	98.85	3
NCCP-80	*Bacillus sp.*	1128	AB715337	*Bacillus methylotrophicus* (EU194897)	99.73	15
NCCP-81	*Bacillus sp.*	1455	AB715338	*Bacillus licheniformis* (AE017333)	98.76	10
NCCP-82	*Bacillus sp.*	1480	AB715339	*Bacillus clausii* (X76440)	99.24	2
NCCP-83	*Bacillus sp.*	1125	AB715340	*Bacillus safensis* (AF234854)	99.08	5
NCCP-84	*Staphylococcus sp.*	1101	AB715341	*Staphylococcus equorum subsp. linens* (AF527483)	99.54	15
NCCP-85	*Bacillus sp.*	1169	AB715342	*Bacillus safensis* (AF234854)	99.91	5
NCCP-86	*Thalasobacillus sp.*	1434	AB698790	*Thalassobacillus devorans* (AJ717299)	98.95	4
NCCP-87	*Halomonas sp.*	790	AB735683	*Halomonas boliviensis* (AY245449)	99.49	6
NCCP-88	*Bacillus sp.*	967	AB715343	*Bacillus safensis* (AF234854)	99.69	5
NCCP-91	*Bacillus sp.*	1421	AB698793	*Bacillus vietnamensis* (AB099708)	98.45	3
NCCP-92	*Brevibacterium sp.*	1406	AB698794	*Brevibacteriumcasei* (X76564)	99.10	8
NCCP-93	*Bacillus sp.*	1418	AB698795	*Bacillus endophyticus* (AF295302)	99.50	1
NCCP-166	*Bacillus sp.*	1432	AB698798	*Bacillus clausii* (X76440)	98.81	2
NCCP-168	*Bacillus sp.*	1410	AB618147	*Bacillus seohaeanensis* (AY667495)	97.06	1
NCCP-170	*Gracilibacillus sp.*	1453	AB698801	*Gracilibacillus dipsosauri* (X82436)	98.81	2
NCCP-171	*Halomonas sp.*	1427	AB698802	*Halomonas elongate* (FN869568)	99.43	6
NCCP-172	*Bacillus sp.*	1423	AB698803	*Bacillus safensis* (AF234854)	99.86	5
NCCP-173	*Bacillus sp.*	1422	AB698804	*Bacillus safensis* (AF234854)	99.93	5
NCCP-174	*Bacillus sp.*	1433	AB698805	*Bacillus licheniformis* (AE017333)	98.32	14
NCCP-175	*Kocuria sp.*	1404	AB698806	*Kocuria sediminis* (JF896464)	99.78	9
NCCP-176	*Halobacillus sp.*	1480	AB698807	*Halobacillus profundi* (AB189298)	95.00	0
NCCP-184	*Staphylococcus sp.*	1050	AB735684	*Staphylococcus equorum subsp. equorum*(AB009939)	99.90	23
NCCP-204	*Staphylococcus sp.*	1420	AB698815	*Staphylococcus equorum subsp. equorum*(AB009939)	98.44	10
NCCP-701	*Salinivibrio sp.*	1459	AB715344	*Salinivibrio sharmensis* (AM279734)	97.82	1
NCCP-704	*Staphylococcus sp.*	1147	AB715345	*Staphylococcus equorum subsp. equorum*(AB009939)	100.0	23
NCCP-705	*Salinicoccus sp.*	1175	AB715346	*Salinicoccus roseus* (X94559)	98.97	4
NCCP-706	*Bacillus sp.*	1042	AB715347	*Bacillus sonorensis* (AF302118)	98.46	13
NCCP-708	*Salinivibrio sp.*	1100	AB735685	*Salinivibrio costicola subsp. Alkaliphilus* (AJ640132)	100.00	6
NCCP-710	*Salinicoccus sp.*	1311		*Salinicoccus roseus* (X94559)	98.62	3
NCCP-713	*Halomonas sp.*	868	AB735686	*Halomonasalk aliantarctica* (AJ564880)	99.42	9
NCCP-716	*Bacillus sp.*	1172	AB735687	*Bacillus safensis* (AF234854)	100.0	6
NCCP-717	*Jeotgalicoccus sp.*	1201	AB735688	*Jeotgalicoccus halotolerans* (AY028925)	99.42	6
NCCP-718	*Kocuria sp.*	1055	AB735689	*Kocuria rosea* (X87756)	99.53	7
NCCP-721	*Oceanobacillus sp.*	985	AB735690	*Oceanobacillus onchorhynchi subsp. Incaldanensid* (AJ640134)	99.90	4
NCCP-722	*Staphylococcus sp.*	1097	AB735691	*Staphylococcus equorum subsp. equorum*(AB009939)	100.0	21
NCCP-724	*Oceanobacillus sp.*	1172	AB735692	*Oceanobacillus picturae* (AJ315060)	100.0	4
NCCP-726	*Bacillus sp.*	1081	AB735693	*Bacillus aerophilus* (AJ831844)	99.54	5
NCCP-727	*Bacillus sp.*	1117	AB735694	*Bacillus aerophilus* (AJ831844)	100.0	5
NCCP-729	*Bacillus sp.*	966	AB735695	*Bacillus endophyticus* (AF295302)	99.79	1
NCCP-730	*Staphylococcus sp.*	1095	AB735696	*Staphylococcus equorum subsp. Equorum* (AB009939)	99.91	18
NCCP-731	*Bacillus sp.*	1134	AB735697	*Bacillus safensis* (AF234854)	99.65	5
NCCP-732	*Bacillus sp.*	1096	AB735698	*Bacillus sonorensis* (AF302118)	99.18	16
NCCP-734	*Bacillus sp.*	1176	AB735699	*Bacillus sonorensis*(AF302118)	99.23	16
NCCP-735	*Halomonas sp.*	1149	AB735700	*Halomonas alkaliphila* (AJ640133)	99.91	14

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).

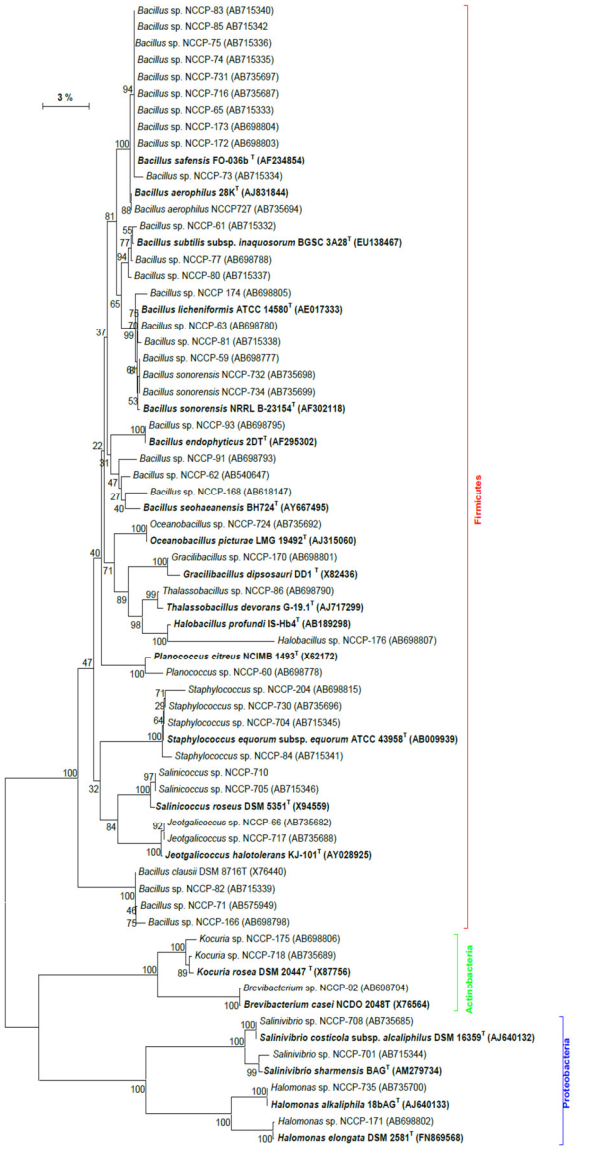

Fig. 2: Phylogenetic tree showing the interrelationships of isolated halotolerant/halophilic strains along with their closely related taxa belonging to *Firmicutes*, *Actinobacteria* and *Proteobacteria* inferred from 16SrRNA gene sequence. Data with gaps were removed after alignment by CLUXTAL X. The rooted tree was constructed using the neighbour-joining method contained in the MEGA5 software (Tamura et al., 2011). Bootstrap values, expressed as percentages of 1000 replications, are given at branching points. Closely related type species were presented in bold letters and the 3% bar shows sequence divergence

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

Ahmed, I., A. Yokota and T. Fujiwara, 2007. A novel highly boron tolerant bacterium, bacillus boroniphilus sp. Nov., isolated from soil, that requires boron for its growth. *Extremophiles*, 11: 217–224

Antón, J., R. Rosselló–Mora, F. Rodríguez–Valera and R. Amann, 2000. Extremely halophilic bacteria in crystallizer ponds from solar salterns. *Appl. Environ. Microbiol.*, 66: 3052–3057

Birbir, M., B. Calli, B. Mertoglu, R. Bardavid, A. Oren, M. Ogmen and A. Ogan, 2007. Extremely halophilic archaea from tuz lake, turkey, and the adjacent kaldirim and kayacik salterns. *World J. Microbiol. Biotechnol.* 23: 309–316

Castillo, A.M., M.C. Gutiérrez, M. Kamekura, Y. Ma, D.A. Cowan, B.E. Jones, W.D. Grant and A. Ventosa, 2006. *Halovivax asiaticus* gen. Nov., sp. Nov., a novel extremely halophilic archaeon isolated from inner mongolia, china. *Int. J. Syst. Evol. Microbiol.,* 56: 765–770

Castillo, A.M., M.C. Gutiérrez, M. Kamekura, Y. Xue, Y. Ma, D.A. Cowan, B.E. Jones, W.D. Grant and A. Ventosa, 2007. *Halorubrum ejinorense* sp. Nov., isolated from lake ejinor, inner mongolia, china. *Int. J. Syst. Evol. Microbiol.*, 57: 2538–2542

Claus, D. and R.C.W. Berkeley, 1986. Genus *Bacillus* 1872. *In: Bergey's Manual of Systematic Bacteriology.* P.H.A. Sneath, PHA, N.S. Mair, M.E. Sharpe and J.G. Holt (ed.). Vol. 2. pp: 1105–1139. Williams and Wilkins, Baltimore, Maryland, USA

Dewhirst, F.E., Z. Shen, M.S. Scimeca, L.N. Stokes, T. Boumenna, T. Chen, B.J. Paster and J.G. Fox, 2005. Discordant 16s and 23s rrna gene phylogenies for the genus helicobacter: implications for phylogenetic inference and systematics. *J. Bacteriol.*, 187: 6106–6118

Euzéby, J.P., 2013. List of prokaryotic names with standing in nomenclature. Accessed on 9 June 2013 http: //www.bacterio.net/h/halobacteriaceae.html

Fritze, D., 2002. Bacillus identification– traditional approaches. *In: Applications and Systematics of Bacillus and Relatives.* Berkeley, R., M. Heyndrickx, N. Logan, P. De Vos (Eds.), pp. 100–122. Blackwell, Oxford, UK

Garzoni, C. and W.L. Kelley, 2009. *Staphylococcus aureus*: New evidence for intracellular persistence. *Trends Microbiol*., 17: 59–65

Graham, J.E. and B.J. Wilkinson, 1992. *Staphylococcus aureus* osmoregulation: roles for choline, glycine betaine, proline, and taurine. *J. Bacteriol.*, 174: 2711–2716

Hall, T.A., 1999. Bioedit: a user–friendly biological sequence alignment editor and analysis program for windows 95/98/nt. *Nucleic Acids Symposium Series*

Johnson, A., L. Thurlow, R. Zwenger and E. Gillock, 2007. Partial characterization of two moderately halophilic bacteria from a kansas salt marsh. *Prairie Nat,* 39: 29–39

Kamekura, M., 1998. Diversity of extremely halophilic bacteria. *Extremophiles*, 2: 289–295

Kushner, D.J., 1985. *The halobacteriaceae*. Academic Press, New York, USA

Kushner, D.J., 1993. *Growth and nutrition of halophilic bacteria.* CRC Press, Boca Raton, Florida, USA

Litchfield, C.D. and P.M. Gillevet, 2002. Microbial diversity and complexity in hypersaline environments: a preliminary assessment. *J. Ind. Microbiol. Biotechnol*., 28: 48–55

Mancinelli, R.L. and L.I. Hochstein, 1986. The occurrence of denitrification in extremely halophilic bacteria. *FEMS Microbiol. Lett.*, 35: 55–58

Morikawa, K., R.L. Ohniwa, T. Ohta, Y. Tanaka, K. Takeyasu and T. Msadek, 2010. Adaptation beyond the stress response: Cell structure dynamics and population heterogeneity in *staphylococcus aureus*. *Microb. Environ*., 25: 75–82

Oren, A., 1993. *Ecology of extremely halophilic microorganisms*. CRC Press, Boca Raton, Florida, USA

Oren, A., 2002. Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. *J. Ind. Microbiol. Biotechnol.*, 28: 56–63

Oren, A. and F.s. Rodríguez–Valera, 2001. The contribution of halophilic bacteria to the red coloration of saltern crystallizer ponds1. *FEMS Microbiol. Ecol*., 36: 123–130

Oren, A., A. Ventosa and W.D. Grant, 1997. Proposed minimal standards for description of new taxa in the order halobacteriales. *Int. J. Syst. Bacteriol*., 47: 233–238

Ozcan, B., G. Ozcengiz, A. Coleri and C. Cokmus, 2007. Diversity of halophilic archaea from six distinct parts of turkey. *J. Microbiol. Biotechnol*., 17: 985–992

PMDC, 2013. *Pakistan mineral development corporation*. Ministry of Petroleum and Natural Resources, Government of Pakistan, Islamabad

Quesada, E., V. Béjar, M. Ferrer, C. Calvo, I. Llamas, F. Martínez–Checa, S. Arias, C. Ruiz–García, R. Páez, M. Martinez–Cánovas and A. del Moral, 2004. *Moderately halophilic, exopolysaccharide–producing bacteria.* Berlin: Springer–Verlag, Berlin, Germany

Quesada, E., A. Ventosa, F. Rodriguez–Valera and A. Ramos–Cormenzana, 1982. Types and properties of some bacteria isolated from hypersaline soils. *J. Appl. Microbiol.*, 53: 155–161

Rohban, R., M. Amoozegar and A. Ventosa, 2009. Screening and isolation of halophilic bacteria producing extracellular hydrolyses from howz soltan lake, Iran. *J. Ind. Microbiol. Biotechnol*., 36: 333–340

Roohi, A., I. Ahmed, M. Iqbal and M. Jamil, 2012. Preliminary isolation and characterization of halotolerant and halophilic bacteria from salt mines of Karak, Pakistan. *Pak. J. Bot.*, 44: 365–370

Singh, S., R. Chandra, D.K. Patel and V. Rai, 2007. Isolation and characterization of novel serratia marcescens (ay927692) for pentachlorophenol degradation from pulp and paper mill waste. *World J. Microbiol. Biotechnol.,*23: 1747–1754

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, 2011. Mega5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.*, 28: 2731–2739

Ventosa, A., E. Mellado, C. Sánchez–Porro and M. Marquez , 2008. *Halophilic and halotolerant micro–organisms from soils*. Berlin Springer–Verlag, Berlin, Germany

Woese, C.R. and G.E. Fox, 1977. Phylogenetic structure of the prokaryotic domain: The primary kingdoms. *Proc. Natl. Acad. Sci. USA*, 74: 5088–5090

(Received 30 September 2013; Accepted 24 February 2014)