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ARTICLE

Diversity of *Bacillus*-like bacterial community in the rhizospheric and non-rhizospheric soil of halophytes (*Salsola stocksii* and *Atriplex amnicola*), and characterization of osmoregulatory genes in halophilic *Bacilli*

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Abstract: Salinity is one of the major abiotic stresses; a total of 3% of the world's land mass is affected by salinity. Approximately 6.3 million hectares of land in Pakistan is affected by salinity to varying degrees, and most of the areas are arid to semiarid with low annual precipitation. The aim of the present study is to identify and characterize *Bacillus* and *Bacillus*-derived bacterial genera from the rhizospheric and non-rhizospheric soil samples from the Khewra Salt Mine, Pakistan, by using culture-independent and -dependent methods. Seven *Bacillus*-like bacterial genera, *Bacillus*, *Halobacillus*, *Virgibacillus*, *Brevibacillus*, *Paenibacillus*, *Tumebacillus*, and *Lysinibacillus*, were detected by using pyrosequencing analysis, whereas only four genera, *Bacillus*, *Halobacillus*, *Oceanobacillus*, and *Virgibacillus*, were identified by culture-dependent methods. Most of the *Bacillus*-like isolates identified in this study were moderately halophilic, alkaliphilic, and mesophilic bacteria and were considered a good source of hydrolytic enzymes because of their ability to degrade proteins, carbohydrates, and lipids. Eight *Bacillus*-like strains from the genera *Bacillus*, *Halobacillus*, *Oceanobacillus*, and *Virgibacillus* showed positive results for the presence of *ectABC* gene cluster (ectoine), six strains could synthesize betaine from choline, and six strains tested positive for the synthesis of proline from either glutamate or ornithine by using proline dehydrogenase enzyme.

Key words: halophilic *Bacilli*, pyrosequencing, osmoregulatory genes, *Salsola stocksii*, *Atriplex amnicola*.

Résumé : La salinité est un des principaux stress abiotiques, 3 % de la masse continentale au total étant affectée par la salinité. Approximativement 6,3 millions d'hectares de terre au Pakistan sont affectés par la salinité à divers degrés et la plupart des régions sont arides ou semi-arides, les précipitations annuelles étant faibles. L'objectif de cette étude était d'identifier et caractériser *Bacillus* et les genres bactériens de type *Bacillus* d'échantillons de sol de la rhizosphère et hors rhizosphère de la mine de sel de Khewra, Pakistan, à l'aide de méthodes dépendantes ou indépendantes de la culture. Sept genres bactériens de type *Bacillus*, *Bacillus*, *Halobacillus*, *Virgibacillus*, *Brevibacillus*, *Paenibacillus*, *Tumebacillus* et *Lysinibacillus* ont été détectés par le pyroséquençage alors que quatre genres seulement, *Bacillus*, *Halobacillus*, *Oceanobacillus* et *Virgibacillus* ont été identifiés par des méthodes dépendantes de la culture. La plupart des isolats de type *Bacillus* identifiés dans cette étude étaient des bactéries modérément halophiles, alcalinophiles et mésophiles et étaient considérés comme une bonne source d'enzymes hydrolytiques à cause de leur capacité à dégrader les protéines, les sucres et les lipides. Huit souches de type *Bacillus* des genres *Bacillus*, *Halobacillus*, *Oceanobacillus* et *Virgibacillus* comportaient la grappe génique *ectABC* (ectoïne), six souches pouvaient synthétiser la bétaïne à partir de la choline et six souches pouvaient synthétiser la proline à partir du glutamate ou de l'ornithine en utilisant une proline déshydrogénase. [Traduit par la Rédaction]

Mots-clés : *Bacillus* halophiles, pyroséquençage, gène d'osmorégulation, *Salsola stocksii*, *Atriplex amnicola*.

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Introduction

High concentrations of salts in the soil change the availability of water and nutrients for both plants and their associated microorganisms, which, directly or indirectly, influences soil stability and organic matter (Mavi et al. 2012). Salinity also affects microbial diversity, which plays a role in maintaining soil structure and biogeochemical cycles (Tripathi et al. 2006). The rich microbial diversity of halophyte rhizospheres helps these plants cope with high salinity and also tolerate drought (Berendsen et al. 2012). Rhizobacteria promote plant growth by increasing the availability and uptake of carbon, nitrogen, and minerals from the soil (Dodd and Pérez-Alfocea 2012); provide protection against plant pathogens; and contribute significantly to the well-being and salinity tolerance of halophytes (Bulgarelli et al. 2012).

The physiology of the moderate and extreme halophilic bacteria is affected by changes in the salt concentration, growth temperature, pH, and nature of available nutrients (Amoozegar et al. 2016). Moderate halophilic bacteria can grow at 0.85–3.4 mol/L NaCl concentrations (Oren 2012; DasSarma and DasSarma 2015). In saline environments, members of the phylum *Firmicutes*, e.g., *Bacillus*, *Virgibacillus*, *Halobacillus*, *Oceanobacillus*, *Paenibacillus*, and *Brevibacillus*, have been found to be more abundant than other bacteria (Liszka et al. 2012). Halophilic *Bacilli* have a wide range of applications in bioenzyme production, biodefense, biofuel production, and bioremediation of organic toxic compounds (Lundberg et al. 2012; Liu et al. 2017). These *Bacilli* are a good source of novel enzymes that function under salt-stress conditions, such as proteases, xylanases, cellulases, and amylases with polyextremophilic properties (Taprig et al. 2013). Proteases, amylases, and lipases have extensive applications in the pharmaceutical, food, textile, and paper industries (Abel-Nabey and Farag 2016). Cellulose, lipid, and pectin degradation by *Bacilli* strains produce different organic compounds like methanol, which are used as a carbon source by other bacteria (Porwal et al. 2008; Knief et al. 2012).

Moderate halophilic bacteria use a "compatible solute" strategy to cope with their external environments by accumulating small, highly water-soluble organic compounds, such as glycine betaine, proline, glutamine, ectoine, potassium, and glutamic acid (Moghaddam et al. 2016; Nath 2016). Ectoine, a cyclic tetrahydropyrimidine, is used as an osmolyte in halotolerant and halophilic bacteria. The biosynthesis and regulation of ectoine has been studied in a large number of halophilic bacteria, especially with detail in *Halomonas* and *Oceanobacillus* (Schubert et al. 2007; Tanimura et al. 2016). Betaine is a natural compound having a negatively charged ion carboxylate group and a positively charged phosphonium ion or ammonium ion. Different halophilic bacteria such as *Halomonas*, *Virgibacillus*, *Oceanobacillus*, and *Kocuria* can synthesize betaine from glycine (Ates et al. 2011; Ying et al. 2016). A large number of halophilic bacteria, e.g., *Bacillus*, *Streptococcus*, and *Escherichia coli*, have the ability to use some amino acids as osmolytes that accumulate in high levels in response to salt and drought stress (Collins and Deming 2013).

Khewra Salt Mine is the world second largest salt mine, located near Pind Dadan Khan Tehsil of Jhelum District, Punjab, Pakistan (32°38′N, 73°10′E). Based on its origin, Khewra Salt Mine like other hypersaline bodies is classified as thalassic because it is derived from evaporation of sea water (Ahmad et al. 2007). It has Na^+ and Cl^- dominating ions and the pH is near neutral to slightly alkaline. Plants like *Suaeda*, *Salsola*, *Atriplex*, and *Justica* are the dominant genera found here. Few studies have been conducted on the microbial diversity in the rhizosphere of halophytes from the Khewra Salt Mine. In this study, we discuss the diversity of the *Bacillus*-like bacterial community in rhizospheric and non-rhizospheric soil of halophytes (*Salsola stocksii* and *Atriplex amnicola*) and hypersaline lake-bank soil samples by 454 pyrosequencing and culture-dependent methods. We also characterized *Bacillus*-like strains phenotypically on the basis of salt, pH, and temperature tolerance and on extracellular enzymes. Halophilic bacteria can tolerate more salinity than halophytes can because of their internal osmotic balance. So, the main focus of this study was to identify and characterize osmoregulatory genes for glycine betaine, ectoine, and proline from halophilic *Bacilli* strains isolated from the rhizosphere and non-rhizospheric soils of halophytes. Osmoregulatory genes identified in this study can be used to develop transgenic salt-tolerant crops.

Materials and methods

Sample collection

We surveyed an area approximately 1.1 km from the Khewra Salt Mines (Table S1[1]; Fig. S1[1]). Rhizospheric soil samples were collected by gently uprooting the plants and collecting the soil adhering to roots. For non-rhizospheric saline soil samples, the upper 8–10 cm of mineral soil was collected. Hypersaline lake soil samples were collected from the bank of a salt lake. At each site, soil samples with approximately 500 g and four replicates each from four spatially separated plants were collected in black sterile polythene bags. These samples were stored at 4 °C for further analysis.

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjm-2017-0544.

Soil physicochemical parameters

Each soil sample (300 g) was thoroughly mixed and sieved through an aperture size of 2 mm. Physical properties (pH, moisture content, salinity, and temperature) of soil samples from the rhizosphere of a variety of plants and non-rhizospheric regions were determined. Moisture (%), temperature, and texture class were measured by the Anderson method (Anderson and Ingram 1993); pH was measured by 1:2.5 (*m*/*v*) soil to water mixture; and electrical conductivity (dS/m) was measured by 1:1 (*m*/*v*) soil to water mixture at 25 °C (Adviento-Borbe et al. 2006). Organic matter (C_{org}) was determined by the Walkley-Black method (1934). Cation exchange capacity (CEC) is the capacity to retain and release cations (Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+}) and sodium adsorption ratio (SAR) is the measure of the sodicity of soil, which is calculated as the ratio of the sodium to the magnesium and calcium.

Diversity analysis of *Bacillus*-like bacterial community from the rhizosphere of *S. stocksii* by 16S rRNA based pyrosequencing

Metagenomic DNA was extracted from 1 g of soil using a FastPrep® instrument (MP Biomedicals, USA) according to the manufacturer's instructions. The concentration of metagenomic DNA was qualitatively determined on 0.8% (*m*/*v*) agarose gel and quantified using Nanodrop (NanoDrop 200c Thermo Scientific, USA). In total, 16 DNA samples (eight rhizospheric, four non-rhizospheric saline, and four hypersaline lake-bank soil samples) were sequenced through high-throughput sequencing.

The V3–V4 region of 16S rRNA gene was amplified using primers F515 (5′-GTGCCAGCMGCCGCGG-3′) and R907 (5′-CCGTCAATTCMTTTRAGTTT-3′), which were linked with unique identifier and adopter sequences (Table S2[1]). The detailed PCR conditions for amplicon sequencing were the same as described previously (Mirza et al. 2014). Briefly, a 50 μL PCR amplification reaction contained 1× buffer, 0.2 μmol/L (each) primers, 1.8 mmol/L $MgCl_2$, 200 μmol/L deoxynucleoside triphosphates (dNTPs), 20 ng of template, and 1 μL of FastStart high-fidelity PCR system enzyme (Roche Applied Sciences). The PCR conditions were 3 min at 95 °C, followed by 30 cycles of denaturation at 94 °C for 45 s, primer annealing at 54 °C for 45 s, extension at 72 °C for 1 min, and final extension for 7 min. Amplified PCR products were purified with Agencourt AMPure beads (Beckman Coulter, Brea, California, USA). Purified PCR products from different samples were pooled in equimolar concentrations. Pyrosequencing was performed on the mixture with the 454 GS FLX sequencer (454 Life Sciences) at the Utah State University Center for Integrated Biosystems.

Sequence data analysis

Sequences were processed and sorted using the default parameters in QIIME 1.3 (Caporaso et al. 2010). An offset of 10 nucleotides was set to remove the first 10 bases of each sequence, and high-quality sequences with an average length of 375 bases were selected. High-quality sequences were clustered into operational taxonomic units (OTUs) with a 3% difference using UCLUST. For the identification of chimeric sequences, Chimera Slayer software was used (DeSantis et al. 2006). The cleaned sequences were analyzed using RDP Classifier (Wang et al. 2007) with a 97% confidence threshold. All *Bacillus*-related sequences (852 from *S. stocksii*, 1163 from *A. amnicola*, 1098 from non-rhizospheric saline soil samples, and 575 from hypersaline lake-bank soil samples) and 18 pure culture isolates of *Bacillus*, *Halobacillus*, *Virgibacillus*, and *Oceanobacillus* were aligned using MUSCLE (Edgar 2004) and clustered in OTUs at 97% DNA identity. Phylogenetic community similarity was calculated by constructing a neighbor-joining tree using MEGA7 (Kumar et al. 2016).

Isolation of *Bacillus*-related strains from the rhizosphere and non-rhizospheric soil of halophytes (*S. stocksii* and *A. amnicola*) and hypersaline lake-bank soil samples

Halophilic medium (HaP) (5 g/L tryptone, 1 g/L yeast extract, 117 g/L NaCl, 5 g/L KCl, 10 g/L $MgSO_4$, 2 g/L K_2HPO_4, and pH 7.2) was used for the isolation and purification of bacteria in saline environments (Schneegurt 2012). Rhizosphere (RS) indicates the soil adhering to the roots. For isolations of *Bacillus*-like bacterial isolates from the rhizosphere of halophytes, non-rhizospheric, and hypersaline lake-bank soil samples, the soil was mixed thoroughly, sieved, and then 10 g of it was suspended in saline solution (1% NaCl), followed by stirring for 30 min (Malik et al. 1997). Serial dilutions (10^{-1}–10^{-10}) were made for all samples (Somasegaran and Hoben 1994). Dilutions from 10^{-3} to 10^{-6} were inoculated onto HaP plates for determining the colony-forming units (CFU) per gram of dry mass. Plates were incubated at 37 °C until the appearance of bacterial colonies, after which they were counted and CFU was calculated. The bacteria were purified by repeated subculturing of single colonies. Single colonies selected were grown in HaP broth and stored in 33% glycerol at −80 °C for further characterization.

Morphological and biochemical characterization of *Bacillus*-related strains

For morphological characterization, colony morphology (color, shape, elevation, size, and margin) and cell morphology (shape, size, motility, and Gram-staining) were studied. Halophilic bacterial strains were biochemically characterized to detect different enzymes (β-galactosidase, arginine deaminase, lysine decarboxylase, tryptophan deaminase, gelatinase, catalase, and oxidase) and carbon sources (glucose, sucrose, mannitol, maltose, arabinose, lactose, and sorbitol) utilization by using QTS 24 strips (DESTO Laboratories, Karachi, Pakistan).

Molecular characterization of *Bacillus*-related strains

Genomic DNA was isolated by the CTAB (cetyltrimethylammonium bromide) method (Winnepenninckx et al. 1993). PCR amplification of 16S rRNA was performed by

using the universal forward and reverse primers P1 (5′-GAGAGTTTGATCCTGGTCAGAACGAAC-3′) and P6 (5′-CGTACGGCTACCTTGTTACGACTTCACC-3′) for prokaryotes (Tan et al. 1997). A PCR reaction of 50 μL was prepared by using 0.5 μL of *Taq* DNA polymerase (5 U), 2 μL of 10× *Taq* buffer, 2.5 μL of 25 mmol/L $MgCl_2$mol/L), 2 μL of dNTPs (2.5 mmol/L), 2 μL each of forward and reverse primer (10 pmol), 36 μL of double-distilled H_2O, and 3 μL of template DNA. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, with a final extension at 72 °C for 10 min, as described by Tan et al. (1997). PCR products were analyzed by using 1% agarose gel. PCR products were purified by using a GeneJET PCR Purification kit (K0702, Thermo Fisher Scientific). Purified PCR products were sequenced by using forward and reverse primers (Eurofins, Germany).

Acquired sequences were assembled and analyzed with the help of Chromas Lite 2.01 sequence analysis software (Technelysium Pty Ltd., Australia). The gene sequences were compared with those deposited in the GenBank nucleotide database using the NCBI BLAST program. Sequences were aligned using the Clustal X 2.1 program, and a phylogenetic tree was constructed using the Neighbor-joining method (Saitou and Nei 1987). Bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the distance tree topologies obtained (Varian 2005). The evolutionary distances were computed using the Neighbor-joining method (Tamura et al. 2004) and units are presented as the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted using MEGA7 (Kumar et al. 2016). There were a total of 1457 positions in the final dataset. The 16S rRNA gene sequence from *Micrococcus luteus* was used as an outgroup.

Screening of *Bacillus*-related strains with respect to their salt, pH, and temperature tolerance ability

Bacterial isolates were grown in the presence of various salt, pH, and temperature conditions by using HaP broth medium. The salt concentrations tested were 1.5–4.5 mol/L NaCl, pH ranged from 4 to 12, and temperature was between 4 and 42 °C. Isolates were cultured in 250 mL flasks at 37 °C with continuous rotatory agitation at 150 r/min for 72 h (Jadhav et al. 2010). During incubation, bacterial growth was measured at an optical density of 600 nm after different time intervals (3, 6, 12, 24, 48, and 72 h).

Enzyme assays for *Bacillus*-related strains

Protease activity was tested on the medium described by Kumar et al. (2009). Amylase and cellulose activities were identified by using 2% iodine solution and spotting a single colony of each bacterial strain on CMC (carboxymethyl cellulose 1%) agar plates (Gupta et al. 2012). Catalase was identified by using H_2O_2 and pure culture colonies from agar plates (MacFadden 1980). Lipase activity was tested by using Luria–Bertani medium with 1% butyrin and Tween 80 hydrolysis assay, as described by Sierra (1957). Oxidase activity was tested by using cytochrome oxidase test strips (MacFadden 1980). The clear zones around the bacterial colonies after 4–12 days of incubation at 37 °C were considered as a positive result of protease, cellulase, and lipase activities.

PCR amplification of osmoregulatory genes

Genes for compatible solutes like ectoine, glycine betaine, and proline have been characterized in this study. For amplification of *ectABC* gene cluster from different halophilic *Bacillus* strains, primer pair EO1 and EO2 (Rajan et al. 2008) was used, and a reaction mixture of 25 μL containing 12 ng of template DNA, 2.5 μL of 10× *Taq* polymerase buffer (Fermentas), 0.5 μL of 10 mmol/L dNTPs (Fermentas), 2 μL of 25 mmol/L $MgCl_2$ (Fermentas), 0.5 μmol/L (each) primers, and 0.2 units of *Taq* DNA polymerase (Fermentas) was prepared in a 0.5 mL thin-walled PCR tube. Amplification was performed in a Nyx Technik Amplitronyx Series 4 (ATC201) Thermal Cycler with the following conditions: initial denaturation at 95 °C for 5 min, followed by 35 repeated cycles of 94 °C for 1 min, 50 °C for 50 s, and 72 °C for 2 min, and final extension at 72 °C for 10 min. For PCR amplification of the *betA* gene, the primer pair bAF and bAR was used (Rajan et al. 2010). The *proDH* gene for proline dehydrogenase has been amplified by using the primer pair PDHPF and PDHPR (Mohammadi and Ominidia 2012). The PCR profile for the *betA* and *proDH* genes was same as that for the *ectABC* gene cluster. Amplified PCR products were run on agarose gel and purified by using a PCR purification kit (Fermentas) according to the standard protocol recommended by the manufacturer. Purified PCR products were sequenced commercially (Eurofins MWG Operon, Huntsville, Alabama, USA) by using forward and reverse primers.

Phylogenetic analysis of *Bacillus*-related strains on the basis of osmoregulatory genes

Eight different *Bacillus*-related strains were phylogenetically analyzed on the basis of the *ectABC* gene cluster by using the same procedure as that for the 16S rRNA gene sequences. Six *Bacillus*-related strains were phylogenetically analyzed on the basis of the *betA* gene sequence and six *Bacillus*-related strains were phylogenetically analyzed on the basis of the *proDH* gene sequence. Sequences of the *ectABC* gene cluster and the *betA* gene from *Halomonas elongata* were used as outgroups in the phylogenetic tree based on the *ectABC* gene cluster and the *betA* gene, respectively. The sequence of the *proDH* gene from *Pseudomonas entomophila* was used as an outgroup in the phylogenetic tree based on the *proDH* gene.

Table 1. Physical and chemical properties of rhizospheric soil samples (*Salsola stocksii* and *Atriplex amnicola*) and non-rhizospheric soil samples.

Parameter	*S. stocksii*	*A. amnicola*	Non-rhizospheric saline soil samples	Lake-bank soil samples
$EC_{1:1}$ (dS/m)	4.68a	5.39ab	5.63ab	6.62b
pH	8.16ab	7.56a	8.22ab	8.49b
Temperature (°C)	23.52a	25.33ab	25.61b	22.23a
Moisture (%)	29ab	23a	25a	39b
Texture class	Sandy loam	Sandy loam	Sandy loam	Sandy loam
OM (g/kg)	37.74b	34.39ab	30.54a	26.61a
P (mg/kg)	3.62b	3.29b	2.80a	2.35a
K (mg/kg)	0.62b	0.55ab	0.38a	0.31b
Ca (mg/kg)	1.36a	1.44b	1.28a	1.17b
Mg (mg/kg)	1.36b	1.41a	1.29b	1.04a
NO^{-3} (mg/kg)	16.11b	12.87ab	11.15a	10.35a
H+Al (mg/kg)	59.24b	52.47ab	50.64ab	42.31a
V (mg/kg)	4.58b	3.16a	3.26a	4.77b
CEC (mg/dm)	75.91a	69.78a	80.18b	72.58a
SAR	12.45b	9.32ab	13.17a	12.45ab

Note: EC, electrical conductivity; OM, organic matter; P, phosphorous; K, potassium; Ca, calcium; Mg, magnesium; NO^{-3}, nitrate ion; H+Al, potential acidity; V, base saturation index; CEC, cation exchange capacity; and SAR, sodium adsorption ratio. Within a row, values followed by a different letter are statistically significantly different at the 5% level.

Statistical analyses

One-way ANOVA (Analysis of variance (ANOVA)) was applied to analyze the differences in physical and chemical properties among rhizospheric and non-rhizospheric soil samples and significance at the 5% level was tested by least significance difference test using STATISTIX software (8.2 version). A nonmetric multidimensional scaling plot was used to show overall patterns of *Bacillus*-related bacterial diversity in different soil samples by using PAST 3.12 (Hammer et al. 2001).

Nucleotide sequence accession numbers

Bacillus-related 16S rRNA sequences identified through pyrosequencing from the rhizosphere and non-rhizospheric soil samples of halophytes have been submitted in the NCBI Sequence Read Archive under ID project PRJNA309754. Sequences for the 16S rRNA gene from pure culture *Bacillus*-related isolates from the rhizosphere and non-rhizospheric soil samples of halophytes were deposited to NCBI GenBank under the accession Nos. LT221128 (HL1HP4), LT635432 (HL1HP11), LT221134 (HL2HP6), LT221138 (HL2RP7), LT635433 (HL2RP13), LT221136 (HL2RP14), LT221155 (HL3HP16), LT221158 (HL4HP3), LT221159 (HL4RP4), LT635434 (HL4HP15), LT221174 (AT2RP3), LT221177 (AT2RP4), LT221187 (AT3HP4), LT221188 (AT3HP15), LT221228 (NRS5HaP2), LT221232 (NRS5HaP13), LT221242 (LK2HaP12), and LT221248 (LK3HaP7). Sequences of the *ectABC* gene cluster have been deposited to NCBI GenBank under the accession Nos. LT883370–LT883377, sequences of the *betA* gene were deposited under the accession Nos. LT883378–LT883383, and sequences of the *proDH* gene have been deposited under the accession Nos. LT883384–LT883389.

Results

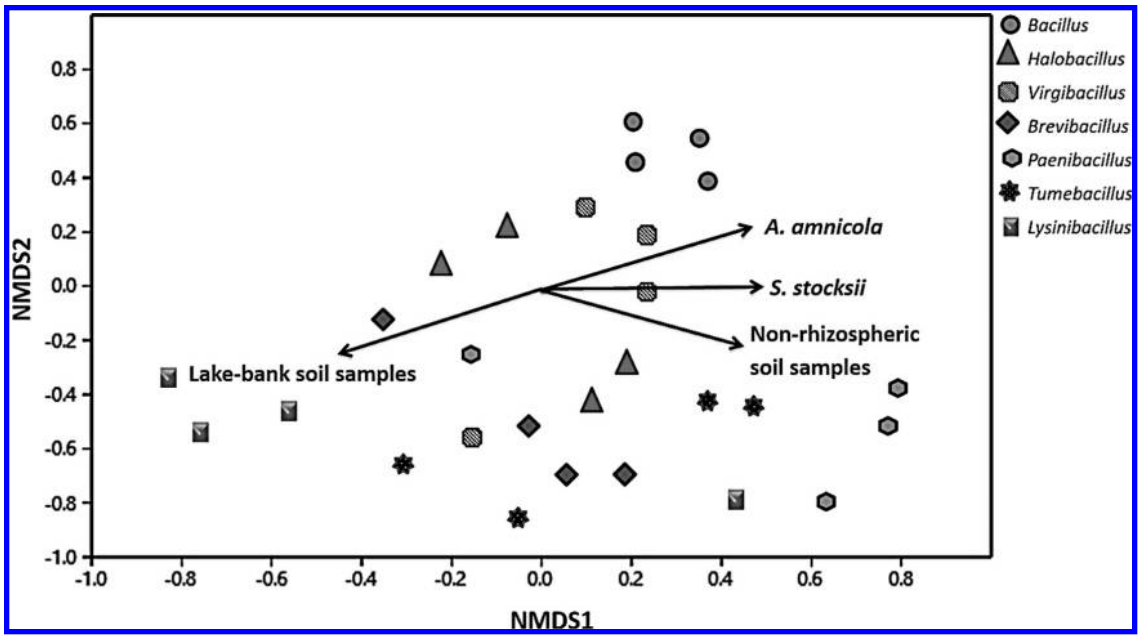
Soil physicochemical analysis

Rhizospheric and non-rhizospheric soil samples of halophytes (*S. stocksii* and *A. amnicola*) and hypersaline lake-bank soil samples were characterized on the basis of physicochemical properties like soil salinity, pH, organic matter, vegetation type, texture class, CEC, and SAR. Electrical conductivity ($EC_{1:1}$) ranged from 4.68 to 6.62 dS/m, with the highest values in the hypersaline lake-bank soil samples and the lowest values in *S. stocksii*. pH values ranged from 7.56 to 8.49, temperature from 22.23 to 25.61 °C, and moisture from 23% to 39%. Total organic matter ranged from 26.61 to 37.74 g/kg. The available P, K, Ca, and Mg contents were different in hypersaline lake-bank soil samples than in the rhizospheric and non-rhizospheric soil samples (Table 1). CEC values ranged from 69.78 to 80.18 mg/dm^3, and SAR values from 9.32 to 13.17, with the highest values in non-rhizospheric saline soil samples and the lowest in *Atriplex* soil samples.

Diversity analysis of *Bacillus*-like bacterial community in the rhizospheric and non-rhizospheric soil samples of halophytes (*S. stocksii* and *A. amnicola*) and hypersaline lake-bank soil samples

Characterization of *Bacillus*-like communities by 16S rRNA gene-based pyrosequencing showed that seven major phylogenetic groups, *Bacillus*, *Halobacillus*, *Virgibacillus*, *Brevibacillus*, *Paenibacillus*, *Tumebacillus*, and *Lysinibacillus*, were identified in the rhizospheres of halophytes (*S. stocksii* and *A. amnicola*), non-rhizospheric soil samples, and hypersaline lake-bank soil samples. Nonmetric multidimensional scaling plot showed that the structure of *Bacillus*-like communities in the rhizospheric and non-rhizospheric soil of halophytes was different from that

Fig. 1. Nonmetric multidimensional scaling representation of the 16S rRNA gene sequence based on the Bray–Curtis similarity index. In this figure, results from analysis of *Bacillus*-like communities in the rhizospheric and non-rhizospheric soil samples of halophytes (*Salsola stocksii* and *Atriplex amnicola*) and lake-bank soil samples is presented. It is based on OTUs represented by >97% similarity.



of *Bacillus*-like communities in hypersaline lake-bank soil samples (Fig. 1). This could be due to a difference in the soil physicochemical properties at different sites. A total of 852 sequences related to *Bacillus*-like bacterial strains in the rhizosphere of *S. stocksii*, 1163 sequences in the rhizosphere of *A. amnicola*, 1098 sequences in non-rhizospheric soil samples, and 575 sequences in hypersaline lake-bank soil samples have been detected in this study. The detailed phylogenetic analysis and distribution of sequences related to *Bacillus*-like bacterial strains is shown in Fig. 2A. *Bacillus*-like communities from the rhizosphere of *S. stocksii* showed a similar diversity pattern, especially for genera *Bacillus* and *Halobacillus*, when studied through pyrosequencing analysis and culture-dependent methods. When overall results from pure culture isolates and culture-independent analysis of *Bacillus*-like communities were compared, the majority of isolates were identified as *Bacillus* strains. Pyrosequencing analysis showed that sequences belonging to *Halobacillus* were more abundant in all the soil samples than sequences from other bacterial genera (Fig. 2).

Biochemical and molecular characterization of *Bacillus* species

From the rhizospheric and non-rhizospheric soil samples of halophytes (*S. stocksii* and *A. amnicola*) and hypersaline lake-bank soil samples, 18 *Bacillus* isolates were selected and identified on the basis of biochemical and molecular characterization (Table 2). Of 18 isolates, 16 were identified as *Bacillus* strains and two as *Oceanobacillus* strains on the basis of biochemical and morphological characterization, whereas 16S rRNA gene analysis demonstrated that seven isolates (HL1HP4, HL2HP6, HL2RP14, HL4HP3, AT2RP4, AT3HP4, and LK2HaP12) were related to different species of the bacterial genus *Bacillus*, five (HL2RP7, HL4HP15, AT3HP15, NRS5HaP13, and LK3HaP7) to *Oceanobacillus*, four (HL1HP11, HL2RP13, HL4RP4, and NRS5HaP2) to *Halobacillus*, and two (HL3HP16 and AT2RP3) to *Virgibacillus* (Table 2; Fig. 2B).

Phenotypic characterization of halophilic *Bacillus* strains

All the strains had the ability to grow at a salt concentration of 3 mol/L NaCl, whereas nine strains could tolerate salt concentrations up to 4 mol/L NaCl (Table 3). Most *Bacillus* strains could grow at pH 9 from all the soil samples, while only six strains (HL2RP7, HL2RP13, HL4HP3, AT3HP15, NRS5HaP2, and LK3HaP7) could grow at pH 11. More than 85% of halophilic *Bacillus*-like strains could grow at 4 °C, and 72% strains were able to grow at 42 °C (Table 3). Most *Bacillus*, *Halobacillus*, and *Oceanobacillus* strains had the ability to degrade proteins, carbohydrates, and lipids. In the case of enzyme profile, most isolates showed catalase and protease activity from all soil samples. Of 18 isolates, 14 showed proteolytic activity, 10 showed positive results for lipase enzyme, five were positive for cellulase activity, six showed positive activity for amylase enzyme, 12 were positive for oxidase, and 17 isolates showed catalase activity (Table 3).

PCR amplification of the *ectABC* gene cluster, *betA* gene, and *proDH* gene

Of 18 isolates from the groups *Bacillus*, *Virgibacillus*, *Halobacillus*, and *Oceanobacillus*, eight strains (HL1HP4, HL2HP6, HL2RP7, AT3HP4, HL2RP14, AT2RP3, HL4HP4, and AT3HP15) showed PCR amplification of the *ectABC* gene cluster (Table 4), six strains (HL1HP4, HL2HP6,

Fig. 2. (A) Molecular phylogenetic analysis and relative abundance of *Bacillus*-related community from the rhizospheric and non-rhizospheric soil samples of halophytes (*S. stocksii* and *A. amnicola*) and lake-bank soil samples. (B) Phylogenetic tree was constructed on the basis of 16S rRNA sequences by using Neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

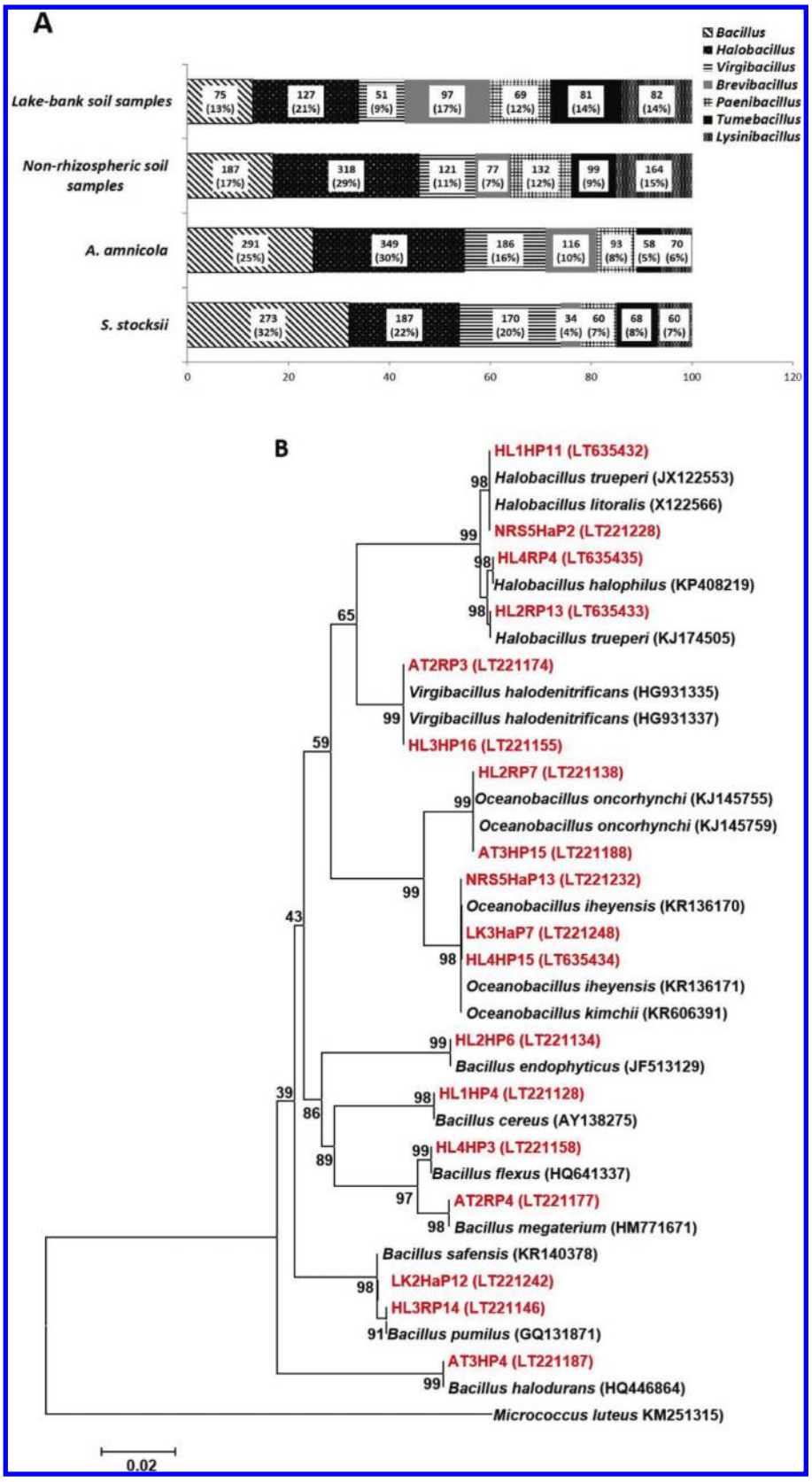


Table 2. Identification of pure culture *Bacillus* isolates from the rhizospheric and non-rhizospheric soil samples of halophytes (*S. stocksii* and *A. amnicola*) and lake-bank soil samples on the basis of QTS 24 bacterial identification kit and 16S rRNA gene sequence analysis.

	Bacillus isolate identified by:			
Isolate	QTS 24	16S rRNA gene sequences	Sequence similarity (%)	Acc. No.
HL1HP4	*Bacillus cereus*	*Bacillus cereus*	99	LT221128
HL1HP11	*Bacillus cereus*	*Halobacillus trueperi*	99	LT635432
HL2HP6	*Bacillus pumilus*	*Bacillus endophyticus*	98	LT221134
HL2RP7	*Oceanobacillus* sp.	*Oceanobacillus oncorhynchi*	99	LT221138
HL2RP13	*Bacillus sphericus*	*Halobacillus trueperi*	99	LT635433
HL2RP14	*Bacillus* sp.	*Bacillus pumilus*	99	LT221136
HL3HP16	*Bacillus* sp.	*Virgibacillus halodenitrificans*	99	LT221155
HL4HP3	*Bacillus sphericus*	*Bacillus flexus*	99	LT221158
HL4RP4	*Bacillus cereus*	*Halobacillus halophilus*	99	LT221159
HL4HP15	*Bacillus* sp.	*Oceanobacillus iheyensis*	98	LT635434
AT2RP3	*Bacillus* sp.	*Virgibacillus halodenitrificans*	100	LT221174
AT2RP4	*Bacillus* sp.	*Bacillus halodurans*	99	LT221177
AT3HP4	*Bacillus* sp.	*Bacillus halodurans*	99	LT221187
AT3HP15	*Oceanobacillus* sp.	*Oceanobacillus oncorhynchi*	99	LT221188
NRS5HaP2	*Bacillus pumilus*	*Halobacillus litoralis*	99	LT221228
NRS5HaP13	*Bacillus pumilus*	*Oceanobacillus kimchii*	99	LT221232
LK2HaP12	*Bacillus megaterium*	*Bacillus safensis*	99	LT221242
LK3HaP7	*Bacillus* sp.	*Oceanobacillus iheyensis*	99	LT221248

Table 3. Phenotypic characterization of halophilic *Bacillus*-like strains from the rhizosphere of *Salsola* and *Atriplex*, non-rhizospheric, and lake-bank soil samples.

	Growth at:											
Isolate	3.0 mol/L NaCl	4.0 mol/L NaCl	pH 9	pH 11	4 °C	42 °C	Protease	Lipase	Cellulase	Amylase	Oxidase	Catalase
HL1HP4	+	−	+	−	+	−	+	+	−	+	−	+
HL1HP11	+	−	+	−	+	+	+	−	−	−	−	+
HL2HP6	+	+	−	−	+	−	+	+	+	−	+	−
HL2RP7	+	−	+	+	+	+	+	−	−	+	+	+
HL2RP13	+	+	+	+	+	+	−	−	+	−	+	+
HL2RP14	+	−	+	−	+	+	+	+	−	−	−	+
HL3HP16	+	−	+	−	−	+	+	+	−	+	+	+
HL4HP3	+	+	+	+	+	+	+	−	+	−	−	+
HL4RP4	+	−	+	−	+	−	+	+	−	−	+	+
HL4HP15	+	+	−	−	+	+	+	+	−	+	+	+
AT2RP3	+	−	+	+	−	+	−	−	−	−	+	+
AT2RP4	+	+	+	−	+	+	−	−	+	−	+	+
AT3HP4	+	+	+	−	+	+	+	+	−	−	+	+
AT3HP15	+	−	+	+	+	+	+	+	−	−	−	+
NRS5HaP2	+	+	+	+	+	+	+	−	−	+	−	+
NRS5HaP13	+	−	+	−	+	−	+	+	−	−	+	+
LK2HaP12	+	+	−	−	+	−	+	+	−	−	+	+
LK3HaP7	+	+	+	+	+	+	−	−	+	+	+	+

AT3HP4, AT2RP3, AT3HP15, and LK3HaP7) showed positive results for *betA* gene amplification (Table 4), and six strains (HL1HP4, HL1HP11, AT3HP4, HL2RP14, HL4RP4, and AT3HP15) showed PCR amplification of the *proDH* gene (Table 4).

Phylogenetic analysis on the basis of osmoregulatory genes

Phylogenetic analysis of the *ectABC* gene cluster showed that three strains had similarity with the genus *Bacillus*, two strains were related to *Oceanobacillus*, two strains were belonging to *Virgibacillus*, and one strain was from the genus *Halobacillus* (Fig. 3A). The results of phylogenetic analysis of the *betA* gene indicated that three isolates showed more than 98% homology with the genus *Bacillus*, two strains were related to *Oceanobacillus*, and one strain was belonging to *Virgibacillus* (Fig. 3B). Phylogenetic analysis on the basis of the *proDH* gene demon-

Table 4. PCR amplification of osmoregulatory genes from halophilic *Bacillus*-like strains isolated from the rhizosphere of *Salsola* and *Atriplex*, non-rhizospheric, and lake-bank soil samples.

	PCR amplification		
Isolate	*ectABC* gene cluster	*betA* gene	*proDH* gene
HL1HP4	+	+	+
HL1HP11	–	–	+
HL2HP6	+	+	–
HL2RP7	+	–	–
HL2RP13	–	–	–
HL2RP14	+	–	+
HL3HP16	+	–	–
HL4HP3	–	–	–
HL4RP4	+	–	–
HL4HP15	–	–	–
AT2RP3	+	+	–
AT2RP4	–	–	–
AT3HP4	–	+	+
AT3HP15	+	+	+
NRS5HaP2	–	–	–
NRS5HaP13	–	–	–
LK2HaP12	–	–	–
LK3HaP7	–	+	+

strated that three strains showed 99% similarity with the genus *Bacillus*, two strains were related to *Oceanobacillus*, and one strain was from the genus *Halobacillus* (Fig. 3C).

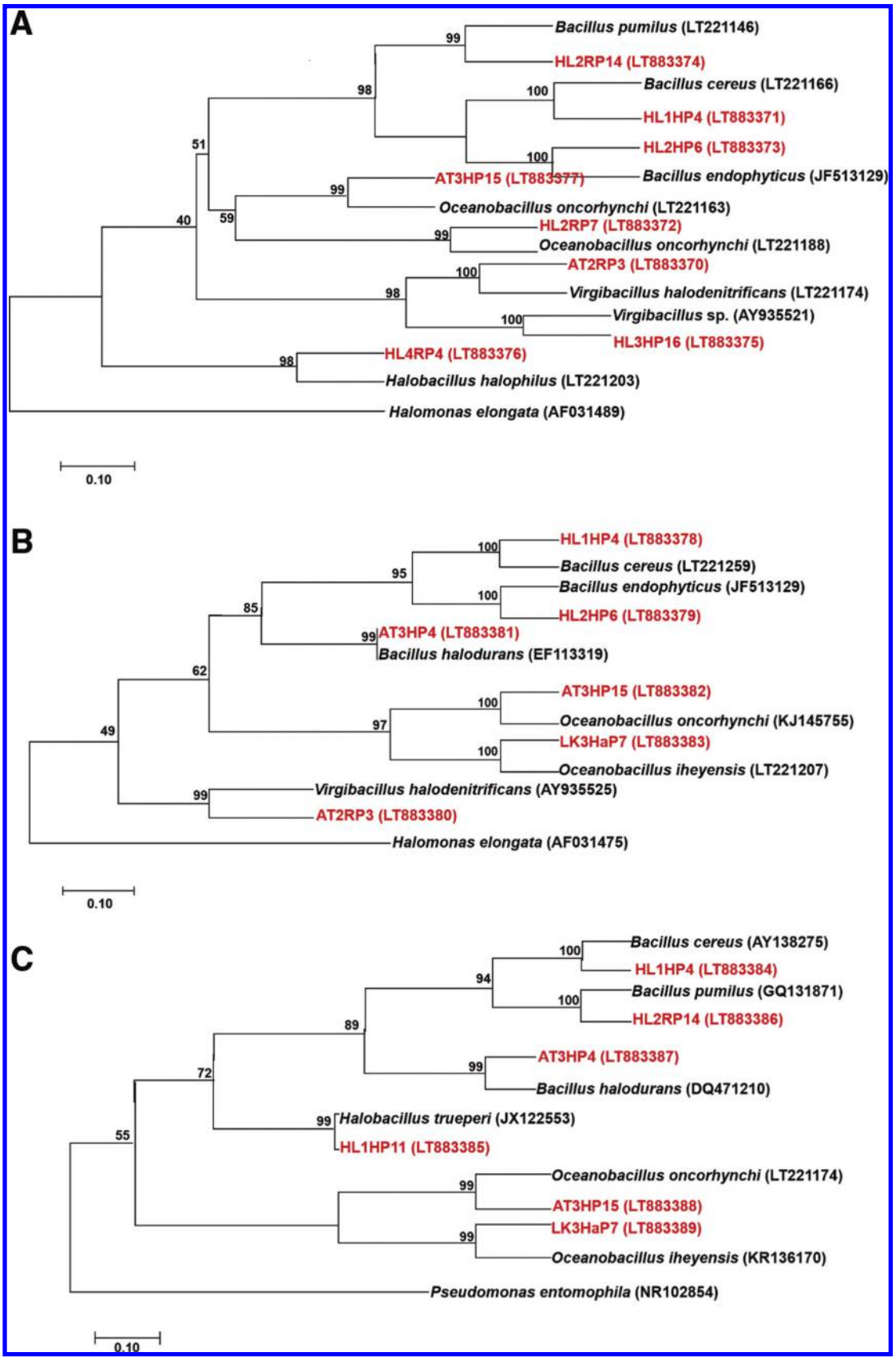
Discussion

Microbial diversity associated with halophytes is a crucial determinant of plant productivity and salt tolerance. This study is the first report of its kind that deals with the diversity analysis of moderately halophilic *Bacillus*-like bacteria from rhizospheric and non-rhizospheric soil of halophytes (*S. stocksii* and *A. amnicola*) by culture-independent and culture-dependent techniques. *Bacillus*-like halophilic bacteria have previously been isolated from various environments like deep-sea hypersaline sediments, glacial ice, saline soils, and inclusions inside materials such as salt crystals (Sass et al. 2008; Larose et al. 2013; Sharma et al. 2015; Yuan et al. 2016).

The results of pyrosequencing analysis of 16S rRNA showed that a total of 3688 sequences were related to seven major phylogenetic groups: *Bacillus*, *Halobacillus*, *Virgibacillus*, *Brevibacillus*, *Paenibacillus*, *Tumebacillus*, and *Lysinibacillus*. From the rhizosphere of *A. amnicola*, the majority of sequences (31.53%) belonging to *Bacillus*-like bacteria have been identified, as compared with sequences from the rhizosphere of *S. stocksii* (23.11%), non-rhizospheric soil samples (29.77%), and hypersaline lake-bank soil samples (15.59%). This could be due to the difference in salinity levels of rhizospheric and non-rhizospheric soil samples. The results of pyrosequencing analysis also suggested that sequences from the genera *Bacillus* and *Halobacillus* were more abundant among various *Bacillus*-like bacterial groups. Although some novel genera, such as *Brevibacillus*, *Paenibacillus*, *Tumebacillus*, and *Lysinibacillus*, were identified from all the soil samples, they were found to be less abundant. The culture-independent techniques allowed the discovery of novel bacterial species from different environmental samples. It is well known that *Bacillus*-like organisms play an important ecological role in biogeochemical cycles in different ecosystems, such as marine waters and saline soils (Taprig et al. 2013; Mukhtar et al. 2017). Halophilic *Bacillus* strains promote plant growth, produce industrially important enzymes (proteases, amylases, cellulases, and lipases), and are involved in bioremediation of different toxic chemicals and pollutants from saline environments (Goswami et al. 2016; Jaisingh et al. 2016). A total of 18 *Bacillus*-like isolates belonging to four phylogenetic groups *Bacillus*, *Halobacillus*, *Virgibacillus*, and *Oceanobacillus* were obtained from rhizospheric and non-rhizospheric soil samples of halophytes (*S. stocksii* and *A. amnicola*). From the marine and saline environments, the *Bacillus*-like bacterial community has been found to be more abundant as compared with other bacteria (Miranda et al. 2008; Irshad et al. 2014). This study showed that more bacterial genera were identified by pyrosequencing analysis than by culture-dependent methods, which suggests that culture-independent techniques are more effective for discovery of unique microbial diversity (Li et al. 2015).

Most of the isolates were moderate halophiles but some were extremely halophilic bacteria. Most bacterial strains were able to grow at pH 9 from rhizospheric and non-rhizospheric soil samples. More than 90% of bacterial isolates grew well at 4 and 42 °C. Previous studies also reported that moderate halophiles and mesophiles are more abundant than extremely halophilic and thermophilic bacteria in different soils (Mwirichia et al. 2010; Mukhtar et al. 2016). Halophilic strains from the groups *Halobacillus*, *Virgibacillus*, and *Oceanobacillus* show optimum growth at salt concentrations of 1–2 mol/L NaCl and 28–37 °C (DasSarma and DasSarma 2015). From the rhizospheric and non-rhizospheric soil samples of halophytes (*S. stocksii* and *A. amnicola*), about 77.78% *Bacillus* strains showed proteolytic activity, 55.57% strains could degrade lipids, 33.34% strains were positive for amylase, and 27.78% strains showed cellulase activity. Moderately halophilic bacteria have been used as a good source of industrially important enzymes, such as proteases, lipases, cellulases, amylases, oxidases, and DNases (Lundberg et al. 2012; Liu et al. 2017). Enzymes produced by halophilic bacteria have a unique structural and catalytic feature to sustain the metabolic and physiological processes under high osmotic stress (Kumar et al. 2012). Protease- and lipase-producing halophilic bacteria have been previously isolated from marine environments and a food source like fish sauce (Phrommao et al. 2011). Bacterial strains from the genera *Bacillus* and *Halobacillus* are known to be a good source of α-amylases

Fig. 3. The phylogenetic analysis of (A) *ectABC* gene cluster, (B) *betA* gene, and (C) *proDH* gene sequences from halophilic *Bacilli* strains. Phylogenetic trees were constructed using the Neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches.



(Ali et al. 2014). Halophilic cellulases have been produced by different lignocellulose-hydrolyzing halophilic bacteria, such as *Bacillus*, *Halobacillus*, *Salibacillus*, and *Halomonas* (de Lourdes Moreno et al. 2013). A number of halophilic *Bacillus* species have been used as biofertilizers and biocontrol agents for different crops, such as wheat, rice, and sugarcane, under salt-stress conditions (Kumar et al. 2011).

Moderately halophilic bacteria maintain their internal osmotic balance by accumulating compatible solutes like ectoine, glycine betaine, proline, and trehalose. Osmoregulatory genes for betaine, ectoine, and proline were also identified and characterized from halophilic *Bacillus* strains isolated from the rhizosphere and non-rhizospheric soil of halophytes. Eight bacterial strains related to *Bacillus*, *Virgibacillus*, *Halobacillus*, and *Oceanobacillus* showed positive results for PCR amplification of the *ectABC* gene cluster (Table 4). Ectoine, a cyclic tetrahydropyrimidine, is considered as a marker for halotolerant and moderately halophilic bacteria and can be synthesized by a number of halophilic bacterial strains related to the genera *Halobacillus* and *Halomonas* (Tanimura et al. 2016). Aspartate aldehyde is used as a precursor molecule in the biosynthesis of ectoine. This molecule is converted into 2,4-diaminobutyric acid, and finally as a result of acetylation, ectoine is formed (Youssef et al. 2014). The choline dehydrogenase (*betA*) was identified and characterized from six isolates belonging to the groups *Bacillus*, *Oceanobacillus*, and *Virgibacillus* from the rhizosphere of halophytes and hypersaline lake-bank soil samples. Different intracellular enzymes are involved for the accumulation of betaine. They maintain internal balance by regulating water inside the cells and, thus, protect the cells from dehydration. Previously, a number of studies have also reported that halophilic bacterial genera, such as *Halomonas*, *Bacillus*, *Oceanobacillus*, and *Staphylococcus*, have ability to synthesize betaine from choline, but amplification and characterization of the *betA* gene from *Oceanobacillus* and *Virgibacillus* are reported for the first time in this study. The betaine operon consists of the genes *betA* (choline dehydrogenase), *betB* (betaine aldehyde dehydrogenase), and *betT* (choline transporter) (Ying et al. 2016; Zou et al. 2016). Six bacterial strains from the groups *Bacillus*, *Oceanobacillus*, and *Halobacillus* showed *proDH* gene detection and identification. Some moderately halophilic bacteria (*E. coli*, *Bacillus*, *Halobacillus*, and *Halomonas*) use proline as a compatible solute to survive under salt-stress environments. These bacteria can synthesize proline by using either glutamate or ornithine as a precursor molecule (Collins and Deming 2013).

Conclusion

To the best of our knowledge, this study is the first report of *Bacillus*-like bacterial diversity from the rhizospheric and non-rhizospheric soil of halophytes (*S. stocksii* and *A. ammicola*) growing in Pakistan (Khewra Salt Mine). Seven major phylogenetic groups, *Bacillus*, *Halobacillus*, *Virgibacillus*, *Brevibacillus*, *Paenibacillus*, *Tumebacillus*, and *Lysinibacillus*, were identified through pyrosequencing analysis, whereas only four genera, *Bacillus*, *Halobacillus*, *Virgibacillus*, and *Oceanobacillus*, were identified by culture-dependent methods. Most *Bacillus* strains isolated in this study were moderately halophilic, alkaliphilic, and mesophilic bacteria. They showed positive results for production of industrially important enzymes, such as proteases, amylases, cellulases, lipases, and oxidases. Osmoregulatory genes for different compatible solutes, such as ectoine, glycine betaine, and proline dehydrogenase, have been identified and characterized from bacterial isolates related to *Bacillus*, *Halobacillus*, *Virgibacillus*, and *Oceanobacillus*. Identification and characterization of *Bacillus* and *Bacillus*-derived genera provides information about the importance of these bacteria as a source of enzymes in industry and as inoculants and biocontrol agents for salt-affected agricultural soils.

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References

Abel-Nabey, H.M., and Farag, A.M. 2016. Production, optimization and characterization of extracellular amylase from halophilic *Bacillus lichineformis* AH214. Afr. J. Biotechnol. **15**: 670–683. doi:10.5897/AJB2015.15073.

Adviento-Borbe, M.A., Doran, J.W., Drijber, R.A., and Dobermann, A. 2006. Soil electrical conductivity and water content affect nitrous oxide and carbon dioxide emissions in intensively managed soils. J. Environ. Qual. **35**: 1999–2010. doi:10.2134/jeq2006.0109. PMID:17071868.

Ahmad, K., Hussain, M., Ashraf, M., Luqman, M., Ashraf, M.Y., and Khan, Z.I. 2007. Indigenous vegetation of Soon valley at the risk of extinction. Pak. J. Bot. **39**: 679–690.

Ali, I., Akbar, A., Yanwisetpakdee, B., Prasongsuk, S., Lotrakul, P., and Punnapayak, H. 2014. Purification, characterization, and potential of saline waste water remediation of a polyextremophilic α-amylase from an obligate halophilic *Aspergillus gracilis*. BioMed Res. Int. **2014**: 106937. PMID: 24949415.

Amoozegar, M.A., Bagheri, M., Makhdoumi, A., Nikou, M.M., Fazeli, S.A.S., Schumann, P., et al. 2016. *Oceanobacillus halophilus* sp. nov., a novel moderately halophilic bacterium from a hypersaline lake. Int. J. Syst. Evol. Microbiol. **66**: 1317–1322. doi:10.1099/ijsem.0.000952. PMID:26869142.

Anderson, J.M., and Ingram, J.S. 1993. Tropical soil biology and fertility: a handbook of methods. 2nd ed. CAB International, Wallingford, UK. pp. 93–94.

Ates, Ö., Oner, E.T., and Arga, K.Y. 2011. Genome-scale reconstruction of metabolic network for a halophilic extremophile, *Chromohalobacter salexigens* DSM 3043. BMC Syst. Biol. **5**: 12. doi:10.1186/1752-0509-5-12. PMID:21251315.

Berendsen, R.L., Pieterse, C.M.J., and Bakker, P.A.H.M. 2012. The

rhizosphere microbiome and plant health. Trends Plant Sci. **17**: 478–486. doi:10.1016/j.tplants.2012.04.001. PMID:22564542.

Jadhav, V.V., Yadav, A., Shouche, Y.S., and Bhadekar, R.K. 2010. Isolation and cellular fatty acid composition of psychrotrophic *Halomonas* strains from Antarctic sea water. Songklanakarin J. Sci. Technol. **35**(3) 287–292.

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., et al. 2012. Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. Nature, **488**: 91–95. doi:10.1038/nature11336. PMID:22859207.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., et al. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods, **7**: 335–336. doi:10.1038/nmeth.f.303. PMID:20383131.

Collins, R.E., and Deming, J.W. 2013. An inter-order horizontal gene transfer event enables the catabolism of compatible solutes by *Colwellia psychrerythraea* 34H. Extremophiles, **17**: 601–610. doi:10.1007/s00792-013-0543-7. PMID:23674353.

DasSarma, S., and DasSarma, P. 2015. Halophiles and their enzymes: negativity put to good use. Curr. Opin. Microbiol. **25**: 120–126. doi:10.1016/j.mib.2015.05.009. PMID:26066288.

de Lourdes Moreno, M., Pérez, D., García, M.T., and Mellado, E. 2013. Halophilic bacteria as a source of novel hydrolytic enzymes. Life, **3**: 38–51. doi:10.3390/life3010038. PMID: 25371331.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., et al. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. **72**: 5069–5072. doi:10.1128/AEM.03006-05. PMID:16820507.

Dodd, I.C., and Pérez-Alfocea, F. 2012. Microbial amelioration of crop salinity stress. J. Exp. Bot. **63**: 3415–3428. doi:10.1093/jxb/ers033. PMID:22403432.

Edgar, R.C. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinf. **5**: 113. doi:10.1186/1471-2105-5-113.

Goswami, D., Thakker, J.N., and Dhandhukia, P.C. 2016. Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. Cogent Food Agric. **2**: 1127500. doi:10.1080/23311932.2015.1127500.

Gupta, P., Samant, K., and Sahu, A. 2012. Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. Int. J. Microbiol. **2012**: 578925. doi:10.1155/2012/578925. PMID:22315612.

Hammer, Ø., Harper, D.A.T., and Ryan, P.D. 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontol. Electron. **4**: 9–13.

Irshad, A., Ahmad, I., and Kim, S.B. 2014. Culturable diversity of halophilic bacteria in foreshore soils. Braz. J. Microbiol. **45**: 563–572. doi:10.1590/S1517-83822014005000050. PMID: 25242943.

Jaisingh, R., Kumar, A., and Dhiman, M. 2016. Isolation and characterization of PGPR from rhizosphere of *Sesame indicum* L. Int. J. Adv. Res. Biol. Sci. **3**: 238–244.

Knief, C., Delmotte, N., Chaffron, S., Stark, M., Innerebner, G., Wassmann, R., et al. 2012. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. ISME J. **6**: 1378–1390. doi:10.1038/ismej.2011.192. PMID: 22189496.

Kumar, A., Prakash, A., and Johri, B.N. 2011. *Bacillus* as PGPR in crop ecosystem. *In* Bacteria in agrobiology: crop ecosystems. *Edited by* D.K. Maheshwari. Springer-Verlag, Berlin, Heidelberg, Germany. pp. 37–59. doi:10.1007/978-3-642-18357-7_2.

Kumar, K.V., Srivastava, S., Singh, N., and Behl, H.M. 2009. Role of metal resistant plant growth promoting bacteria in ameliorating fly ash to the growth of *Brassica juncea*. J. Haz. Mater. **170**: 51–57. doi:10.1016/j.jhazmat.2009.04.132. PMID:19487076.

Kumar, S., Karan, R., Kapoor, S., Singh, S.P., and Khare, S.K. 2012. Screening and isolation of halophilic bacteria producing industrially important enzymes. Braz. J. Microbiol. **43**: 1595–1603. doi:10.1590/S1517-83822012000400044. PMID: 24031991.

Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. **33**: 1870–1874. doi:10.1093/molbev/msw054. PMID:27004904.

Larose, C., Prestat, E., Cecillon, S., Berger, S., Malandain, C., Lyon, D., et al. 2013. Interactions between snow chemistry, mercury inputs and microbial population dynamics in an Arctic snowpack. PLoS One, **8**: e79972. doi:10.1371/journal.pone.0079972. PMID:24282515.

Li, H., Zhong, Q., Wirth, S., Wang, W., Hao, Y., Wu, S., et al. 2015. Diversity of autochthonous bacterial communities in the intestinal mucosa of grass carp (*Ctenopharyngodon idellus*) (Valenciennes) determined by culture-dependent and culture-independent techniques. Aquacult. Res. **46**: 2344–2359. doi:10.1111/are.12391.

Liszka, M.J., Clark, M.E., Schneider, E., and Clark, D.S. 2012. Nature versus nurture: developing enzymes that function under extreme conditions. Annu. Rev. Chem. Biomol. Eng. **3**: 77–102. doi:10.1146/annurev-chembioeng-061010-114239. PMID: 22468597.

Liu, M., Cui, Y., Chen, Y., Lin, X., Huang, H., and Bao, S. 2017. Diversity of Bacillus-like bacterial community in the sediments of the Bamenwan mangrove wetland in Hainan, China. Can. J. Microbiol. **63**: 238–245. doi:10.1139/cjm-2016-0449. PMID:28177800.

Lundberg, D.S., Lebeis, S.L., Paredes, S.H., Yourstone, S., Gehring, J., Malfatti, S., et al. 2012. Defining the core *Arabidopsis thaliana* root microbiome. Nature, **488**: 86–90. doi:10.1038/nature11237. PMID:22859206.

MacFadden, J.F. 1980. Biochemical tests for identification of medical bacteria. Williams and Wilkins, Baltimore, Md., USA.

Malik, K.A., Bilal, R., Mehnaz, S., Rasul, G., Mirza, M.S., and Ali, S. 1997. Association of nitrogen-fixing, plant-growth-promoting rhizobacteria (PGPR) with kallar grass and rice. Plant Soil, **194**: 37–44. doi:10.1023/A:1004295714181.

Mavi, M.S., Sanderman, J., Chittleborough, D.J., Cox, J.W., and Marschner, P. 2012. Sorption of dissolved organic matter in salt-affected soils: effect of salinity, sodicity and texture. Sci. Total Environ. **435–436**: 337–344. doi:10.1016/j.scitotenv.2012.07.009. PMID:22863809.

Miranda, C.A., Martins, O.B., and Clementino, M.M. 2008. Species-level identification of *Bacillus* strains isolates from marine sediments by conventional biochemical, 16S rRNA gene sequencing and inter-tRNA gene sequence lengths analysis. Antonie van Leeuwenhoek, **93**: 297–304. doi:10.1007/s10482-007-9204-0. PMID:17922298.

Mirza, B.S., Muruganandam, S., Meng, X., Sorensen, D.L., Dupont, R.R., and McLean, J.E. 2014. Arsenic(V) reduction in relation to iron(III) transformation and molecular characterization of the structural and functional microbial community in sediments of a basin-fill aquifer in Northern Utah. Appl. Environ. Microbiol. **80**: 3198–3208. doi:10.1128/AEM.00240-14. PMID:24632255.

Moghaddam, J.A., Boehringer, N., Burdziak, A., Kunte, H.J., Galinski, E.A., and Schäberle, T.F. 2016. Different strategies of osmoadaptation in the closely related marine myxobacteria *Enhygromyxa salina* SWB007 and *Plesiocystis pacifica* SIR-1. Microbiology, **162**: 651–661. doi:10.1099/mic.0.000250.

Mohammadi, H.S., and Ominidia, E. 2012. Proline dehydrogenase from *Pseudomonas fluorescens*: gene cloning, purification, characterization and homology modeling. Appl. Biochem. Microbiol. **48**: 167–174. doi:10.1134/S0003683812020081.

Mukhtar, S., Mirza, M.S., Awan, H.A., Maqbool, A., Mehnaz, S., and Malik, K.A. 2016. Microbial diversity and metagenomic analysis of the rhizosphere of para grass (*Urochloa mutica*) growing under saline conditions. Pak. J. Bot. **48**: 779–791.

Mukhtar, S., Ishaq, A., Hassan, S., Mehnaz, S., Mirza, M.S., and Malik, K.A. 2017. Comparison of microbial communities associated with halophyte (*Salsola stocksii*) and non-halophyte (*Triticum aestivum*) using culture-independent approaches. Pol. J. Microbiol. **66**: 353–364. doi:10.5604/01.3001.0010.4866. PMID:29319512.

Mwirichia, R., Muigai, A.W., Tindall, B., Boga, H.I., and Stackebrandt, E. 2010. Isolation and characterization of bacteria from the haloalkaline Lake Elmenteita, Kenya. Extremophiles, **14**: 339–348. doi:10.1007/s00792-010-0311-x. PMID:20401502.

Nath, A. 2016. Insights into the sequence parameters for halophilic adaptation. Amino Acids, **48**: 751–762. doi:10.1007/s00726-015-2123-x. PMID:26520112.

Oren, A. 2012. Taxonomy of the family Halobacteriaceae: a paradigm for changing concepts in prokaryote systematics. Int. J. Syst. Evol. Microbiol. **62**: 263–271. doi:10.1099/ijs.0.038653-0. PMID:22155757.

Phrommao, E., Rodtong, S., and Yongsawatdigul, J. 2011. Identification of novel halotolerant bacillopeptidase F-like proteinases from a moderately halophilic bacterium, *Virgibacillus* sp. SK37. J. Appl. Microbiol. **110**: 191–201. doi:10.1111/j.1365-2672.2010.04871.x. PMID:20955191.

Porwal, S., Kumar, T., Lal, S., Rani, A., Kumar, S., Cheema, S., et al. 2008. Hydrogen and polyhydroxybutyrate producing abilities of microbes from diverse habitats by dark fermentative process. Bioresour. Technol. **99**: 5444–5451. doi:10.1016/j.biortech.2007.11.011. PMID:18083024.

Rajan, A.L., Joseph, T.C., Thampuran, N., James, R., Kumar, K.A., Viswanathan, C., and Bansal, K.C. 2008. Cloning and heterologous expression of ectoine biosynthesis genes from *Bacillus halodurans* in *Escherichia coli*. Biotechnol. Lett. **30**: 1403–1407. doi:10.1007/s10529-008-9688-3. PMID:18488150.

Rajan, A.L., Toms, C.J., Nirmala, T., Roswin, J., Viswanathan, C., and Kailash, C.B. 2010. Functional characterization and sequence analysis of choline dehydrogenase from *Escherichia coli*. Genet. Eng. Biotechnol. J. **2010**: GEBJ-12.

Saitou, N., and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**: 406–425. PMID:3447015.

Sass, A.M., McKew, B.A., Sass, H., Fichtel, J., Timmis, K.N., and McGenity, T.J. 2008. Diversity of Bacillus-like organisms isolated from deep-sea hypersaline anoxic sediments. Saline Syst. **4**: 8. doi:10.1186/1746-1448-4-8. PMID:18541011.

Schneegurt, M.A. 2012. Media and conditions for the growth of halophilic and halotolerant bacteria and archaea. *In* Advances in understanding the biology of halophilic microorganisms. *Edited by* R.H. Vreeland. Springer, Dordrecht, Netherlands. pp. 35–58. doi:10.1007/978-94-007-5539-0_2.

Schubert, T., Maskow, T., Benndorf, D., Harms, H., and Breuer, U. 2007. Continuous synthesis and excretion of the compatible solute ectoine by a transgenic, nonhalophilic bacterium. Appl. Environ. Microbiol. **73**: 3343–3347. doi:10.1128/AEM.02482-06. PMID:17369334.

Sharma, A., Singh, P., Kumar, S., Kashyap, P.L., Srivastava, A.K., Chakdar, H., et al. 2015. Deciphering diversity of salt-tolerant *Bacilli* from saline soils of Eastern Indo-gangetic Plains of India. Geomicrobiol. J. **32**: 170–180. doi:10.1080/01490451.2014.938205.

Sierra, G. 1957. A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. Antonie van Leeuwenhoek, **23**: 15–22. doi:10.1007/BF02545855. PMID:13425509.

Somasegaran, P., and Hoben, H.J. 1994. Handbook for *Rhizobia*: methods in legume–*Rhizobium* technology. Springer-Verlag, New York. doi:10.1007/978-1-4613-8375-8.

Tamura, K., Nei, M., and Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. U.S.A. **101**: 11030–11035. doi:10.1073/pnas.0404206101. PMID:15258291.

Tan, Z.Y., Xu, X.D., Wang, E.T., Gao, J.L., Martinez-Romero, E., and Chen, W.X. 1997. Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related *Rhizobia*. Int. J. Syst. Bacteriol. **47**: 874–879. doi:10.1099/00207713-47-3-874. PMID:9226921.

Tanimura, K., Matsumoto, T., Nakayama, H., Tanaka, T., and Kondo, A. 2016. Improvement of ectoine productivity by using sugar transporter-overexpressing *Halomonas elongata*. Enzyme Microb. Technol. **89**: 63–68. doi:10.1016/j.enzmictec.2016.03.006. PMID:27233128.

Taprig, T., Akaracharanya, A., Sitdhipol, J., Visessanguan, W., and Tanasupawat, S. 2013. Screening and characterization of protease-producing *Virgibacillus*, *Halobacillus* and *Oceanobacillus* strains from Thai fermented fish. J. Appl. Pharm. Sci. **3**: 025–030. doi:10.7324/JAPS.2013.30205.

Tripathi, S., Kumari, S., Chakraborty, A., Gupta, A., Chakrabarti, K., and Bandyapadhyay, B.K. 2006. Microbial biomass and its activities in salt-affected coastal soils. Biol. Fertil. Soils, **42**: 273–277. doi:10.1007/s00374-005-0037-6.

Varian, H. 2005. Bootstrap tutorial. Math. J. **9**: 768–775.

Walkley, A., and Black, I.A. 1934. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci. **37**: 29–38. doi:10.1097/00010694-193401000-00003.

Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. **73**: 5261–5267. doi:10.1128/AEM.00062-07. PMID:17586664.

Winnepenninckx, B., Backeljau, T., and de Wachter, R. 1993. Extraction of high molecular weight DNA from molluscs. Trends Genet. **9**: 407–412. doi:10.1016/0168-9525(93)90102-N. PMID:8122306.

Ying, X., Liu, Y., Xu, B., Wang, D., and Jiang, W. 2016. Characterization and application of *Halomonas shantousis* SWA25, a halotolerant bacterium with multiple biogenic amine degradation activity. Food Addit. Contam. **33**: 674–682. doi:10.1080/19440049.2016.1147086.

Youssef, N.H., Savage-Ashlock, K.N., McCully, A.L., Luedtke, B., Shaw, E.I., Hoff, W.D., and Elshahed, M.S. 2014. Trehalose/2-sulfotrehalose biosynthesis and glycine–betaine uptake are widely spread mechanisms for osmoadaptation in the Halobacteriales. ISME J. **8**: 636–649. doi:10.1038/ismej.2013.165. PMID:24048226.

Yuan, Z., Druzhinina, I.S., Labbé, J., Redman, R., Qin, Y., Rodriguez, R., et al. 2016. Specialized microbiome of a halophyte and its role in helping non-host plants to withstand salinity. Sci. Rep. **6**: 32467. doi:10.1038/srep32467. PMID:27572178.

Zou, H., Chen, N., Shi, M., Xian, M., Song, Y., and Liu, J. 2016. The metabolism and biotechnological application of betaine in microorganism. Appl. Microbiol. Biotechnol. **100**: 3865–3876. doi:10.1007/s00253-016-7462-3. PMID:27005411.