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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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Short title: Diversity of *Bacillus*-like community in the rhizosphere of halophytes

Abstract

Salinity is one of the major abiotic stresses, with a total of 3% of the world's land mass being 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 as well as culture-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 *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*

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 help 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 Perez-Alfocea 2012) and 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 to 3.4M 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 found to be more abundant as compared to 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 pharmaceutical, food, textile and paper industries (Abel-Nabey and Farag 2016). Cellulose, lipids and pectin degradation by *Bacilli* strains produce different organic compounds like methanol which are used as a carbon source by other bacteria (Knief et al. 2012).

Moderate halophilic bacteria use ‘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). 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 *E. coli* have ability to use some amino acids as osmolytes that accumulate in high levels in response to salt and drought stress (Collins et al. 2016).

Khewra Salt Mine is the world second largest salt mine, located near Pind Dadan Khan Tehsil of Jhelum District, Punjab, Pakistan (32° 38′ North, 73°10′ East). 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 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 have also discussed about the diversity of *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 based on salt, pH and temperature tolerance and extracellular enzymes. Halophilic bacteria can tolerate more salinity as compare to halophytes 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.

Material and Methods

Sample collection

We surveyed an area approximately 1.1 km from the Khewra Salt Mines (Table S1 and Fig. S1). 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 ℃ for further analysis.

Soil physicochemical parameters

Each soil sample (300 g) was thoroughly mixed and sieved through a aperture size of 2 mm. Physical properties (pH, moisture content, salinity and temperature) of soil samples from rhizosphere of a variety of plants and non-rhizospheric regions were determined. Moisture (%), temperature and texture class were measured by the Anderson method (Anderson et al. 1993); pH was measured by 1:2.5 (w/v) soil to water mixture and electrical conductivity (dS/m) was measured by 1:1 (w/v) soil to water mixture at 25 ℃ (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 instructions. The concentration of metagenomic DNA was qualitatively determined on 0.8% (w/v) agarose gel and quantified using Nanodrop (NanoDrop 200c Thermo Scientific, USA). In total, 16 DNA samples (8 rhizospheric, 4 non-rhizospheric saline and 4 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). 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 1X buffer, 0.2 µM of each primer, 1.8 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates (dNTPs), 20 ng of template, and 1µl FastStart high-fidelity PCR system enzyme (Roche Applied Sciences). The PCR conditions were 3 min at 95 ℃, followed by 30 cycles of denaturation at 94 ℃ for 45 s, primer annealing at 54 ℃ for 45 s, extension at 72 ℃ for 1 min, and final extension for 7 min.

Amplified PCR products were purified with Agencourt AMPure beads (Beckman Coulter, Brea, CA). 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 in order 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 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 operational taxonomic units (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) (Tryptone 5 g/l, Yeast Extract 1 g/l, NaCl 117 g/l, 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 grams of it was suspended in saline solution (1% NaCl), followed by stirring for 30 minutes (Malik et al. 1997). Serial dilutions (10^{-1}-10^{-10}) were made for all samples (Somasegaran 1994). Dilutions from 10^{-3} to 10^{-6} were inoculated onto HaP plates for determining the colony forming units (CFU) per gram of dry weight.

Plates were incubated at 37 ℃ until the appearance of bacterial colonies, after which they were counted and CFU was calculated. The bacteria were purified by repeated sub-culturing of single colonies. Single colonies selected were grown in HaP broth and stored in 33% glycerol at -80 ℃ 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 CTAB method (Winnepenninckx et al. 1993). PCR amplification of 16S rRNA were performed by using universal forward and reverse primers P1 (5'-GAGAGTTTGATCCTGGTCAGAACGAAC-3'), P6 (5'CGTACGGCTACCTTGTTACGACTTCACC-3') for prokaryotes (Tan et al. 1997). A PCR reaction of 50 µl was prepared by using Taq polymerase (5U) 0.5 µl, Taq buffer (10X) 2 µl, MgCl2 (25 mM) 2.5 µl, dNTPS (2.5 mM) 2 µl, 2 µl each of forward and reverse primer (10 pmol), 36 µl of dd.H2O and 3 µl of template DNA. First denaturation step at 95 ℃ for 5 min followed by 35 cycles of 94 ℃ for 1 min, 55 ℃ for 1 min and 72 ℃ for 2 min and a final extension step was at 72 ℃ 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 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 analysed with the help of Chromas Lite 2.01 sequence analysis software (Technelysium Pty Ltd. Australia). The gene sequences were compared to those deposited in the GenBank nucleotide database using the NCBI BLAST program. Sequences were aligned using Clustal X 2.1 program and phylogenetic tree was constructed using 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 are in the units of 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. Sequence of 16S rRNA gene from *Micrococcus luteus* was sued 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 varying salt, pH and temperature conditions by using HaP broth medium. The salt concentrations tested were 1.5 - 4.5M NaCl, pH ranged from 4 - 12 and temperature between 4 - 42 ℃. Isolates were cultured in 250 ml flasks at 37 ℃ with continuous rotatory agitation at 150 rpm for 72 h (hours) (Bhadekar et al. 2010). During incubation, bacterial growth in terms of optical density (OD 600) was measured after different time intervals (3h, 6h, 12h, 24h, 48h and 72h).

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 single colony of the bacterial strains on CMC (carboxymethyl cellulose 1%) agar plates respectively (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 LB 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 ℃ 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 e*ctABC* gene cluster from different halophilic *Bacillus* strains, a pair of primers EO1 and EO2 (Rajan et al. 2008) was used and a reaction mixture of 25 µL containing 12 ng template DNA, 2.5 µL 10X *Taq* polymerase buffer (Fermentas), 0.5 µL 10 mM dNTPs (Fermentas), 2 µL of 25 mM MgCl2 (Fermentas), 0.5 µM each of primers and 0.2 units *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 ℃ for 5 min, followed by 35 repeated cycles of 94 ℃ for 1 min, 50 ℃ for 50 sec and 72 ℃ for 2 min and final extension at 72 ℃ for 10 min. For PCR amplification of *betA* gene, a primer pair bAF and bAR was used (Rajan et al. 2010). *ProDH* gene for proline dehydrogenase has been amplified by using a primer pair PDHPF and PDHPR (Mohammadi and Ominidia, 2012). PCR profile for *betA* and *ProDH* genes was same as in case of the e*ctABC* gene cluster. Amplified PCR products were run on agarose gel and purified by using 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 analysed on the basis of e*ctABC* gene cluster by using the same procedure as in case for the 16S rRNA gene sequences. Six *Bacillus*-related strains were phylogenetically analyzed on the basis of *betA* gene and six *Bacillus*-related strains were phylogenetically analyzed on the basis of *ProDH* gene sequences. Sequences of e*ctABC* gene cluster and *betA* gene from *Halomonas elongata* were used as outgroups in the phylogenetic tree based on e*ctABC* gene cluster and *betA* gene respectively. Sequence of *ProDH* gene from *Pseudomonas entomophila* was used as outgroup in the phylogenetic tree based on ProDH gene.

Statistical analyses

One-way ANOVA (Analysis of variance) 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 (LSDT) using STATISTIX software (8.2 version). 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 (SRA) 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 numbers of 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 *ectABC* gene cluster have been deposited to NCBI GenBank under the accession numbers of LT883370 - LT883377, sequences of *betA* gene were deposited under the accession numbers of LT883378 - LT883383 and sequences of *ProDH* gene have been deposited under the accession numbers of 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 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 ℃ 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 7 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 compared to *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 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, maximum isolates were identified as *Bacillus* strains. Pyrosequencing analysis showed that sequences belonging to *Halobacillus* were more abundant in all the soil samples as compared to 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). Out of 18 isolates, sixteen isolates were identified as *Bacillus* strains and two isolates identified 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 bacterial genus *Bacillus*, five isolates (HL2RP7, HL4HP15, AT3HP15, NRS5HaP13 and LK3HaP7) were belonging to *Oceanobacillus*, four isolates were related to *Halobacillus* (HL1HP11, HL2RP13, HL4RP4 and NRS5HaP2) and two isolates (HL3HP16 and AT2RP3) were belonging to *Virgibacillus* (Table 2 and Fig. 2B).

Phenotypic characterization of halophilic *Bacillus* strains

All the strains had the ability to grow at salt concentrations of 3M NaCl whereas nine strains could tolerate salt concentrations up to 4M NaCl (Table 3). Mostly *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% halophilic *Bacillus*-like strains could grow at 4 ℃ and 72% strains were able to grow at 42 ℃ (Table 3). Mostly *Bacillus, Halobacillus* and *Oceanobacillus* strains had the ability to degrade proteins, carbohydrates and lipids. In the case of enzymes profile, maximum isolates showed catalase and protease activity from all soil samples. Out of eighteen, fourteen isolates showed proteolytic activity, ten isolates showed positive results for lipase enzyme, five isolates were positive for cellulase activity, six isolates showed positive activity for amylase enzyme, twelve strains were positive for oxidase test and seventeen isolates showed catalase activity (Table 3).

PCR amplification of *ectABC* gene cluster, *betA* gene and *ProDH* gene

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

Phylogenetic analysis on the basis of osmoregulatory genes

Phylogenetic analysis of *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 from the genus *Halobacillus* (Fig. 3a). The results on phylogenetic analysis of *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 *ProDH* gene demonstrated that three strains showed 99% similarity with the genus *Bacillus*, two strains were related to *Oceanobacillus* and one strain 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 as well as culture-dependent techniques. *Bacillus*-like halophilic bacteria have been previously 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; Yuan et al. 2016).

The results of pyrosequencing analysis of 16S rRNA showed that a total of 3688 sequences were related to 7 major phylogenetic groups *Bacillus, Halobacillus, Virgibacillus, Brevibacillus, Paenibacillus, Tumebacillus* and *Lysinibacillus.* From the rhizosphere of *A. amnicola,* maximum sequences (31.53%) belonging to *Bacillus*-like bacteria have been identified as compared to 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 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 involved in bioremediation of different toxic chemicals and pollutants from saline environments. A total of 18 *Bacillus*-like isolates belonged to four phylogenetic groups *Bacillus, Halobacillus, Virgibacillus* and *Oceanobacillus* have been obtained from rhizospheric and non-rhizospheric soil samples of halophytes (*S. stocksii* and *A. amnicola*). From the marine and saline environments, *Bacillus*-like bacterial community has been found to more abundant as compared to other bacteria (Miranda et al. 2008; Irshad et al. 2014). This study showed that more bacterial genera were identified by using pyrosequencing analysis as compared to culture-dependent methods which suggests that culture-independent techniques are more effective for discovery of unique microbial diversity (Li et al. 2014).

Most of the isolates were moderate halophiles but some are extremely halophilic bacteria. Maximum 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 ℃. Previous studies also reported that moderately halophiles and mesophiles are more abundant as compare to 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 concentration 1 – 2M NaCl and 28 – 37 ℃ (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 had ability to degrade lipids, 33.34% strains showed positive results for amylase enzyme and 27.78% strains showed cellulase activity. Moderately halophilic 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 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 environment and food source like fish sauce (Phrommao et al. 2010). Bacterial strains from the genera *Bacillus* and *Halobacillus* are known to be a good source of α-amylases (Ali et al. 2014). Halophilic cellulases have been produced by different lignocellulose hydrolyzing halophilic bacteria such as *Bacillus, Halobacillus, Salibacillus* and *Halomonas* (De Lourdes 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 accumulation of compatible solutes such as 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 *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 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 regulation of 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 *betA* gene from *Oceanobacillus* and *Virgibacillus* reported for the first time in this study. The betaine operon consists of *betA* gene (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 et al. 2016).

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. amnicola*) 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. Mostly *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 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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Conflict of interest

The authors declared that they have no conflict of interest in the publication.

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642 # Figure Legends

643

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

648 **Fig. 2. (A)** Molecular phylogenetic analysis and relative abundance of *Bacillus*-related community from
649 the rhizospheric and non-rhizospheric soil samples of halophytes (*S. stocksii* and *A. amnicola*) and lake-
650 bank soil samples. **(B)** Phylogenetic tree was constructed on the basis of 16S rRNA sequences by using
651 Neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered
652 together in the bootstrap test (1,000 replicates) was shown next to the branches.

653 **Fig. 3.** The phylogenetic analysis of **(a)** *ectABC* gene cluster, **(b)** *betA* and **(c)** proDH gene sequences
654 from halophilic *Bacilli* strains. Phylogenetic tree was constructed on the basis of Neighbor-joining
655 method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap
656 test (1,000 replicates) was shown next to the branches.

Tables

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

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

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 SA (*Sodium adsorption ratio*). Alphabets in the column represent statistically significant values at 5% level.

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.

Isolates	Identification Methods		Sequence similarity (%)	Accession No.
	QTS 24	16S rRNA gene sequences		
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.

Isolates	Growth at						Protease	Lipase	Cellulase	Amylase	Oxidase	Catalase
	3.0M NaCl	4.0M NaCl	pH 9	pH 11	4 ℃	42 ℃						
HL1HP4	+	-	+	-	+	-	+	+	-	+	-	+
HL1HP11	+	-	+	-	+	+	+	-	-	-	-	+
HL2HP6	+	+	-	-	+	-	+	+	+	-	+	-
HL2RP7	+	-	+	+	+	+	+	-	-	+	+	+
HL2RP13	+	+	+	+	+	+	-	-	+	-	+	+
HL2RP14	+	-	+	-	+	+	+	+	-	-	-	+
HL3HP16	+	-	+	-	-	+	+	+	-	+	+	+
HL4HP3	+	+	+	+	+	+	+	-	+	-	-	+
HL4RP4	+	-	+	-	+	-	+	+	-	-	+	+
HL4HP15	+	+	-	-	+	+	+	+	-	+	+	+
AT2RP3	+	-	+	+	+	-	-	-	-	-	+	+
AT2RP4	+	+	+	-	+	+	-	-	+	-	+	+
AT3HP4	+	+	+	-	+	+	+	+	-	-	+	+
AT3HP15	+	-	+	+	+	+	+	+	-	-	-	+
NRS5HaP2	+	+	+	+	+	+	+	-	-	+	-	+
NRS5HaP13	+	-	+	-	+	-	+	+	-	-	+	+
LK2HaP12	+	+	-	-	+	-	+	+	-	-	+	+
LK3HaP7	+	+	+	+	+	+	-	-	+	+	+	+

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

Isolates	PCR amplification		
	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	-	+	+

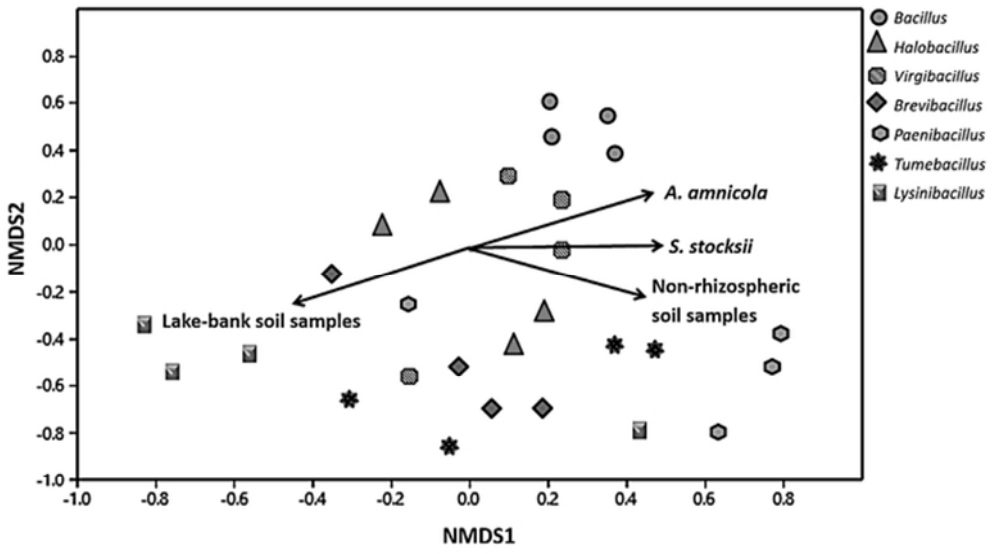
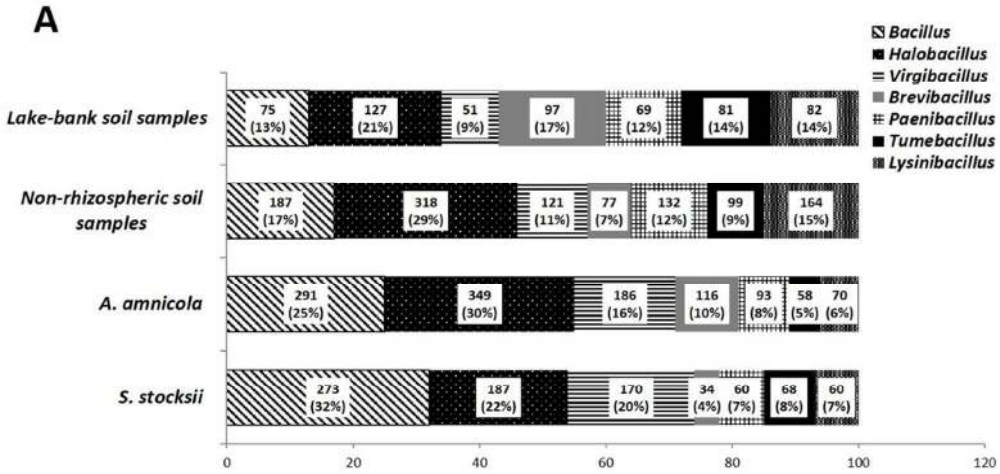
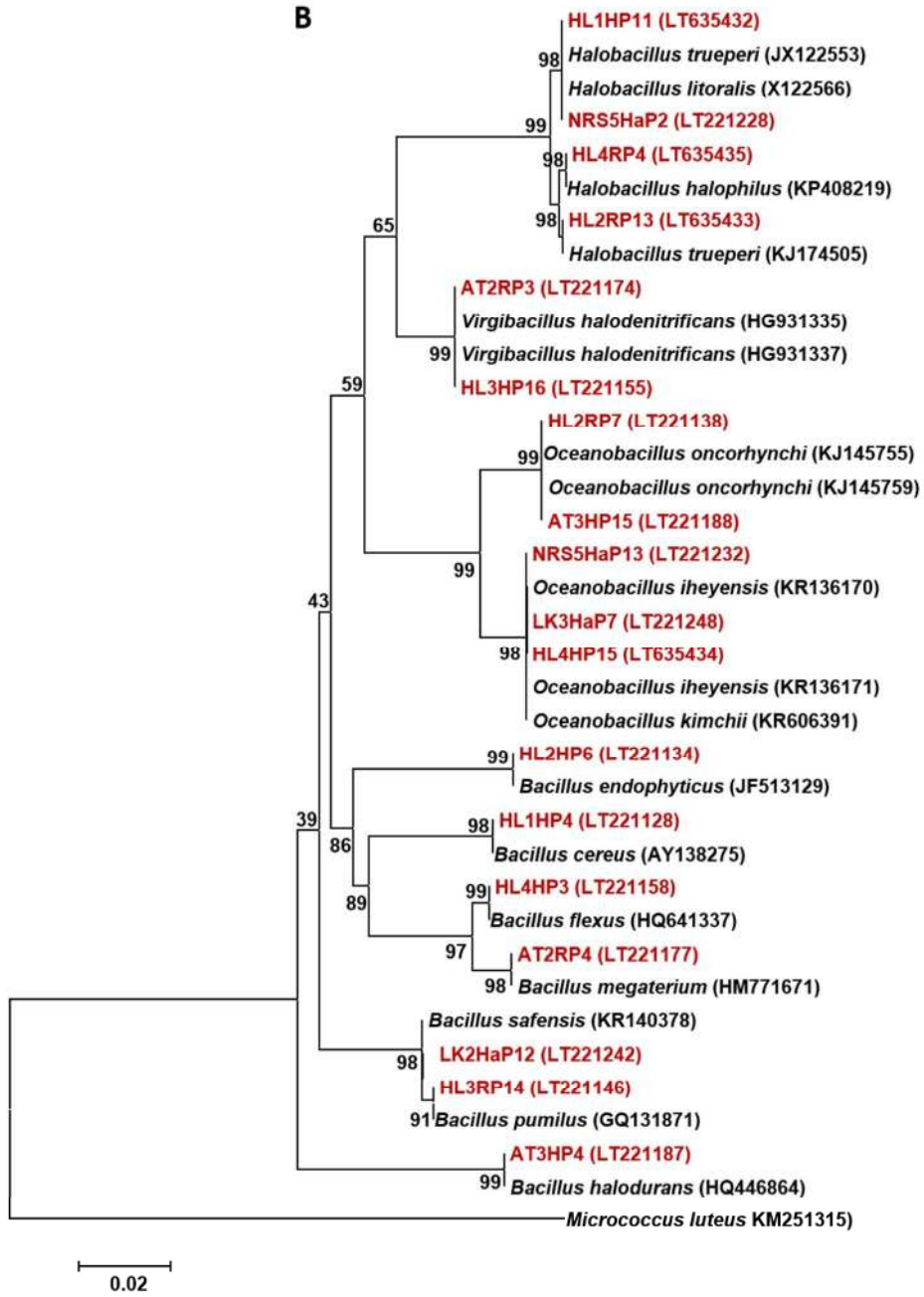


Fig. 1. Nonmetric multidimensional scaling representation of the 16S rRNA gene sequence based on the Bray-Curtis similarity index. In this figure, analysis of Bacillus-like communities in the rhizospheric and non-rhizospheric soil samples of halophytes (S. stocksii and A. amnicola) and lake-bank soil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

55x33mm (300 x 300 DPI)


A
Bacillus
Halobacillus
Virgibacillus
Brevibacillus
Paenibacillus
Tumebacillus
Lysinibacillus
Lake-bank soil samples
Non-rhizospheric soil samples
A. amnicola
S. stocksii
75 (13%)
127 (21%)
51 (9%)
97 (17%)
69 (12%)
81 (14%)
82 (14%)
187 (17%)
318 (29%)
121 (11%)
77 (7%)
132 (12%)
99 (9%)
164 (15%)
291 (25%)
349 (30%)
186 (16%)
116 (10%)
93 (8%)
58 (5%)
70 (6%)
273 (32%)
187 (22%)
170 (20%)
34 (4%)
60 (7%)
68 (8%)
60 (7%)
0
20
40
60
80
100
120


B
HL1HP11 (LT635432)
Halobacillus trueperi (JX122553)
Halobacillus litoralis (X122566)
NRS5HaP2 (LT221228)
HL4RP4 (LT635435)
Halobacillus halophilus (KP408219)
HL2RP13 (LT635433)
Halobacillus trueperi (KJ174505)
AT2RP3 (LT221174)
Virgibacillus halodenitrificans (HG931335)
Virgibacillus halodenitrificans (HG931337)
HL3HP16 (LT221155)
HL2RP7 (LT221138)
Oceanobacillus oncorhynchi (KJ145755)
Oceanobacillus oncorhynchi (KJ145759)
AT3HP15 (LT221188)
NRS5HaP13 (LT221232)
Oceanobacillus iheyensis (KR136170)
LK3HaP7 (LT221248)
HL4HP15 (LT635434)
Oceanobacillus iheyensis (KR136171)
Oceanobacillus kimchii (KR606391)
HL2HP6 (LT221134)
Bacillus endophyticus (JF513129)
HL1HP4 (LT221128)
Bacillus cereus (AY138275)
HL4HP3 (LT221158)
Bacillus flexus (HQ641337)
AT2RP4 (LT221177)
Bacillus megaterium (HM771671)
Bacillus safensis (KR140378)
LK2HaP12 (LT221242)
HL3RP14 (LT221146)
Bacillus pumilus (GQ131871)
AT3HP4 (LT221187)
Bacillus halodurans (HQ446864)
Micrococcus luteus KM251315)
0.02

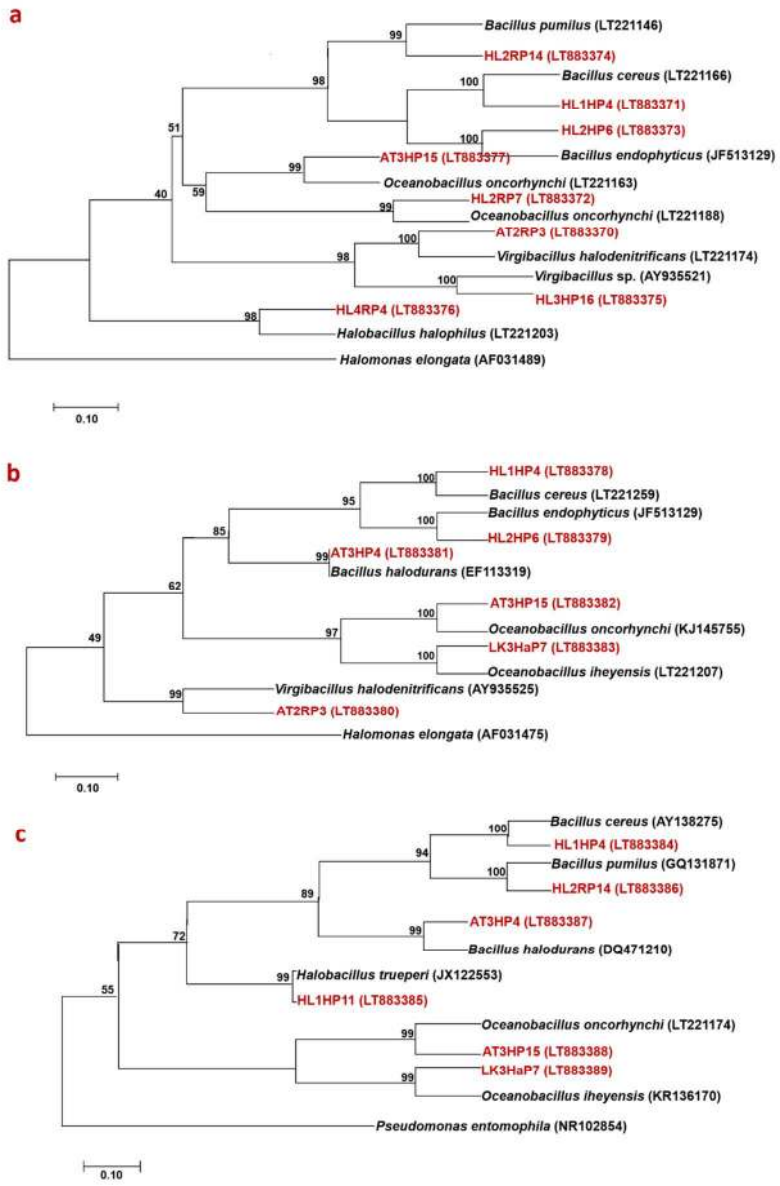


Fig. 3. The phylogenetic analysis of (a) ectABC gene cluster, (b) betA and (c) proDH gene sequences from halophilic Bacilli strains. Phylogenetic tree was constructed on the basis of Neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was shown next to the branches.

119x163mm (300 x 300 DPI)