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Isolation and Characteristics of Biotechnologically Important Antagonistic Thermophilic Bacteria from Rhizosphere of *Haloxylon salicornicum*

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Abstract

Rhizobacteria are an active part of microbial population in the rhizosphere of plants. In this study, twenty rhizobacteria were isolated from the rhizosphere of a perennial grass, *Haloxylon salicornicum,* found in Cholistan desert, an arid landmass near Bahawalpur Pakistan, in one set of experimental conditions. Colony characteristics, biochemical and molecular analyses of these isolates were performed. All isolates were bacilli, gram positive with off-white colonies and exhibited typical bacilli colony morphology. None of the isolates was gelatinase, urease, indole, H_2S and catalase producer. Eleven isolates were amylase producers and 8 isolates were acid producers. All isolates fermented glucose, 3 fermented lactose and 19 fermented fructose. Molecular data revealed that out of twenty isolates, 14 isolates showed 91–99% identity with *Brevibacillus borstelensis*, 4 with *Bacillus subtilis* (97–98%) and 2 with *Bacillus licheniformis* (94–99%) through BLAST analysis. All identified bacterial isolates cladded with their respective groups in the phylogenetic tree. Many (11–15 out of 20) of the isolates were more effective in inhibiting growth of the tested bacterial strains as compared to the positive control (Ampicillin 50 μg/disc). We conclude that bacilli are the predominant form populating rhizosphere of this desert grass. Among the isolated bacteria *Brevibacillus borstelensis*, *Bacillus subtilis* and *Bacillus licheniformis* are the most predominant species.

Key words: *Haloxylon salicornicum*, rhizobacteria, 16S rRNA for phylogenic analysis, rhizosphere on Cholistan desert

Introduction

Rhizobacteria can promote plant growth directly or indirectly and are found in rhizosphere in association with plant roots. A great number of bacteria have been reported for their plant growth promoting abilities such as *Bacillus, Azotobacter*, *Pseudomonas*, *Klebsiella*, *Alcaligenes*, *Arthrobacter*, *Serratia*, *Burkholderia*, *Azospirillum*, *Enterobacter* and *Rhizobium* (Kumar *et al.*, 2012). Rhizosphere has been defined as the soil volume that is directly influenced by presence of roots of living plants. The rhizosphere thus supports an active microbial population (Ahmad *et al.*, 2008). A number of studies have revealed that due to the presence of rhizodeposits and root exudates, the soil environment of root system is a favorable place of microbial abundance and activity (Hartmann *et al.*, 2008; Smalla *et al.*, 2006). Rhizobacteria are of great interest for their applications as biofertilizers or pesticides in agriculture and for phytoremediation. The plant growth promoting rhizobacteria (PGPR) can enhance plant growth and yield, and reduce chances of pathogen infection and biotic or abiotic plant stresses (Lugtenberg and Kamilova, 2009; van Loon and Bakker, 2006). In a number of cases, the required effects of PGPR are not attained in the field probably due to insufficient rhizosphere or plant colonization required for revealing beneficial effects (Lugtenberg *et al.*, 2001), and the lack of thorough understanding of the mechanisms responsible to promote plant growth.

Haloxylon salicornicum (vernacular name: *Lana* or *Khar*) belongs to Chenopodiaceae family and is found in Cholistan desert of Pakistan as a common shrub (Shafi *et al.*, 2002). It is much branched, erect leafless and woody at base perennial shrub. Its stem and branches are pale yellow, flowers and fruits are

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not found, and joints produce into two short triangular points in the form of leaves and are woolly within. The plant is used as fodder for domestic animals and is mostly grazed by camels. It has high salt contents and therefore, better to retrieve the desert soil. The extract of this plant is used for washing clothes (Arshad *et al.*, 2002). The plant has medicinal importance and is taken as tea by women who are experiencing problems during pregnancy and to relieve dysmenorrhea (Saleem *et al.*, 2013). Because of its unique characteristics, this plant is expected to host biotechnologically important microbes in its rhizosphere.

Ribosomal RNA sequences especially 16S rRNA are the most important targets for bacterial identification to study evolution, ecology and to determine phylogenetic relationships among various taxa. These sequences also serve for the exploration of bacterial diversity present in an environment and quantification of relative abundance of taxa of different ranks (Hugenholtz *et al.*, 1998). The16S rRNA gene is suitable for bacterial identification because it allows studying phylogenetic relationships among distant taxa. The gene of 16S rRNA is functionally indispensable part of the core gene set (Daubin *et al.*, 2003) that promotes its importance for studying phylogenetic relationships. It is subject to variations, especially in the variable regions, and allows enough diversification for bacterial classification. The suitable PCR primers have been enabled due to the presence of conserved regions in 16S rRNA gene to study different taxa at various taxonomic levels that range from individual bacterial strains to whole phyla (Head *et al.*, 1998).

The current study was carried out to isolate some thermophilic bacterial strains from the rhizospheric soil of *H. salicornicum* of Cholistan desert. Morphological and biochemical properties of the isolated bacteria are reported. The 16S rRNA gene was amplified to identify these bacterial isolates and to study their phylogenetic relationships with other strains.

Experimental

Material and Methods

Study area. Cholistan desert spreads over an area of 26,000 km^2 and is situated in Southern Punjab of Pakistan. It is located between 27°42′ and 29°45′ N latitude and 69°52′ and 75°24′ E longitude (Wariss *et al.*, 2013). It is sandy and hot desert with mean annual rainfall of 100–150 mm. The rain usually falls during the period of monsoon, spring and winter seasons. The temperature reaches above 50°C in summer (Jamil *et al.*, 2013). The inconsistent rainfall promotes only spiny, leafless, small and grazed shrubs in addition to some trees. The soils of this desert are mostly alkaline, saline, gypsiferous and often dunned (Akbar *et al.*, 1996).

Collection of rhizospheric soil. Soil samples (5–10 g) from the rhizosphere of *H. salicornicum* growing in Cholistan were collected at various places in extreme summer (June and July), winter (December and January) and rainy seasons (August and September), mixed to get random samples. Samples were collected in sterile sealed plastic bags on dry ice using ethanol flamed spatula, preserved in properly labeled sterile plastic jars and stored in a dark and cool place. Some soil samples were also stored at 4°C and processed within a week for biochemical and molecular analyses.

Growth of cultivable bacteria in sterile normal saline/Ringer solution. Rhizospheric microbial suspension was made from the soil sample (1 g) including pieces of plant roots, about 3.5 cm in length, in 30 ml sterile ringer solution in a sterile cotton plugged 250 ml flask. After continuous swirling on shaker a homogeneous suspension was obtained that was used as stock to prepare serial dilutions.

The original suspension was serially diluted to get 6 dilutions ranging from 10^{-1} to 10^{-6}. For bacterial growth, 100 µl of soil suspension (10^{-3} to 10^{-6} dilutions) was taken and poured/spread on LB agar plates. This step was followed by incubation of the plates at 30°C for 16–24 hrs. Several colonies were observed and colonies seen to be inhibiting the growth of another bacterial colony and having clear zone around (data not shown) were considered as antagonistic to each other and were isolated.

To get purified isolates, individual bacterial colonies grown on agar plates were picked and transferred to fresh LB agar plates. Repeated streaking and transfer was attempted to obtain pure strains. Following isolation, pure cultivable bacteria were stored as LB glycerol stocks (LB: glycerol, 50:50) at –70°C till further analysis.

For analyses, overnight LB broth cultures of bacterial isolates were grown using LB glycerol stocks as seed cultures.

Determination of antibacterial activity of the isolates. Disc diffusion method was used to evaluate the antibacterial potential of the isolates. Broth cultures of the isolates were grown for 16 hours at 50°C and 40 µl of the culture's supernatant was applied on the filter paper discs (5 mm diameter) in installments of 10 µl. Discs were tested against 4 Gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi,* and *Shigella sonnei*) and 2 Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*).

Physical and biochemical analysis of cultivable bacteria. Colony and staining characteristics like gram staining, motility and cell morphology *etc.* of bacterial isolates were observed under the microscope. Biochemical analysis included tests for the production of amylase, acid, urease, gelatinase, catalase, protease,

H_2S and antibiotic production. Analysis was further extended to MR, VP, motility, and glucose, lactose and fructose fermentation tests. All analyses were carried out using standard reagents and following optimized procedures (Cappuccino and Sherman, 1999).

Extraction of genomic DNA from bacterial isolates. LB broth cultures of purified bacterial isolates were used for DNA isolation using standard protocol consisting of four stages including disruption, lysis, removal of proteins and contaminants, and finally recovery of DNA. In this study, SDS based method was employed (Vivantis, Kit. Catalogue No. GF-BA-100 preps) as suggested by the manufacturer.

Amplification of 16S rRNA gene. For the amplification of 16S rRNA gene of bacterial isolates, PCR conditions were optimized and reactions were carried out in a thermocycler (MY $GENE_{Tm}$ Model MG-96+). Universal forward and reverse primers specifically designed and reported for bacterial 16S rRNA genes in earlier studies (Porteous and Armstrong, 1993) were used for PCR amplification. Forward and reverse primers used in this study are shown below.

Sense primer: 5'-AACACATGCAAGTGGAAC-3'
Antisense primer: 5'-ACGGGCGGTGTGTACAAG-3'

Expected size of PCR amplicons using *E. coli* DNA was calculated to be 1357 bp. Each tube was loaded with 50 µl final volume of a solution containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 100 µM dNTPs mix, 0.3 µl of each primer (10 pmol), 1.25U of *Taq* DNA polymerase and an appropriate amount of chromosomal DNA. PCR amplifications were carried out as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles each of 94°C for 30 s, 50°C for 60 s and 72°C for 60 s with a final extension step of 72°C for 10 min.

RFLP analysis of 16S rRNA amplicons using four cutter restriction enzymes. Five types of 4 base cutter restriction enzymes, *Rsa*I, *TaqI, HpaII, HinfI,* and *HhaI*, were used in this study for RFLP of 16S rRNA gene PCR amplicons. The documented gels were labeled using 1 and 0 to indicate the presence/absence of band on gel. Data was transferred to excel sheet for preparation of dendrograms by PAST-3 software. Dendrograms helped to identify the pattern of similarity and dissimilarity within the group and among the other groups of rhizospheric soils bacteria.

Phylogenetic analysis of 16S rRNA genes. PCR amplified 16S rRNA genes of the isolates were selected based on dendrograms analyses. At least 2–3 members of each group were randomly selected and sequenced (Macrogen, South Korea). The sequences of 16S rRNA genes were assembled and analyzed using BLAST (Altschul *et al.*, 1990). Along with our twenty sequences for 16S rRNA, twelve sequences of the most nearest relatives were also retrieved from GenBank for the comparison study. All the sequences were aligned using ClustalX and imported into the Bioedit program for manual alignment. Neighbor joining phylogenetic tree for 16S rRNA gene was constructed and the evolutionary analyses were conducted using MEGA7 (Kumar *et al.*, 2016) with 100 bootstrap replicates. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 33 nucleotide sequences. All positions containing gaps and missing data were eliminated. The final dataset consisted a total of 682 positions.

Results

Determination of the antibacterial activity. Discs prepared using cultures of the isolated bacteria exhibited antibacterial activity against all tested microorganisms. Ampicillin was used as standard and tested at the concentration of 50 µg/disc. It is obvious from the results (Table I) that some isolates (HSHM1105, HSHM1109, HSHM1111, HSHM1118, HSHM1207, HSHM1212-HSHM1215, HSHM1219) were more effective against *E. coli* as compared to ampicillin. Isolates HSHM1103-1105, HSHM1117, HSHM1120, HSHM1202, HSHM1206, HSHM1208, HSHM1212, HSHM1214-1216, HSHM1219 were found to possess higher antibacterial potential against *P. aeruginosa* than the standard. Similarly, all isolates except HSHM1109, HSHM1110 and HSHM1216 exhibited a an antagonistic behavior against *S. typhi* higher than the standard. However, against *S. sonnei* 12 isolates (HSHM1111, HSHM1117, HSHM1118, HSHM1120, HSHM1201, HSHM1202, HSHM1206-1208, HSHM1213-1215) exhibited antibacterial activity higher than ampicillin. Multiple isolates were examined to be more active against *S. aureus* (HSHM1118, HSHM1120, HSHM1201, HSHM1207, HSHM1208, HSHM1212-1216, HSHM1219) and *B. subtilis* (HSHM1103, HSHM1104, HSHM1111, HSHM1118, HSHM1120, HSHM1202, HSHM1206-1208, HSHM1212, HSHM1214-1216, HSHM1219) as compared to ampicillin.

Phenotypic characterization of bacterial isolates. Total 20 rhizobacteria were isolated on agar plates from the rhizosphere of *H. salicornicum.* The bacterial isolates showed various phenotypic properties including different morphological traits, fermentable carbohydrates profiles and enzyme production patterns (Table II). In all cases, colonies were off-white, round and irregular after 15 and 24 hours, respectively. No chromogenesis and odor was observed in any bacterial isolate. All isolates were found to be gram-positive bacteria that belonged to bacilli class that is an extremely

Table I
Antibacterial activity of selected bacteria isolated from rhizosphere of *Haloxylon salicornicum* (HS)*.

Sr.	Tested isolate	Zone of inhibition (mm) against					
		Gram-negative bacteria			Gram-positive bacteria		
		E. coli Mean ± SD	*P. aeruginosa* Mean ± SD	*S. typhi* Mean ± SD	*S. sonnei* Mean ± SD	*S. aureus* Mean ± SD	*B. subtilis* Mean ± SD
1	HSHM1201	11.33 ± 0.57	13.00 ± 0.00	**18.66±1.125**	**18.33 ± 1.00**	**20.00 ± 1.00**	10.00 ± 0.00
2	HSHM1202	13.33 ± 0.57	**19.00 ± 0.00**	**17.00 ± 0.00**	**21.00 ± 1.00**	12.33 ± 0.57	**16.66 ± 0.57**
3	HSHM1103	11.33 ± 0.57	**16.00 ± 0.00**	**15.00 ± 0.00**	8.66 ± 0.57	9.33 ± 0.57	**14.00 ± 1.00**
4	HSHM1104	11.00 ± 0.00	**16.66 ± 0.57**	**15.00 ± 1.00**	8.63 ± 0.57	9.33 ± 0.57	**14.00 ± 0.00**
5	HSHM1105	**17.33 ± 1.13**	**17.33 ± 1.13**	**18.66 ± 0.57**	11.00 ± 1.00	12.00 ± 0.00	12.33 ± 0.57
6	HSHM1206	12.00 ± 1.00	**22.33 ± 0.57**	**22.00 ± 0.00**	**21.00 ± 1.00**	12.00 ± 0.00	**16.66 ± 0.57**
7	HSHM1207	**20.00 ± 0.00**	9.00 ± 0.00	**17.33 ± 0.57**	**22.33 ± 0.57**	**18.33 ± 1.13**	**21.33 ± 1.13**
8	HSHM1208	12.33 ± 0.57	**21.33 ± 0.57**	**23.66 ± 0.57**	**14.00 ± 0.00**	**20.00 ± 0.00**	**15.00 ± 1.00**
9	HSHM1109	**20.00 ± 0.00**	8.00 ± 0.00	8.33 ± 0.57	9.00 ± 1.00	9.00 ± 1.00	13.00 ± 0.00
10	HSHM1110	**15.66 ± 0.57**	8.66 ± 0.57	8.00 ± 0.00	9.00 ± 0.00	9.33 ± 0.57	13.33 ± 0.57
11	HSHM1111	**15.00 ± 0.00**	9.33 ± 0.57	**15.33 ± 0.57**	**18.33 ± 0.57**	8.00 ± 0.00	**15.33 ± 1.13**
12	HSHM1212	**17.33 ± 0.57**	**25.00 ± 0.00**	**16.66 ± 0.57**	12.33 ± 0.57	**21.00 ± 0.00**	**17.33 ± 0.57**
13	HSHM1213	**20.00 ± 0.00**	11.00 ± 1.00	**15.33 ± 0.57**	**18.66 ± 0.57**	**18.33 ± 0.57**	12.33 ± 0.57
14	HSHM1214	**20.66 ± 0.57**	**22.33 ± 0.57**	**23.00 ± 0.00**	**20.00 ± 0.00**	**20.00 ± 1.00**	**21.00 ± 1.00**
15	HSHM1215	**20.33 ± 1.13**	**22.33 ± 0.57**	**23.33 ± 1.13**	**22.00 ± 1.00**	**18.33 ± 0.57**	**21.00 ± 0.00**
16	HSHM1216	13.33 ± 1.13	15.33 ± 0.57	10.33 ± 0.57	9.00 ± 0.00	**20.00 ± 1.00**	**20.00 ± 0.00**
17	HSHM1117	13.33 ± 0.57	**17.00 ± 1.00**	13.66 ± 0.57	**19.33 ± 0.57**	8.00 ± 0.00	13.00 ± 0.00
18	HSHM1118	**25.00 ± 1.00**	13.33 ± 0.57	**15.00 ± 1.00**	**18.66 ± 0.57**	**17.33 ± 1.13**	**15.00 ± 0.00**
19	HSHM1219	**17.33 ± 0.57**	**22.00 ± 0.00**	**15.00 ± 1.00**	12.33 ± 0.57	**15.33 ± 1.13**	**17.00 ± 0.00**
20	HSHM1120	11.00 ± 1.00	**18.33 ± 0.57**	13.33 ± 0.57	**18.66 ± 0.57**	**18.00 ± 0.00**	**22.33 ± 1.13**
21	Ampicillin	13.22 ± 0.58	13.15 ± 0.65	13.11 ± 1.22	12.99 ± 0.59	13.01 ± 1.23	13.45 ± 0.89
		11	12	15	12	11	14

diverse group of bacteria. Production of urease, gelatinase, indole and hydrogen sulfide was not seen in these isolates. Catalase production and glucose fermentation were observed in all selected bacterial isolates.

Analysis of 16S rRNA sequences of isolates. The selected isolates showed varying percentage identity with different bacteria through nucleotide BLAST search of partial sequences (~ 1100 bp) of the 16S rRNA. Although, it has been suggested that RFLP can help differentiate bacterial strains up to species level (Dec *et al.*, 2016), in the present study, phylogenetic trees developed on the basis of RFLP and base sequences exhibited some differences. This could be attributed to the number of restriction enzymes used for RFLP analyses.

Nearest relatives of selected bacteria isolated from rhizosphere of *H. salicornicum* are given (Table III) along with their percentage identity. Out of twenty nearest relative isolates, 14 isolates showed 91–99% identity with *Brevibacillus borstelensis*, 4 with *Bacillus subtilis* (97–98%) and 2 isolates with *Bacillus licheniformis* (94–99%). The partial nucleotide 16S rRNA gene sequences of all bacterial isolates were deposited in GenBank have been allotted accession numbers (Table III).

A neighbor-joining (NJ) phylogenetic tree was generated through aligning 16S rRNA sequences of the bacterial isolates examined in this study and their nearest relatives taken from GenBank, NCBI (Fig. 1) to study their evolutionary relationships and cladding pattern. Three main groups of bacteria were observed in this phylogeny on the basis of which bacteria fell in different clades. *HS*1219, *HS*1215, *HS*1214, *HS*1208, *HS*1202, *HS*1119, *HS*1118, *HS*1110, *HS*1201, *HS*1120, *HS*1103, *HS*1207, *HS*1105 and *HS*1109 grouped with *B. borstelensis*, showing their homology with other *B. borstelensis* species. Only two isolates, that is, *HS*1206 and *HS*1213 were found in the clade of *B. licheniformis* with ESR26 and PF4H_1 strains of *B. licheniformis*. Four isolates were identified as *Bacillus subtilis* species *HS*1111, *HS*1212, *HS*1104 and *HS*1216) but the interesting observation in this clade was the unusual branching of *HS*1111 that was not cladded with other species of *B. subtilis*. The nearest relative of *HS*1111 (accession number: DQ420172) was also found as a separate branch above *HS*1111 that was showing evolutionary divergence of both sequences from other *B. subtilis* species. Phenotypic characteristics showed that *HS*1111 is

Table II
Phenotypic characteristics of selected bacteria isolated from rhizosphere of *Haloxylon salicornicum* (HS)*.

Sr. No.	Tested Parameter	Result	Samples % (n)	Sample ID
(a) Morphological characteristics				
1	Color	Off-white	100 (20)	All
2	Margin	Branching	10 (2)	*HS*1103, *HS*1111
		Non-branching	90 (18)	*HS*1104, *HS*1105, *HS*1109, *HS*1110, *HS*1118, *HS*1119, *HS*1120, *HS*1201, *HS*1202, *HS*1206, *HS*1207, *HS*1208, *HS*1212, *HS*1213, *HS*1214, *HS*1215, *HS*1216, *HS*1219
3	Elevation	+ve	10 (2)	*HS*1103, *HS*1111
		−ve	90 (18)	*HS*1104, *HS*1105, *HS*1109, *HS*1110, *HS*1118, *HS*1119, *HS*1120, *HS*1201, *HS*1202, *HS*1206, *HS*1207, *HS*1208, *HS*1212, *HS*1213, *HS*1214, *HS*1215, *HS*1216, *HS*1219
4	Configuration after 15 hrs	Round	100 (20)	All
5	Configuration after 24 hrs	Irregular	100 (20)	All
6	Chromogenesis	−ve	100 (20)	All
7	Opacity	+ve	10 (2)	*HS*1103, *HS*1111
		−ve	90 (18)	*HS*1104, *HS*1105, *HS*1109, *HS*1110, *HS*1118, *HS*1119, *HS*1120, *HS*1201, *HS*1202, *HS*1206, *HS*1207, *HS*1208, *HS*1212, *HS*1213, *HS*1214, *HS*1215, *HS*1216, *HS*1219
8	Odor	No odor	100 (20)	All
9	Gram Staining	+ve	100 (20)	All
10	Cell Morphology	Bacilli	100 (20)	All
(b) Biochemical characteristics				
1	Amylase production	+ve	55 (11)	*HS*1201, *HS*1202, *HS*1206, *HS*1212, *HS*1214, *HS*1216, *HS*1103, *HS*1104, *HS*1111, *HS*1119, *HS*1118
		−ve	45 (9)	*HS*1105, *HS*1109, *HS*1110, *HS*1120, *HS*1207, *HS*1208, *HS*1213, *HS*1215, *HS*1219
2	Acid production	+ve	45 (8)	*HS*1105, *HS*1109, *HS*1110, *HS*1120, *HS*1208, *HS*1213, *HS*1215, *HS*1219
		−ve	55 (12)	*HS*1103, *HS*1104, *HS*1111, *HS*1118, *HS*1119, *HS*1201, *HS*1202, *HS*1206, *HS*1207, *HS*1212, *HS*1214, *HS*1216
3	Urease production	−ve	100 (20)	All
4	Gelatinase production	−ve	100 (20)	All
5	Motility test	+ve	15 (3)	*HS*1118, *HS*1208, *HS*1215
		−ve	85 (17)	*HS*1103, *HS*1104, *HS*1105, *HS*1109, *HS*1110, *HS*1111, *HS*1119, *HS*1120, *HS*1201, *HS*1202, *HS*1206, *HS*1207, *HS*1212, *HS*1213, *HS*1214, *HS*1216, *HS*1219
6	MR	−ve	100 (20)	All
7	VP	−ve	100 (20)	All
8	Indol production	−ve	100 (20)	All
9	H_2S production	−ve	100 (20)	All
10	Catalase production	+ve	100 (20)	All
11	Glucose fermentation	+ve	100 (20)	All
12	Lactose fermentation	+ve	15 (3)	*HS*1208, *HS*1213, *HS*1215
		−ve	85 (17)	*HS*1103, *HS*1104, *HS*1105, *HS*1109, *HS*1110, *HS*1111, *HS*1118, *HS*1119, *HS*1120, *HS*1201, *HS*1202, *HS*1206, *HS*1207, *HS*1212, *HS*1214, *HS*1216, *HS*1219
13	Fructose fermentation	+ve	95 (19)	*HS*1103, *HS*1104, *HS*1105, *HS*1109, *HS*1110, *HS*1111, *HS*1118, *HS*1119, *HS*1120, *HS*1201, *HS*1202, *HS*1206, *HS*1207, *HS*1208, *HS*1213, *HS*1214, *HS*1215, *HS*1216, *HS*1219
		−ve	5 (1)	*HS*1212
14	Litmus (Milk)	−ve	100 (20)	All

Total number of isolates = 20, n = number of samples, + ve = Gram-positive, – ve = Gram-negative,
* Names of the isolates have been abbreviated as HS for convenience. Names of the in-lab stocks start with HSHM.

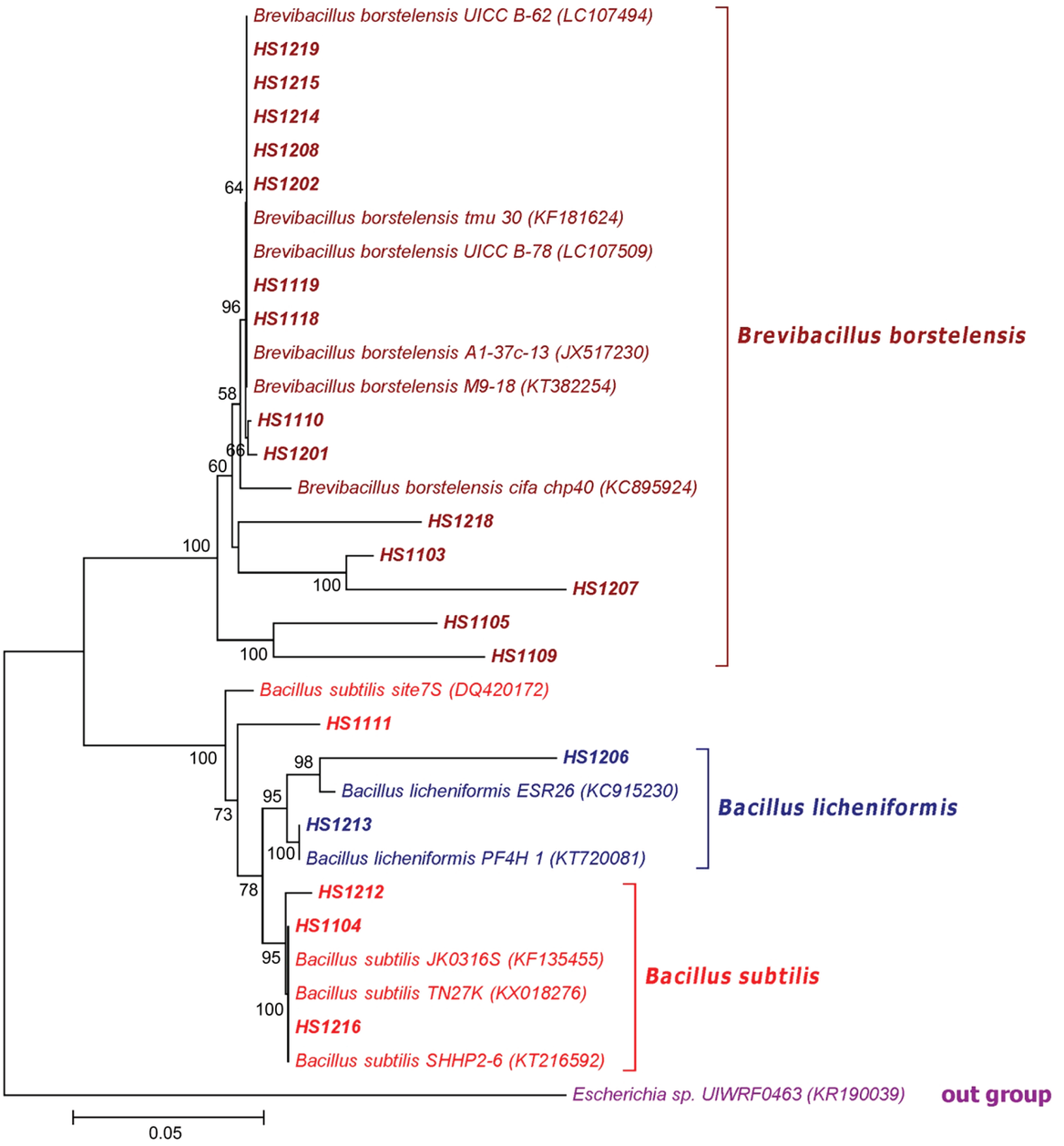

Fig. 1. Neighbour-joining phylogenetic tree of the selected bacterial isolates.

The tree was rooted with *Escherichia* spp. as an out-group. The scale bar represents the sequence divergence. Bootstrap values (100 replicates) are shown at the nodes. The optimal tree with the sum of branch length = 0.65614117 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

different from other *B. subtilis* species in terms of margin, elevation and opacity as it had branching filaments with positive values for colony elevation and opacity.

RFLP and phylogenetic analysis. RFLP analysis of the 20 selected bacterial isolates using 16S rRNA products identified 5 distinct patterns. Representative patterns are shown in Fig. 2. These patterns were found to be consistent and absolutely reproducible. Among 222 16S rRNA restriction fragments recorded, *RsaI* and *HinfI* each gave 20 shared fragments (18% of total), 16 fragments were unique among accessions (7.2%) and remaining 166 fragments were phylogenetically informative (74.7%). A dendrogram was constructed based on RFLP analysis of 16S rRNA gene of bacteria isolated from rhizosphere of *H. salicornicum* (Fig. 3). The RFLP dendrogram showed two main clades (labeled with letters A and B).

Table III
Nearest relatives of selected isolates of bacteria.

Isolate	Allotted Accession Number	Nearest relative	Accession Number	Identity (%)
*HS*1103	KX426601	*Brevibacillus borstelensis* M9-18	KT382254.1	93
*HS*1104	KX426602	*Bacillus subtilis* JK0316S	KF135455	98
*HS*1105	KX426603	*Brevibacillus borstelensis* A1-37c-13	JX517230.1	93
*HS*1109	KX426604	*Brevibacillus borstelensis* cifa_chp40	KC895924.1	96
*HS*1110	KX426605	*Brevibacillus borstelensis* M9-18	KT382254.1	97
*HS*1111	KX426606	*Bacillus subtilis* site7S	DQ420172.1	97
*HS*1118	KX426607	*Brevibacillus borstelensis* M9-18	KT382254.1	99
*HS*1119	KX426608	*Brevibacillus borstelensis* UICC B-78	LC107509.1	99
*HS*1120	KX426609	*Brevibacillus borstelensis*tmu30	KF181624.1	95
*HS*1201	KX426610	*Brevibacillus borstelensis* M9-18	KT382254.1	96
*HS*1202	KX426611	*Brevibacillus borstelensis* UICC B-78	LC107509.1	98
*HS*1206	KX426612	*Bacillus lecheniformis* ESR26	KC915230.1	94
*HS*1207	KX426613	*Brevibacillus borstelensis* A1-37c-13	JX517230.1	91
*HS*1208	KX426614	*Brevibacillus borstelensis* M9-18	KT382254.1	98
*HS*1212	KX426615	*Bacillus subtilis* TN27K	KX018276.1	98
*HS*1213	KX426616	*Bacillus lecheniformis* PF4H_1	KT720081.1	99
*HS*1214	KX426617	*Brevibacillus borstelensis* M9-18	KT382254	98
*HS*1215	KX426618	*Brevibacillus borstelensis* UICC B-62	LC107494.1	97
*HS*1216	KX426619	*Bacillus subtilis* SHHP2-6	KT216592	98
*HS*1219	KX426620	*Brevibacillus borstelensis* M9-18	KT382254.1	99

Clade B has only five isolates, that is, *HS*1109, *HS*1119, *HS*1202, *HS*1208 (all have identity with *B. borstelensis*) and *HS*1213 (that has identity with *B. licheniformis*). Clade A was further divided into 2 subclades (labeled with a1 and a2). Only three isolates, that is, *HS*1214, *HS*1215 (had identity with *B. borstelensis*) and *HS*1216 (identity with *B. subtilis*) fell into subclades a2. Species of the same genera did not appear together in RFLP dendrograms revealing that identification of bacterial isolates on the basis of RFLP is not a reliable technique.

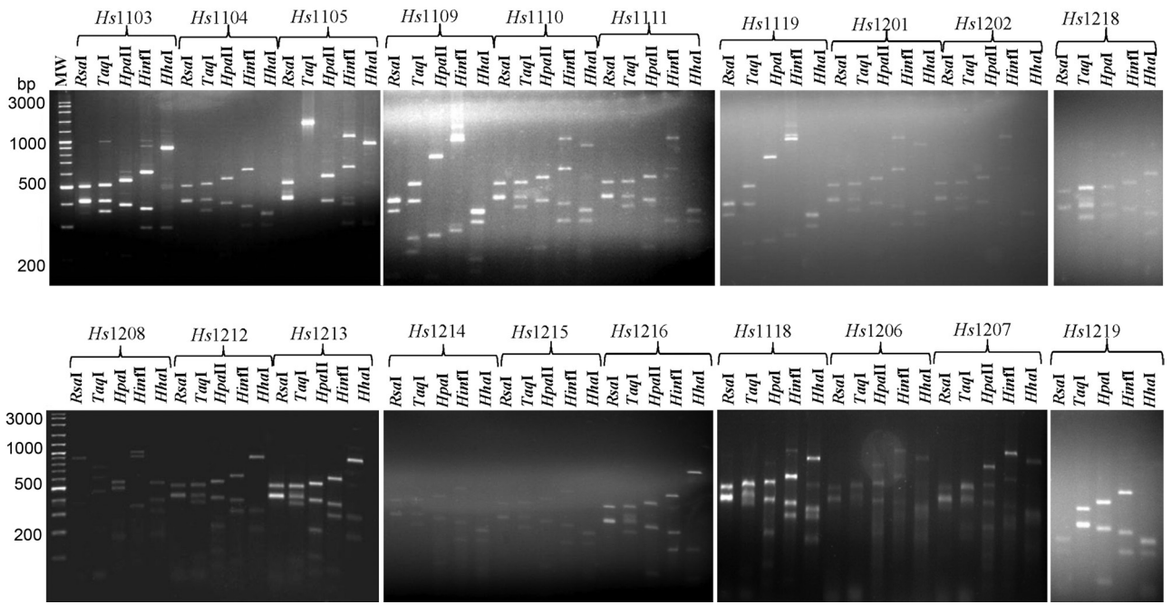

Fig. 2. RFLP analysis of the selected bacterial isolates using 5 different restriction endonucleases.

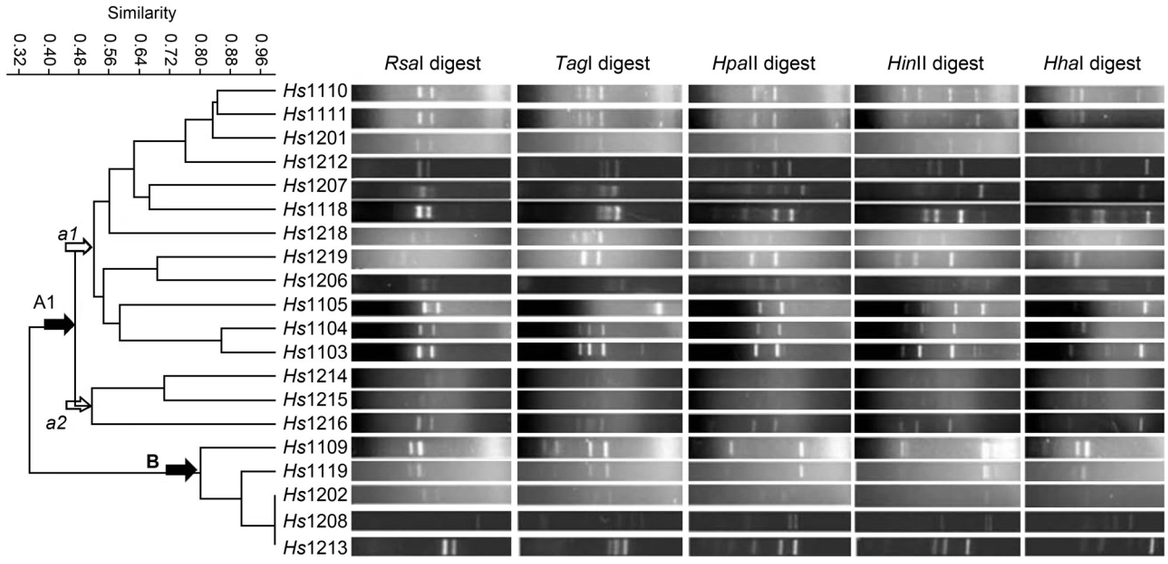

Fig. 3. Dendrogram constructed based on RFLP analysis of 16S rRNA gene of bacteria isolated from rhizosphere of *H. salicornicum*.

Discussion

A number of plant-associated rhizobacteria are known for their role in promotion of plant growth and to increase plant resistance against different diseases and abiotic stresses. Cholistan desert has not been explored for its rhizobacterial diversity associated with its plants. Therefore, the present study represents the very first study of its kind that has been performed to isolate and identify rhizobacteria from a representative plant *H. salicornicum* from Cholistan desert, a desert having unique soil with high salinity and basic pH (Hameed *et al.*, 2011). Based on biochemical characterization these isolates were found to be catalase positive and glucose fermentive; 19 were found positive for fructose fermentation and 11 were positive for amylase production. Amylase producers are more important industrially because bacterial enzymes are obtained in high yield and they possess high thermostability. A thermostable α-amylase from *B. licheniformis* has also been reported (Morgan and Priest, 1981).

Bacterial classification is accomplished when morphological and biochemical methods are used along with molecular methods of analysis. The amplified fragment length polymorphism (AFLP) technique of DNA fingerprinting has been shown to be useful for the identification of different bacterial species (Duim *et al.*, 2001) but it is expensive and laborious. Another technique, whole-genome DNA-DNA hybridization allows identification of bacterial species but is not routinely used (Stackebrandt and Goebel, 1994). Community structures, metabolic function, composition and ecological roles are the characteristics of different microbial communities. The 16S rRNA gene has been employed to investigate environmental microbial diversity that does not require isolation and cultivation of bacteria to offer phylogenetic taxonomic classification. Because of heterogeneity of 16S phylogenetic marker among operons of same genome or lack of its resolution at species level, the use of this technique is often criticized but the technique is still believed to be a standard for bacterial identification (Pontes *et al.*, 2007). The 16S rRNA based identification of bacteria has therefore come up as a potential and useful alternative.

In the present studies, 14 *Brevibacillus* and 6 *Bacillus* (4 *B. subtilis* and 2 *B. licheniformis*) species were identified from rhizosphere of *H. salicornicum*. The isolate *HS*1111 depicted a divergence through different branching pattern in the phylogenetic tree because it didn't clade with other strains of *B. subtilis* (Fig. 1). The importance of *Bacillus* species in plant growth promotion is widely accepted and a large number including *B. subtilis* have been reported for their bio-control because of the production of various antibiotics. Plant growth promotion is also supported through phytohormones production, phosphate solubilization and release of ammonia from nitrogenous organic matter (Hayat *et al.*, 2010). *B. subtilis* and *B. licheniformis* are also reported for producing two important plant hormones, indole-3-acetic acid and indole-3-butyric acid (Lim and Kim, 2009). It has also been observed that the colonization of *B. subtilis* at root hairs of plants is accompanied with morphological changes of these root hairs (Huang *et al.*, 2011). The PGPR strain of *B. licheniformis* has been found capable of survival under drought stress conditions and the PGPR treated plants, therefore, continue to accumulate plant growth when exposed to drought stress (Lim and Kim, 2013). In other studies, it

is also revealed that *B. licheniformis* has great resistance against abiotic stress including drought stress (Cheng *et al.*, 2007; Kloepper *et al.*, 2007; Sziderics *et al.*, 2007).

Bacillus species are aerobic endospore forming plant growth promoting rhizobacteria which are known to produce many valuable enzymes including proteases, amylases, laccase and lipases. In addition, the *Bacillus* species have potential to degrade complex carbohydrates including cellulose, xylulose and oligosaccharides like arabinogalactan, stachyose and raffinose (Ahmad *et al.*, 2008; Amoa-Awua and Jakobsen, 1995; Larsen *et al.*, 2014; Ouoba *et al.*, 2003a; Ouoba *et al.*, 2003b; Ouoba *et al.*, 2003c; Ouoba *et al.*, 2007; Reiss *et al.*, 2011). Many *Bacillus* species have been identified to have antimicrobial potential. Antimicrobial potential of thermophilic bacterial species including *Brevibacillus borstelensis* and *B. licheniformis* has been documented against gram positive bacteria (*Micrococcus luteus* and *Staphylococcus aureus*) and gram negative bacteria including *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumonia* (Muhammad *et al.*, 2009). *B. subtilis* is capable of producing an amocaumacin-A like secondary metabolite that has antimicrobial potential against *Candida albicans*, *Ustilago maydis, Cryptococcus neoformans*, *S. aureus* and *E. coli* (Esikova *et al.*, 2002; Shankarrao *et al.*, 2014). *B. borstelensis* and *B. licheniformis* induce systemic resistance in plants and inhibit many phytopathogens (Kloepper *et al.*, 2004; Sharma *et al.*, 2014). Contrary to these positive effects, few studies have reported the pathogenic properties of *B. licheniformis* against humans (Salkinoja-Salonen *et al.*, 1999). Moreover, *Brevibacillus* species have potential of degrading many toxic chemicals like Toluidine Blue dye and carbendazim, a known fungicide (Alhassani *et al.*, 2007; Arya and Sharma, 2014; Mallick *et al.*, 2014).

In conclusion, the morphological, biochemical and the molecular approaches described above present the first ever study carried out under given experimental conditions to identify and characterize 20 rhizobacteria from a representative plant of Cholistan desert. The phylogenetic significance of the presence of all bacilli from three species in the rhizosphere of one desert plant remains to be determined. Work is in progress on the molecular analysis of rhizobacteria from other plants of Cholistan desert in this regard. These bacteria on further characterization can be explored for the production of valuable molecules and can be manipulated to induce tolerance efficiently against both biotic and abiotic stress in economically important plants.

Availability of data and materials. Ribosomal DNA sequences are presented in the GenBank data as per accession numbers presented in Table II.

Conflict of interests

The authors declare that they have no financial or non-financial competing interests.

Author's contributions

FHN was the PI and HEC grant recipient. M Ashraf, JR and MSC were the collaborators who contributed through sample identification, collection and analyses. M Aslam, RR and HM performed the bench work. SE, FHN, M Ashraf, M Aslam and JR were involved in the data analyses and manuscript writing. M Aslam and GM prepared the dendrograms and conducted the related analysis.

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