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SHORT COMMUNICATION

16S rRNA gene sequence analysis of halophilic and halotolerant bacteria isolated from a hypersaline pond in Sichuan, China

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Abstract One hundred and twenty bacterial isolates were obtained from a hypersaline pond (c. 22% salinity) in Sichuan, China. Bacteria were isolated from hypersaline water, sediment and soil samples using three culture media and an incubation temperature of 37°C. Of these isolates, 47 were selected and examined by phylogenetic analysis of 16S rRNA gene sequences and by tests of salt tolerance. The phylogenetic analysis placed the 47 bacterial isolates either in the phylum *Firmicutes* or in the class *Gammaproteobacteria* and showed that they were affiliated with the genera *Salimicrobium*, *Halalkalibacillus*, *Virgibacillus*, *Alkalibacillus, Marinococcus*, *Halobacillus, Halomonas*, *Idiomarina*, *Chromohalobacter* and *Halovibrio*. All tested isolates were either halophilic or halotolerant and several were capable of growth in the presence of 30% (w/v) NaCl.

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Introduction

Hypersaline environments are defined as those in which salt concentrations exceed that of seawater (Grant 2004). They include artificial and naturally occurring solar salterns in arid areas such as hypersaline lakes and the Dead Sea, underground brines originating from marine evaporite deposits, hypersaline soils, and brines in deep ocean zones. Because water availability is limited by high salt concentration, hypersaline environments are considered to be extreme environments for microbial life (Oren 2008). Despite this fact, diverse taxa of halophilic (i.e requiring salt for growth) and halotolerant bacteria have been recovered from a wide variety of hypersaline environments (Caton et al. 2004; Grant 2004; Jiang et al. 2006; Tsiamis et al. 2008; Xiang et al. 2008). Moreover, bacteria such as *Salinibacter ruber* have been found inhabiting solar crystallization ponds where hypersaline brines approach saturation, conditions once considered suitable only for the *Archaea* (Antón et al. 2000, 2002; Litchfield et al. 2009; Tsiamis et al. 2008).

The isolation and characterization of halotolerant and halophilic bacteria from hypersaline environments is of practical importance because these bacteria have biotechnological potential with regard to the production of useful biomolecules, such as osmolytes (compatible solutes), hydrolytic enzymes and exopolysaccharides (Margesin and Schinner 2001).

Hypersaline subterranean aquifers in the Sichuan basin of China are located at depths of between 50 and 3,000 m and occur within sedimentary rocks ranging in age from the

Sinian to the Cretaceous and especially the Triassic (Zhou et al. 1997). These hypersaline brines are of marine origin (thalassohaline) and have been exploited by man for more than 2,000 years by deep-well drilling and harvesting the salt by boiling (Kuhn 2004). The hypersaline environment investigated in this work is located in the Sichuan Basin and is a reservoir-storage pond of brines (c. 22% salinity) used in the commercial extraction of salts and other chemicals. The pond is maintained year round with warm (c. 40°C) geothermal brines pumped from a deep well (depth >1,000 m).

The aim of this work was to isolate and characterize bacteria from the hypersaline pond ecosystem with a view to screening for halotolerant and halophilic types. Bacterial isolates were obtained by plating on three agar media and by liquid enrichment. Characterization was achieved by analysis of 16S rRNA gene sequences.

Materials and methods

Site description and sample collection

The hypersaline pond (30°36′04.56″N, 105°15′22.79″E, altitude 326 m) has a maximum depth of 2.5 m and a surface area of c. 1,200 m^2. The pond is protected from rain by means of a plastic roof and is maintained year round by being supplied with geothermal brines from a subterranean aquifer. The warm brines (temperature c. 40°C) derive from a deep well and are fed into the hypersaline pond to maintain levels. The climate at this location is of the humid subtropical monsoon type with average annual rainfall exceeding 900 mm.

All water, soil and sediment sampling was carried out during May 2008. Air temperature at the time of sampling varied between 25 and 35°C, while water temperature (0.5 m depth) varied between 25 and 30°C.

Sixteen soil samples were randomly collected to 15 cm depth from unvegetated areas within a 0.5-m border at the edge of the pond. Water samples (500 ml) were collected at 0.5, 1 and 1.5 m depth at each of 12 randomly selected locations, and 19 randomly selected samples of sediment were collected from the bottom of the pond. Each of the soil, sediment and water samples was pooled so as to provide respective composite samples. Sampling was done with aseptic precautions.

Samples were transported to the laboratory and analyses performed immediately or within 3 days. Portions of the composite water sample were sent to a commercial laboratory (Sichuan University, China) for chemical analysis. Cations were determined by Inductively Coupled Plasma Mass Spectrometry (model: OptiMass 9500; Australia) and anions determined by Ion Chromatography (model: ICS 3000; USA).

Isolation of bacteria and tests of salt tolerance

Bacterial isolation was performed using SP medium (Caton et al. 2004), Ma medium (Maturrano et al. 2006) and CM medium (Li et al. 2002), at four levels of NaCl (w/v): 10, 22, 30 and 35%. The final pH of each medium was adjusted to ~7.2.

To isolate bacteria from hypersaline water, replicate aliquots of dilutions were surface plated (0.1 ml/plate) onto the three media containing agar (15 g/l). Bacteria were isolated from sediment and soil samples by adding 1 g of each sample to 300 ml liquid SP, Ma and CM medium (each at four levels of salt) in Erlenmeyer flasks, incubating for 3 days on an orbital shaker, and then plating dilutions (0.1 ml) onto the respective agar media. Plates were incubated for 10–14 days and individual bacterial colonies selected on the basis of differences in morphology and Gram-staining reaction. Single colonies were streaked on agar media and repicked.

Incubation of plates and liquid media were done at 37°C, because this temperature is widely used for the isolation of diverse bacteria from hypersaline environments (e.g., Maturrano et al. 2006; Wen et al. 2009; Wu et al. 2006) and because warm (about 40°C) subterranean brines are fed to the hypersaline pond. Bacterial isolates were subjected to Gram-staining using a Gram-staining kit (Fisher Diagnostics, USA), according to the manufacturer's instructions. Purified cultures were maintained at −80°C in 50% (w/v) glycerol.

Salt tolerance for growth was tested by streaking fresh bacterial cultures in duplicate on SP agar medium containing 0, 1, 5, 10, 15, 20, 25, 30 and 40% (w/v) NaCl. Growth optima were assessed as described by Caton et al. (2004) and plates were incubated for 10 days at 37°C.

Genomic DNA extraction, PCR amplification of 16S rRNA genes and nucleotide sequencing

Bacterial genomic DNA was extracted and purified using a Takara MiniBEST Bacterial Genomic DNA Extraction kit Ver.2.0 (China) according to the manufacturer's instructions. Purified genomic DNAs were subjected to electrophoresis on 1% agarose gels, followed by ethidium bromide staining and visualization under UV light. 16S rRNA genes were amplified from bacterial genomic DNA using bacterial universal primers (forward primer: 5′-AGAGTTTGATCCTGGCTCAG-3′, reverse primer: 5′-ACGGCAACCTTGTTACGACT-3′) (Edwards et al. 1989). The forward primer corresponds to positions 8–27 and the reverse primer to positions 1,493–1,512 of the 16S rRNA gene sequence of *E. coli* (Accession no. U00096). PCR was performed using a thermal cycler (Thermo Scientific, Model Px2) with a 50-μl reaction containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 10 μM of each dNTP, 0.1 μM of each primer, and 1 U of EX Taq DNA polymerase (Takara, Japan). PCR cycling conditions con-

sisted of denaturation at 95°C for 2 min, 35 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 1 min 30 s, and final extension at 72°C for 10 min.

Amplified PCR products were separated by 1% agarose gel electrophoresis. DNA fragments of the correct size (c. 1,500 bases) were excised from the gel and purified using a TIANgel Midi purification Kit (TIANGEN Inc., China) according to the manufacturer's protocol. PCR products were verified by agarose gel electrophoresis.

For 16S rRNA gene sequencing, purified PCR products were ligated into vector pMD18 (Takara Inc., Japan), and transformed into *Escherichia coli*. Clones were screened by PCR for inserts of the correct size (c. 1,500 bases) using universal primers (Edwards et al. 1989). Inserts were commercially sequenced (Invitrogen, China) to provide full-length 16S rRNA gene sequences.

Phylogenetic analysis

16S rRNA gene sequences of the bacterial reference strains, type strains and closest phylogenetic relatives were selected from GenBank by subjecting the nucleotide sequences of bacterial isolates to similarity searches using BLASTn (http://www.ncbi.nlm.nih.gov/blast) and SeqMatch (Release 10 Update17, Ribosomal Database Project) (Cole et al. 2009). Multiple alignments of sequences were done using ClustalW as implemented in MEGALIGN (DNASTAR Lasergene v.8.0; Madison, USA).

A phylogenetic tree was reconstructed using MEGA 4 (Tamura et al. 2007). The model of nucleotide substitution was selected on the basis of the Akaike information criterion implemented in Model Test 3.7 (Posada 2006). Evolutionary histories were inferred using the neighbor-joining method (Saitou and Nei 1987) and bootstrap consensus trees inferred from 1,000 permutations of the datasets (Felsenstein 1985). Evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei 1993) as the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter =4.44404). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Nucleotide sequences generated in this study were deposited in GenBank and their accession numbers are shown in the phylogenetic tree.

Results

Analysis of the chemical composition of hypersaline water (g/l ± SE) indicated that Na^+ and Cl^- were the most abundant ions (63.58±3.14 and 94.42±3.58, respectively), followed by SO_4^{2-} (18.64±1.37), Mg^{2+} (12.73±2.92), K^+ (11.29±2.31), Ca^{2+} (6.78±1.54), $HC0^{3-}$ (3.62±1.02), and CO_3^{2-} (2.38±0.93) and by trace amounts of Br^- (0.03±0.02) and Mn^{2+} (0.013±0.009); pH 7.5±0.3.

A total of 120 isolates were obtained from hypersaline water, sediment and soil samples. Forty-seven of these isolates were selected on the basis of differences in colony morphology and Gram-staining reaction for further analysis by 16S rRNA sequencing. The range of different colony morphologies of bacterial isolates recovered from each of the hypersaline water, sediment and soil samples were generally similar. Although both Gram-positive and Gram-negative bacterial isolates were obtained from all samples, the majority (60%) were Gram-positive.

The phylogenetic tree reconstructed from full length 16S rRNA gene sequences of the 47 bacterial isolates recovered from hypersaline water, sediment and soil samples is shown in Fig. 1. Data for the source of isolation, NaCl tolerance and closest phylogenetic relative (type strains) of these bacterial isolates are given in Table 1. Analysis of 16S rRNA gene sequences divided the bacterial isolates into the families, *Bacillaceae* (phylum *Firmicutes*), *Halomonadaceae* and *Idiomarinaceae* (class *Gammaproteobacteria*). The *Bacillaceae* accounted for the majority of the isolates (c. 72%) whereas the *Halomonadaceae* and *Idiomarinaceae* accounted for c. 21% and c. 6% of the isolates, respectively.

Among bacteria assigned to the *Firmicutes*, a cluster of 15 isolates was closely related to the type strain of *Salimicrobium luteum* (98.5–99.0% similarity) whereas one isolate (C8-1) grouped with, and was closely related to, the type strain of *Salimicrobium halophilum*. Isolates S25-1 and S15-3 grouped closely with the type strain of *Halobacillus hunanensis* and *Halobacillus alkaliphilus*, respectively, whereas the nearest neighbor of isolates S27-1, S30-2, S101-1 and S105-2 was the type strain of *Halobacillus trueperi* (98.7–99.8% similarity). These four isolates were also closely related to the non-type strain, *Halobacillus* sp., QW1018 (GenBank Accession no. EU124360) isolated from hypersaline well water in China (98.8–99.9% similarity).

Several isolates clustered with the type strain of a species belonging to the genus *Virgibacillus* (three isolates), *Halalkalibacillus* (one isolate), *Alkalibacillus* (four isolates) and the genus *Marinococcus* (four isolates).

The isolates placed in the class *Gammaproteobacteria* were subdivided into four genera. The nearest neighbor of isolates S35-1, S83-1 and S96-2 was the type strain of *Halomonas janggokensis* (99.7–99.9% similarity). These three isolates also grouped closely with the type strain of *Halomonas subterraneum* (GenBank Accession no. EF144148) from saline well water in China. Isolate S78-2 was closely related to the type strains of *Halomonas aquamarina* and *Halomonas meridiana* at the same level of sequence similarity (99.1%). The nearest neighbors of isolate S74-1, isolates S15-2 and S24-1, and isolates S55-1

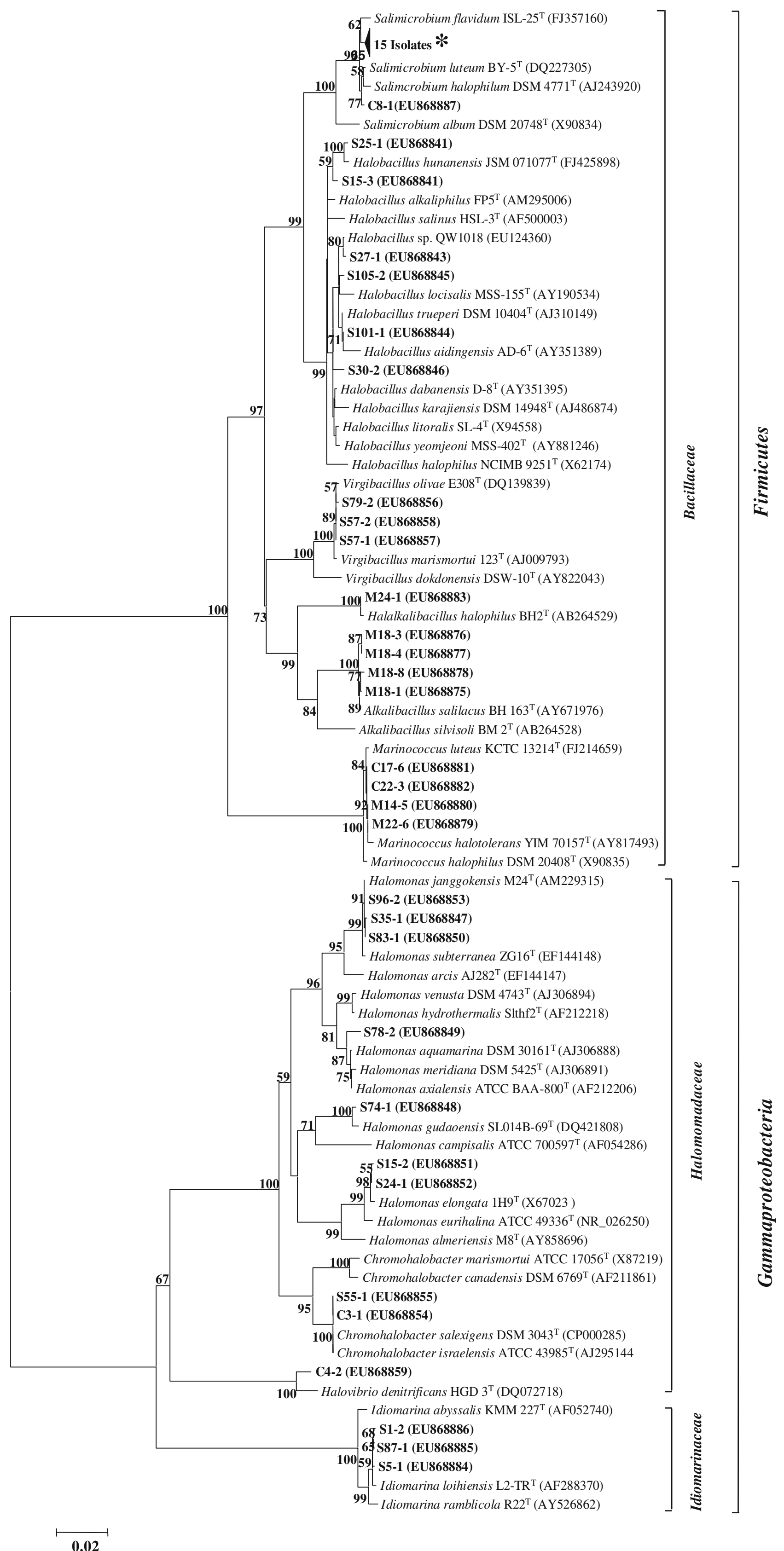

Fig. 1 Phylogenetic relationships of halophilic and halotolerant bacteria isolated from a hypersaline pond in Sichuan, China. The phylogenetic tree, based on 16S rRNA gene sequences, was constructed by the neighbor-joining method. Numerals at nodes indicate bootstrap percentages derived from 1,000 replications. *Bar* represents 2% base substitutions. Strain designations and accession numbers in *bold* are from this study. The cluster of 15 isolates from this study designated with an *asterisk* are C11-3, C13-2, M13-3, M13-5, C14-2, C22-2, M22-4, M25-10, C15-8, M17-8, M22-5, M23-6, M1-1, M14-4 and C15-4 with Gen-Bank Accession numbers EU868860 through EU868874, respectively

Table 1 The isolation source, NaCl tolerance and closest phylogenetic relatives of bacterial isolates from a hypersaline pond in Sichuan, China

Isolate No.[a]	Isolation source	NaCl tolerance[b]	Closest phylogenetic relative (type strain) [c]	Sequence similarity (%)	Isolation source	Reference - Accession No.
Bacillaceae, Bacilliales, Firmicutes						
M1-1	Water	0–30 (15)	*Salimicrobium luteum* BY-5^T	98.5–99.0	Saltern sediments (Korea)	DQ227305
C15-8; C11-3; C13-2 ; C14-2; C15-4; M13-3; M13-5; M14-4	Sediments	0–30 (15)				
M22-4; C22-2; M17-8; M22-5; M23-6; M25-10	Soil	0–30 (10)				
C8-1	Water	1–30 (25)	*Salimicrobium halophilum* DSM 4771^T	99.1	Solar saltern (Korea)	AJ243920
S25-1	Soil	1–25 (10)	*Halobacillus hunanensis* JSM071077^T	99.5	Brine, salt mine (China)	FJ425898
S15-3	Soil	0–25 (10)	*Halobacillus alkaliphilus* FP5^T	99.3	Solar saltern (Spain)	AM295006
S105-2;	Water	0–25 (5)	*Halobacillus trueperi* DSM 10404^T	98.7–99.8	Sediments (Great Salt Lake, USA)	AJ310149
S30-2; **S27-1; S101-1**	Water	0–20 (5)				
S57-1; S57-2; S79-2	Water	1–15 (5)	*Virgibacillus marismortui* 123^T	99.7–99.9	Water (Dead Sea)	AJ009793
M24-1	Soil	5–25 (10)	*Halalkalibacillus halophilus* BH2^T	99.8	Non-saline soil (Japan)	AB264529
M18-1; M18-3; M18-4; M18-8	Soil	5–25 (15)	*Alkalibacillus salilacus* BH163^T	99.6	Sediments, salt lake (China)	AY671976
M14-5	Sediments	0–30 (15)	*Marinococcus luteus* KCTC 13214^T	99.8–99.9	Saline soil (Barkol Lake, China)	FJ214659
C22-3; C17-6; M22-6	Soil	0–30 (10)				
Halomonadaceae, Oceanospirillales, Gammaproteobacteria, Proteobacteria						
S35-1; S83-1; S96-2	Water	0–15 (5)	*Halomonas janggokensis* M24^T	99.7–99.9	Solar saltern (Korea)	AM229315
S78-2	Water	0–20 (10)	*Halomonas aquamarina* DSM 30161^T and *Halomonas meridiana* DSM 5425^T	99.1	Sea water (Hawaii) Saline lake (Antarctica)	AJ306888 AJ306891
S74-1	Water	0–20 (10)	*Halomonas gudaoensis* SL014B-69^T	99.4	Contaminated saline soil (China)	DQ421808
S15-2	Sediments	0–20 (10)	*Halomonas elongata* IH9^T	99.0–99.2	Solar saltern (Bonaire, Antilles)	X67023
S24-1	Soil	0–20 (10)				
C3-1; S55-1	Water	0–30 (20)	*Chromohalobacter salexigens* DSM 3043^T	99.7–99.9	Solar saltern (Bonaire, Antilles)	CP000285
C4-2	Water	10–30 (15)	*Halovibrio denitrificans* HGD 3^T	98.5	Sediments, salt lake (Mongolia)	DQ072718
Idiomarinaceae, Alteromonadales, Gammaproteobacteria, Proteobacteria						
S1-2; S5-1; S87-1	Water	1–25 (10)	*Idiomarina loihiensis* L2-TR^T	99.4–99.5	Deep sea hydrothermal vent (Hawaii)	AF288370

[a] Isolates tested for NaCl tolerance are shown in bold

[b] Range of NaCl concentrations (w/v%) at which bacterial growth was recorded; values in parentheses represent salt concentrations for optimal growth

[c] Type strains of validly published species

and C3-1, were the type strains of *Halomonas gudaoensis*, *Halomonas elongata* and *Chromohalobacter salexigens*, respectively. Isolate C4-2 clustered with the type strain of *Halovibrio denitrificans* (98.5% similarity) whereas isolates S1-2, S5-1 and S87-1 grouped with the type strain of *Idiomarina loihiensis* isolated from a deep sea hydrothermal vent in Hawaii.

Data for the salt tolerance of 22 representative isolates (Table 1) indicate that all were either halotolerant or halophilic and were capable of growth on agar media containing between 15 and 30% (w/v) NaCl.

The halotolerant isolates belonged to three genera of the *Firmicutes* and to two genera of the *Gammaproteobacteria*. Of the halotolerant bacteria, only isolate S57-1 (genus *Virgibacillus*) and isolate S35-1(genus *Halomonas*) did not grow at salt concentrations above 15% w/v NaCl. The remaining isolates grew at salt levels exceeding 15% (w/v).

Isolates representing five genera of the *Firmicutes* and two genera of the *Gammaproteobacteria* were halophilic and required between 1 and 10% (w/v) NaCl for growth. Optimal growth of the 22 halotolerant and halophilic isolates was between 5 and 25% (w/v) NaCl.

Discussion

In this work, a culture-dependant approach was used to isolate diverse halophilic and halotolerant bacterial representatives of the genera *Halalkalibacillus*, *Virgibacillus*, *Marinococcus*, *Salimicrobium*, *Halobacillus* and *Alkalibacillus* (phylum *Firmicutes*) and *Halomonas*, *Idiomarina*, *Chromohalobacter* and *Halovibrio* (class *Gammaproteobacteria*) from a hypersaline environment in China.

A comparison of these findings with other cultivation dependant studies suggests that the *Firmicutes* and *Gammaproteobacteria* are indeed predominant among members of the cultivable bacterial community in a wide variety of hypersaline habitats worldwide (Baati et al. 2010; Hedi et al. 2009; Xiang et al. 2008; Yeon et al. 2005). However, a number of other studies have also reported the isolation of members of the *Actinobacteria* (Jiang et al. 2006; Tsiamis et al. 2008; Wu et al. 2006), *Bacteroidetes* (Benlloch et al. 2002; Caton et al. 2004; Oren 2008) and the *Alphaproteobacteria* (Benlloch et al. 2002) from different hypersaline ecosystems.

Because many of the bacteria inhabiting saline environments are intractable to cultivation, it is perhaps not surprising that culture-independent approaches, such as oligonucleotide microarrays and sequencing 16S rRNA genes from denaturing gradient gel electrophoresis (DGGE) and clone libraries, have identified far greater bacterial diversity than has been achieved using cultivation-based methods (Benlloch et al. 2002; Jiang et al. 2006; Lefebvre et al. 2006; Perreault et al. 2007; Tsiamis et al. 2008). However, culture-independent approaches have the disadvantage that bacterial isolates are not obtained for further investigation. There is an urgent need for new media and approaches for culturing halophilic and halotolerant bacteria from hypersaline environments.

Xiang et al (2008) reported the isolation of bacteria related to the genera *Halomonas* (class *Gammaproteobacteria*), *Planococcus, Halobacillus, Oceanobacillus* and *Virgibacillus* (phylum *Firmicutes*) from subterranean hypersaline well water (20–25% salinity) in Zigong, Sichuan Province, China. In contrast, we isolated bacteria affiliated with four genera of the *Gammaproteobacteria* and with six genera of the *Firmicutes* from a hypersaline ecosystem that is also supplied with brines from a subterranean aquifer in Sichuan Province. Of these bacteria, only representatives of the genera *Halobacillus*, *Halomonas* and *Virgibacillus* were common to both studies. A notable difference between the studies was our isolation and identification of bacterial representatives of the genera *Salimicrobium, Halalkalibacillus* and *Halovibrio*. To our knowledge, this is the first report of the isolation of bacteria related to these three genera from a hypersaline environment in China.

Interestingly, *Halobacillus* sp. strain QW1018 that was isolated from hypersaline well water in Sichuan (Xiang et al. 2008), is closely related to isolates S27-1, S101-1, S105-2 and S30-2 in the present study. Moreover, the type strain of *Halomonas subterranea* (ZG16^T), also isolated from saline well water in Sichuan (Xu et al. 2007), is closely related to our isolates S83-1, S35-1 and S96-2.

According to Schleifer (2009), bacteria with 98.7% or less 16S rRNA gene sequence similarity may be considered to be different species. Several of our bacterial isolates affiliated with the genera *Salimicrobium, Halobacillicillus* and *Halovibrio* showed 98.7% or less sequence similarity to the type strain of the closest relative. Further work is required to ascertain the species identity of these isolates.

The majority of bacterial isolates in this study grew on media containing NaCl at concentrations of between 15 and 30% and can be considered to be extremely halotolerant (Margesin and Schinner 2001). The remaining isolates were halophilic and required salt for growth. Most of these halotolerant and halophilic isolates are related to bacterial genera that have the ability to colonize and survive in diverse habitats. For example, several bacterial isolates are affiliated with different *Halomonas* species that have been isolated from contrasting saline environments including soda lakes, solar salterns, mineral pools, marine habitats, animals, mural paintings and from sewage treatments (Xu et al. 2007). Isolate M24-1 was isolated from hypersaline soil whereas the closest phylogenetic relative (*Halalkalibacillus halophilus*, BH2^T) was originally isolated from non-saline soil in Japan (Echigo et al. 2007). The recovery of isolates related to the genera *Salimicrobium* and *Halomonas* from hypersaline water, sediment and from soil samples further emphasizes the ability of these bacteria to adapt to differing saline environments.

Several bacterial isolates identified in this work may have strong biotechnological potential. For example, members of the genera *Halomonas* and *Marinococcus* have been reported to have the ability to degrade phenol and oil pollutants (Nicholson and Fathepure 2004), whereas bacterial representatives of the genus *Idiomarina* have been reported to produce phytases that have potential applications in food processing and the improvement of crop plant nutrition in agriculture (Jorquera et al. 2008).

Research is underway to assess the biotechnological potential of the halophilic and halotolerant bacterial isolates obtained in this work.

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