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The Diversity of Culturable Organotrophic Bacteria from Local Solar Salterns

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We isolated and cultured bacteria inhabiting solar saltern ponds in Taean-Gun, Chungnam Province, Korea. All of the isolated 64 strains were found to be moderately halophilic bacteria, growing in a salt range of 2-20 %, with an optimal concentration of 5% salt. Bacterial diversity among the isolated halophiles was evaluated via RFLP analyses of PCR-amplified 16S rDNAs, followed by phylogenetic analysis of the partial 16S rDNA sequences. The combination of restriction enzyme digestions with *Hae*III, *Cfo*I, *Msp*I and *Rsa*I generated 54 distinct patterns. A neighbor-joining tree of the partial 16S rDNA sequences resulted in the division of the 64 strains into 2 major groups, 45 strains of γ-*Proteobacteria* (70.3%) and 19 strains of *Firmicutes* (29.7%). The α-*Proteobacteria* and *Cytophaga-Flavobacterium-Bacterioides* groups, which were repeatedly found to exist in thalassohaline environments, were not represented in our isolates. The γ-*Proteobacteria* group consisted of several subgroups of the *Vibrionaceae* (37.5%), *Pseudoalteromonadaceae* (10.9%), *Halomonadaceae* (7.8%), *Alteromonadaceae* (7.8%), and *Idiomarinaceae* (6.3%). Members of *Salinivibrio costicola* (29.7%) were the most predominant species among all of the isolates, followed by *Halobacillus treperi* (12.5%). Additionally, three new species candidates were found, based on similarities of the 16S rDNA sequences to those of previously published species.

Key words: bacterial diversity, halophilic bacteria, solar saltern, 16S rDNA

Lately, many studies have reported that the assessment of bacterial diversity by cultivation-dependent methods generates erroneous information with regard to bacterial diversity, owing to the existence of a host of unculturable bacterial species (Britschi and Giovannoni, 1991). The analysis of microbial diversity has, therefore, shifted in the last two decades, from cultivation-dependent approaches to 16S rRNA-based cultivation-independent approaches. This shift in method has resulted in the discovery of many novel microbial taxa. Nevertheless, this approach also has important limitations, and is often confined to naming 16S rDNA clones or DGGE (denaturing gradient gel electrophoresis) bands based on sequence similarity, and speculation as to the ecophysiology of species, on the grounds of such similarities. Therefore, cultivation remains the preferred method for the acquisition of an accurate picture of the physiology and complex ecological interactions in which microorganisms engage.

Cultivation-dependent methods can be also technologically augmented by molecular techniques. DNA technology has provided useful approaches toward the delineation of genetic differences within the bacterial community (Fry, 2004). Polymerase chain reaction (PCR)-based methods have recently gained significant popularity. It has been reported that PCR-RFLP (restriction fragment length polymorphism) analysis of a rDNA repeat can readily distinguish a given species or strain from others (Yoon *et al.*, 2003).

Hypersaline environments, including crystallizer ponds (i.e., ponds where sodium chloride precipitates) of multipond solar salterns, have been demonstrated to exhibit very low prokaryotic diversity, as compared to other environments (Guixa-Boixereu *et al.*, 1996). However, they still harbor considerable diversity with regard to the extremely halophilic archaea and halotolerant bacteria. Crystallizer ponds also constitute a good habitat for moderately halophilic bacteria. Moderately halophilic bacteria, which manifest optimal growth in media containing 3-15% (w/v) NaCl, and are widely distributed in various marine environments, have been frequently isolated in crystallizer ponds (Rodríguez-Valera *et al.*, 1985; Rodríguez-Valera, 1988; Kis-Papo and Oren, 2000; Oren, 2002; Müller and Oren, 2003)

Halophilic bacteria are of great interest, as these bacteria

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bear great biotechnological potential for the production of compatible solutes or hydrolytic enzymes or exopolysaccharides (Margesin and Schinner, 2001; Lee *et al.*, 2003). Despite the biotechnological potential and diversity of halophilic bacteria which inhabit saltern ponds, little information is currently available regarding the distribution and abundance of bacteria in local saltern ponds in Korea. Furthermore, although many reports have been issued on new species found in saltern ponds, many species remain to be identified (Ventosa *et al.*, 1998a; 1998b).

In this study, we attempted to determine the culturable bacterial diversity of solar saltern ponds in Korea, using PCR-RFLP followed by sequencing analyses. Additionally, we engage in a short discussion of the usefulness of the PCR-RFLP method in the analysis of bacterial communities. This study is the first to describe a systematic study of bacterial diversity in Korean saltern ponds.

Materials and Methods

Sampling of salt water

Water samples were collected in July 2002 from the multipond solar salterns, "Pyung-Wha" and "Sin-Eun-Li", both of which are located in Taean-Gun, in the Chungnam Province of Korea. Samples were stored at 4°C for transport to the laboratory. Water temperature was measured with a thermometer, and recorded to be 35°C at both sites. Salinity and pH were determined to be 8.0% and 8.5, at both sites.

Isolation of bacteria

500 μl of the sample water was spread onto Marine agar (Difco, USA) supplemented with additional 3.0% NaCl, then incubated in darkness for two days at 30°C. Based on the colony characteristics, single colonies were selected and stored in 15% glycerol at -80°C, for later characterization and identification. Marine agar or Marine broth was used for maintenance and phenotypic tests.

Phenotypic characterization

Cell morphology and motility were assessed on freshly prepared wet mounts, by phase-contrast microscopy of the exponentially growing liquid cultures. Gram staining was performed according to the standard protocols. Growth at different concentrations of salt was evaluated using maintenance medium, which had been prepared with salt concentrations of 0, 2, 5, 10, 15, 20, and 25% (w/v) (Quesada *et al.*, 1990).

DNA extraction

DNA was extracted from the isolated bacteria as follows. The bacterial cells grown on solid media were transferred to an Eppendorf tube, which contained 100 μl of STES buffer [500 mM NaCl, 200 mM Tris-HCl (pH 7.6), 10 mM EDTA, and 1% SDS]. The mixture was then vortexed for 5 min with a TOMY microtube mixer (TOMY, Japan), at which time, 200 μl of TE buffer (pH 8.0) was added. The DNA was then purified with an equal volume of TE-saturated phenol/chloroform/isoamyl alcohol (25 : 24 : 1) solution. RNA was removed via 3 h of RNase A treatment at 37°C. The purified DNA was then precipitated with 0.1 volumes of 3.0 M sodium acetate, and 2 volumes of cold 95% ethanol, then centrifuged for 20 min at 12,000 rpm at room temperature. The supernatant was removed and the pellet was washed with 70% ethanol, air-dried, and resuspended in 50 μl of TE. The DNA was stored at -20°C until required for later use.

PCR amplification of the 16S rRNA gene

The 16S rRNA gene was amplified with two general bacterial 16S rDNA primers: 1f (5'-AGAGTTTGATCMTGGCTCAG-3'; *Escherichia coli* position 1 to 20) and 1492R (5'-TACGGHTACCTTGTTACGACT-3'; *E. coli* position 1492-1512) (Chèneby *et al.*, 2000). A PCR machine *i*-cycler (BIO-RAD, USA) was used for this amplification. Amplification reactions contained 20 pmole of each primer, 250 μM dNTP, 10 mM Tris-HCl (pH 8.0), 40 mM KCl, 1.5 mM $MgCl_2$, 2 units of Taq-DNA polymerase (Bioneer, Korea), and 100 ng of template DNA, in a final volume of 50 μl. The following conditions were used in the amplification of the 16S rRNA genes: 94°C for 5 min, followed by 30 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 1 min, with a final 10 min extension at 72°C. The PCR products were then checked on agarose gel with ethidium bromide staining.

PCR-RFLP

RFLP analysis was performed in order to differentiate the bacterial strains. 10 μl of the PCR-amplified products were digested with 0.2 U of each of four 4-base recognizing restriction endonucleases (*Cfo* I, *Hae* III, *Msp* I, and *Rsa* I). The mixture was then digested overnight at 37°C, and the resulting RFLP products were separated by gel electrophoresis. Electrophoresis was accomplished in 3.0% SEAKEM (3 : 1) agarose (FMC Bioproducts, USA) with 1X TAE. The gels were stained with 0.5 mg ethidium bromide/ml, and visualized under UV transillumination at 254 nm.

The patterns were normalized and further processed with the GelCompar Version 4.1 pattern analysis software (Applied-Maths, Belgium) (Vauterin and Vauterin, 1992). Strains were grouped using the Pearson product-moment correlation coefficient (r), as well as cluster analysis by UPGMA (unweighted pair group method with arithmetic average). The normalized densitometric traces obtained using each of the four restriction enzymes were then assembled to produce a single combined densitometric trace for each isolate, and analyzed as a single cumulative DNA.

Sequencing

PCR product purification was conducted using a Wizard

PCR prep kit (Promega, USA). Purified double-stranded PCR fragments were sequenced directly with BigDye terminator cycle sequencing kits (Applied Biosystems, USA.), according to the manufacturer's instructions. The primers, 341f (5'-CCTACGGGAGGCAGCAG-3'; *E. coli* position 341-357) and 926r (5'-CCGTCAATTCMTTT RAGTTT-3'; *E. coli* position 926-945), were then used to determine the partial sequences of the PCR-amplified DNA double strands (Chèneby *et al*, 2000). Gel electrophoresis and data collection were performed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA).

Phylogenetic analysis of partial rDNA sequences

Sequences were compared to the 16S rRNA gene sequences in the GenBank database, via BLAST searches. The sequences were initially aligned with sequences retrieved from GenBank using the CLUSTAL X program (Thompson *et al.*, 1997), and then alignment was manually refined with the PHYDIT program, version 3.0 (Chun, 1995; available at http://plaza.snu.ac.kr/~jchun/phydit). Ambiguously aligned regions were excluded from the following analyses. A neighbor-joining tree was reconstructed with Kimura's 2-parameter distance model (Kimura, 1980), using the PHYLIP 3.57c package (Felsenstein, 1985). Bootstrap analyses of 1,000 replicates were performed in order to assess the relative stability of the branches.

Results

Isolation and Phenotypic characterization

The aerobic or facultative anaerobic and heterotrophic bacteria, expressed as the number of CFU (colony-forming units) which grew on Marine agar, were in the range of 3.0 - 7.0 $\times$ 10^2/ml.

A total of 64 culturable isolates, exhibiting distinct colony characteristics (size, pigmentation, opacity, texture, form, elevation, margin, and surface) were selected for further molecular typing. More than half of the isolates were cream-colored. The others formed yellow, white, or red colonies on solid media. The majority of the isolates (45 strains, 70.3%) were found to be Gram-negative, while 19 isolates (29.7%) were Gram-positive.

None of the isolates grew in NaCl concentrations of over 25% (w/v), and none grew in the absence of NaCl. The optimal concentration of NaCl was determined to be 5%, and so all of our isolates were considered to be moderate halophiles. No exceptions to this designation were found among our isolates.

RFLP analyses

The procedure used to obtain the crude DNA extracts yielded good results for all of the isolates in PCR amplification. The 1.5 kb DNA fragments which corresponded to nearly complete 16S rDNAs were amplified from all strains, by PCR using the primers 1f and 1492R.

Restriction digestion produced 16, 16, 13, and 31 fragments, different in size, for *Msp*I, *Hae*III, *Rsa*I, and *Cfo*I, respectively. A total of 27, 28, 29, and 16 RFLP patterns were identified from the 16S rDNA restriction fragments, *Msp*I, *Hae*III, *Rsa*I, and *Cfo*I, respectively. The combination of four RFLPs produced 54 different patterns. A UPGMA dendrogram from the combined restriction fragment pattern is shown in Fig. 1. Five RFLP patterns were represented more than once among 15 strains. Forty-nine strains exhibited unique RFLP patterns.

Identification with partial 16S rDNA sequences

Bacterial isolates were identified at the species level based on the partial sequence analysis of their 16S rRNA genes. Phylogenetic relationships among strains from saltern ponds are illustrated in Fig. 2. The distribution of strains within major taxa is shown in Table 1.

Discussion

RFLP analyses

Any given RFLP pattern may represent sequences from multiple phylogenetic groups and may, therefore, not be representative of a true phylotype in the traditional sense (Priemé *et al.*, 2002). However, if more restriction enzymes or rapidly-evolving genes are used, the false similarities can be dissolved. RFLP would be an appropriate technique for the fine-structure analysis of specific components of the structure of the microbial community if such conditions were satisfied (Savelkoul *et al.*, 1999).

The total number of different RFLP patterns among the 64 strains was 54, which outnumbered that of the produced sequence types, which was 46. Moreover, the dendrogram of RFLP patterns produced a phylogeny which was almost identical to those observed with the 16S rRNA genes (figs. 1 and 2). This means that RFLP pattern analyses will tend to produce random errors and systematic errors at low rates, similar to the low rates associated with phylogenetic analysis based on nucleotide sequences. RFLP analysis, then, has proven quite useful for the first steps of grouping the bacterial isolates. Representatives of each group can then be subjected to further analyses, including sequencing and fatty acid analysis. In cases in which a preliminary database of RFLP patterns has been built, RFLP pattern analyses should enable us to identify bacterial strains readily, even at the species level, as the same sample types will exhibit similar bacterial communities.

Identification of bacterial isolates

The 16S rDNAs of 64 bacterial strains were sequenced (Table 1). Forty-six different sequences were observed, and among these, 10 sequences were represented by more than two strains. Phylogenetic analysis using 16S rDNA revealed that the 64 strains could be further allocated into two clades, 45 strains of γ-*Proteobacteria* (70.3%), and 19 strains of *Firmicutes* (29.7%), without any strains

belonging to any other groups.

γ- *Proteobacteria, Vibrionaceae*

One frequently encountered group, which was found to consist of 24 strains (37.5%), was apparently affiliated with the family *Vibrionaceae*, evidencing a 97% bootstrap value. The group was further divided into two genera, 17 *Salinivibrio* spp. and 4 *Vibrio* spp..

Salinivibrio costicola is capable of growing in a range

Fig. 1. Dendrogram showing the relationship among the halophilic bacterial isolates, based on the 16S rDNA-RFLP profiles. Family names were determined by comparing partial 16s rDNA sequences in the GenBank database.

of water activities between 0.98 (close to fresh water) to 0.86 (close to saturated NaCl) (Kushner, 1978), and representatives of the species have been isolated in a variety of saline environments (Ventosa *et al.*, 1998a; 1998b).

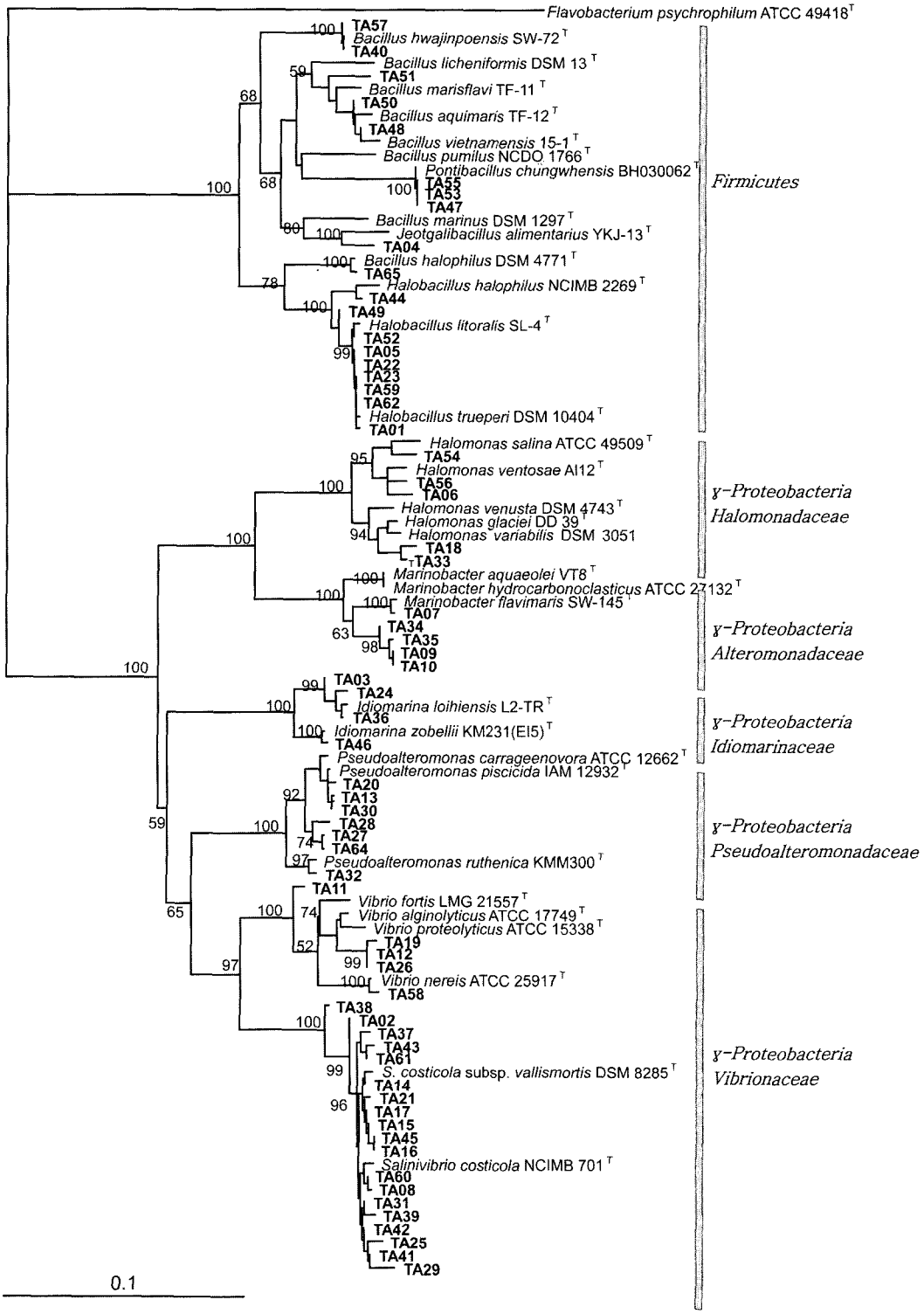

Fig. 2. Phylogenetic association of the 16S rDNA sequences in this study, as well as for those which were closely related among previously published sequences in the database. The phylogenetic tree was built by the neighbour-joining method, using Kimura's 2 parameter distance from the partial 16S rDNA sequences (*E. coli* positions, 344-915). The numbers at the nodes are bootstrap confidence values, and are expressed as percentages of 1,000 bootstrap replications. Bootstrap values higher than 50% are indicated at the main nodes. *Flavobacterium psychrophilum*, a member of the *Cytophaga-Flavobacterium-Bacteroides* group, served as the outgroup.

Members of the *Salinivibrio* clade (TA02, TA08, TA14, TA15, TA16, TA17, TA21, TA25, TA29, TA31, TA37, TA38, TA39, TA41, TA42, TA43, TA45, TA60 and TA61) exhibit 98.0 - 99.5% homology with *S. costicola* subsp. *costicola* or *S. costicola* supsp. *vallismortis* (Huang *et al.*, 2000).

The other group was composed of strains which are clearly closely related to *Vibrio* spp.. Strain TA58 could be identified as *V. nereis,* evidencing 99.5% similarity. Strains TA12, TA19, TA11, and TA26 exhibited 98.0 - 99.1% similarity with the type strain of *V. alginolyticus*, which is involved with the mass mortality of abalone, and has been extensively studied with regard to its pathology and mechanisms (Liu *et al.*, 2001).

γ - *Proteobacteria, Pseudoalteromonadaceae*

Eight isolates of the genus *Pseudoalteromonas* are observed. Strains TA13, TA30, and TA20 showed sequence similarity values of 99.5 - 99.7% to *P. piscicida.* Strains TA32 showed 99.4% homlogy to the strains of *P. ruthenica* isolated from marine mussel and scallop (Ivanova *et al.*, 2002), members of which were known to produce a number of antimicrobial compounds. Strains TA27, TA38, and TA64 showed 97.9 - 98.7% similarity to *P. carrageenovor.*

γ - *Proteobacteria, Halomonadaceae*

This group, consisting of five strains (TA54, TA56, TA06, TA18, and TA33) was associated with the genus *Halomonas* (98.0 - 99.7% similarity), evidencing a 100% bootstrap confidence value. Strain TA54 was identified as *H. salina,* exhibiting 97.9% similarity with the species. Strains TA06 and TA56 were determined to be members of *H. ventosa* by their 98.2% and 98.4% measures of similarity, respectively. *H. ventosae* was reported to produce exopolysaccharides (Martinez-Canovas *et al.*, 2004). Strain TA33 was left unidentified, as the strain was clustered outside of the group consisting of *H. glacei* and *H. variabilis.*

γ - *Proteobacteria, Alteromonadaceae*

A total of 4 strains were assigned to the family *Alteromonadaceae.* Strain TA07 was identified as *Marinobacter flavimaris.* It exhibited 99.68% similarity with the type strain of *M. flavimaris*, which was also isolated from seawater in the Yellow Sea (Yoon *et al.*, 2004b). Strains TA09, TA10, TA34, and TA35 exhibited 99.8 - 100.0% similarity with one another. The 4 strains, which were grouped concretely with a 98% bootstrap value, may constitute a new species, as we were unable to cluster them into any validly published species, and they exhibited rather low similarity values (97.1 - 97.4%) with their apparently closest relative, *M. flavimaris.*

γ - *Proteobacteria, Idiomarinaceae*

The *Idiomarinaceae* were represented by four isolates. Strains TA03, TA24, and TA36 shared 99.4 - 99.8% similarity with the type strain of *Idiomarina loihiensis*, which was isolated in hypothermal vents (Donachie *et al.* 2003). Strain TA46 exhibited 99.7% similarity with the type

Table 1. List of bacterial species isolated from solar saltern ponds

Strain No.	Accession No.	Group	Closest relative based on partial sequence homology[1]	Similarity (%)
TA02	AY839759	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* NCIMB 701^T (X95527)	99.5
TA08	AY839765	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* NCIMB 701^T (X95527)	99.4
TA14	AY839771	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* NCIMB 701^T (X95527)	99.1
TA25	AY839782	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* NCIMB 701^T (X95527)	98.9
TA29	AY839786	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* NCIMB 701^T (X95527)	97.9
TA31	AY839787	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* NCIMB 701^T (X95527)	99.5
TA38	AY839794	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* NCIMB 701^T (X95527)	98.0
TA39	AY839795	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* NCIMB 701^T (X95527)	99.0
TA41	AY839797	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* NCIMB 701^T (X95527)	99.5
TA42	AY839798	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* NCIMB 701^T (X95527)	99.5
TA60	AY839816	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* NCIMB 701^T (X95527)	99.5
TA61	AY839817	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* NCIMB 701^T (X95527)	99.3
TA15	AY839772	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* subsp. *vallismortis* DSM 8285^T (AF057016)	99.5
TA16	AY839773	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* subsp. *vallismortis* DSM 8285^T (AF057016)	99.2
TA17	AY839774	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* subsp. *vallismortis* DSM 8285^T (AF057016)	99.5
TA21	AY839778	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* subsp. *vallismortis* DSM 8285^T (AF057016)	99.4
TA37	AY839793	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* subsp. *vallismortis* DSM 8285^T (AF057016)	98.9
TA43	AY839799	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* subsp. *vallismortis* DSM 8285^T (AF057016)	98.5
TA45	AY839801	γ - *Proteobacteria, Vibrionaceae*	*Salinivibrio costicola* subsp. *vallismortis* DSM 8285^T (AF057016)	99.2

Table 1. Continued.

Strain No.	Accession No.	Group	Closest relative based on partial sequence homology[1]	Similarity (%)
TA11	AY839768	γ - *Proteobacteria, Vibrionaceae*	*Vibrio alginolyticus* ATCC 17749^T (X56576)	99.1
TA12	AY839769	γ - *Proteobacteria, Vibrionaceae*	*Vibrio alginolyticus* ATCC 17749^T (X56576)	98.4
TA19	AY839776	γ - *Proteobacteria, Vibrionaceae*	*Vibrio alginolyticus* ATCC 17749^T (X56576)	98.0
TA26	AY839783	γ - *Proteobacteria, Vibrionaceae*	*Vibrio alginolyticus* ATCC 17749^T (X56576)	98.4
TA58	AY839814	γ - *Proteobacteria, Vibrionaceae*	*Vibrio nereis* ATCC 25917^T (X74716)	99.5
TA27	AY839784	γ - *Proteobacteria, PseudoAlteromonadaceae*	*Pseudoalteromonas carrageenovora* ATCC 12662^T (X82136)	98.6
TA28	AY839785	γ - *Proteobacteria, PseudoAlteromonadaceae*	*Pseudoalteromonas carrageenovora* ATCC 12662^T (X82136)	97.9
TA64	AY839819	γ - *Proteobacteria, PseudoAlteromonadaceae*	*Pseudoalteromonas carrageenovora* ATCC 12662^T (X82136)	98.7
TA13	AY839770	γ - *Proteobacteria, PseudoAlteromonadaceae*	*Pseudoalteromonas piscicida* IAM 12932^T (AB090232)	99.5
TA20	AY839777	γ - *Proteobacteria, PseudoAlteromonadaceae*	*Pseudoalteromonas piscicida* IAM 12932^T (AB090232)	99.5
TA30	AY842286	γ - *Proteobacteria, PseudoAlteromonadaceae*	*Pseudoalteromonas piscicida* IAM 12932^T (AB090232)	99.7
TA32	AY839788	γ - *Proteobacteria, PseudoAlteromonadaceae*	*Pseudoalteromonas ruthenica* KMM300^T (AF316891)	99.4
TA18	AY839775	γ - *Proteobacteria, Halomonadaceae*	*Halomonas glaciei* DD 39^T (AJ431369)	98.0
TA33	AY839789	γ - *Proteobacteria, Halomonadaceae*	*Halomonas glaciei* DD 39^T (AJ431369)	98.1
TA54	AY839810	γ - *Proteobacteria, Halomonadaceae*	*Halomonas salina* ATCC 49509^T (AJ243447)	97.9
TA06	AY839763	γ - *Proteobacteria, Halomonadaceae*	*Halomonas ventosae* Al12^T (AY268080)	98.2
TA56	AY839812	γ - *Proteobacteria, Halomonadaceae*	*Halomonas ventosae* Al12^T (AY268080)	98.4
TA07	AY839764	γ - *Proteobacteria, Altermonadaceae*	*Marinobacter flavimaris* SW-145^T (AY517632)	99.7
TA09	AY839766	γ - *Proteobacteria, Altermonadaceae*	*Marinobacter flavimaris* SW-145^T (AY517632)	97.4
TA10	AY839767	γ - *Proteobacteria, Altermonadaceae*	*Marinobacter flavimaris* SW-145^T (AY517632)	97.4
TA34	AY839790	γ - *Proteobacteria, Altermonadaceae*	*Marinobacter flavimaris* SW-145^T (AY517632)	97.3
TA35	AY839791	γ - *Proteobacteria, Altermonadaceae*	*Marinobacter flavimaris* SW-145^T (AY517632)	97.1
TA03	AY839760	γ - *Proteobacteria, Idiomarinaceae*	*Idiomarina loihiensis* L2-TR^T (AF288370)	99.8
TA24	AY839781	γ - *Proteobacteria, Idiomarinaceae*	*Idiomarina loihiensis* L2-TR^T (AF288370)	99.4
TA36	AY839792	γ - *Proteobacteria, Idiomarinaceae*	*Idiomarina loihiensis* L2-TR^T (AF288370)	99.7
TA46	AY839802	γ - *Proteobacteria, Idiomarinaceae*	*Idiomarina zobellii* KM231(E15)^T (AF052741)	99.7
TA65	AY839820	*Firmicutes*	*Bacillus halophilus* DSM 4771^T (AB021188)	99.5
TA40	AY839796	*Firmicutes*	*Bacillus hwajinpoensis* SW-72^T (AF541966)	100.0
TA57	AY839813	*Firmicutes*	*Bacillus hwajinpoensis* SW-72^T (AF541966)	100.0
TA48	AY839804	*Firmicutes*	*Bacillus vietnamensis* 15-1^T (AB099708)	99.7
TA50	AY839806	*Firmicutes*	*Bacillus vietnamensis* 15-1^T (AB099708)	99.5
TA51	AY839807	*Firmicutes*	*Bacillus vietnamensis* 15-1^T (AB099708)	96.7
TA44	AY839800	*Firmicutes*	*Halobacillus halophilus* NCIMB 2269^T (X62174)	98.9
TA01	AY839758	*Firmicutes*	*Halobacillus trueperi* DSM 10404^T (AJ310149)	99.7
TA05	AY839762	*Firmicutes*	*Halobacillus trueperi* DSM 10404^T (AJ310149)	99.8
TA22	AY839779	*Firmicutes*	*Halobacillus trueperi* DSM 10404^T (AJ310149)	99.8
TA23	AY839780	*Firmicutes*	*Halobacillus trueperi* DSM 10404^T (AJ310149)	99.8
TA49	AY839805	*Firmicutes*	*Halobacillus trueperi* DSM 10404^T (AJ310149)	99.8
TA52	AY839808	*Firmicutes*	*Halobacillus trueperi* DSM 10404^T (AJ310149)	99.8
TA59	AY839815	*Firmicutes*	*Halobacillus trueperi* DSM 10404^T (AJ310149)	99.8
TA62	AY839818	*Firmicutes*	*Halobacillus trueperi* DSM 10404^T (AJ310149)	99.8
TA04	AY839761	*Firmicutes*	*Jeotgalibacillus alimentarius* YKJ-13^T (AF281158)	96.8
TA47	AY839803	*Firmicutes*	*Pontibacillus chungwhensis* BH030062^T (AY553296)	100.0
TA53	AY839809	*Firmicutes*	*Pontibacillus chungwhensis* BH030062^T (AY553296)	100.0
TA55	AY839811	*Firmicutes*	*Pontibacillus chungwhensis* BH030062^T (AY553296)	100.0

[1]The closest matching sequences of the cultivated and characterized strains were identified using BLAST searches.

strain of *I. zobellii*, a novel deep-sea bacterium indigenous to the Pacific Ocean (Ivanova *et al.*, 2000).

Firmicutes, Bacillaceae

The *Firmicutes* were represented by 17 isolates. Strains TA01, TA05, TA22, TA23, TA49, TA52, TA59, and TA62 exhibited similarities to *H. trueperi* in the range of 99.7 to 99.8%, while TA44 was found to exhibit a 98.9% similarity with *H. halophilus*. Strain TA65 was most profoundly similar to a *H. halophilus* soil isolate, with a 99.5% similarity value. The type strains of *H. halophilus* and *H. trueperi* were both isolated from the Great Salt Lake (Spring *et al.*, 1996).

Strains TA40 and TA57 could be assigned to *Bacillus hwajinpoenesis,* and exhibited 100% similarity with the species. Two *B. hwajinpoenesis* strains were previously isolated from seawater, from the Yellow Sea and the East Sea in Korea (Yoon *et al.*, 2004a). Strains TA48 and TA50 exhibited 99.7% and 99.5% similarity with the type strain of *B. vietnamensis*. However, Strain TA51 displayed a 96.7% similarity with its closest relative, *B. vietnamensis*. Such low degrees of sequence similarity suggest that a new *Bacillus* species may need to be created in the near future.

Strains TA47, 53, and 55 exhibited 100% similarity with strains of *Pontibacillus chungwhensis* (Lim *et al*, 2004) which had been previously isolated from a Korean solar saltern.

Strain TA04 was loosely related to the previously published sequences, and displayed the highest similarity to *Jeotgalibacillus alimentarius* (96.8%). This strain merits a novel species status, due to its low degree of similarity with all of the validated species.

Bacterial Diversity of local solar saltern ponds

We failed to find extremely halophilic archaea, which have been frequently identified as the dominant phylotypes in a variety of hypersaline environments, such as saltern ponds (Oren, 1994; Anton *et al.*, 2000). The brines in saltern crystallizer ponds are generally an intense red color, due to the presence of dense communities of orange-red pigmented microorganisms belonging to the family *Halobacteriaceae* of the domain *Archaea* (Kushner, 1985; Kushner & Kamekura, 1988; Norton, 1992).

Until recently, it has been assumed that representatives of the domain *Bacteria* do not significantly contribute to the microbial community of hypersaline brines at or approaching NaCl saturation. As a result, the bacterial list shown in Table 1 cannot provide actual information regarding the structural diversity of the solar saltern ponds, as the *Archaea* dominant in such an environment cannot be isolated by traditional cultivation approaches, as mentioned earlier in this study.

However, the domain *Bacteria* may indeed constitute a major contributor to the microbial community in the local saltern ponds, considering the clear color of the sampled water. The sampled water from the solar saltern ponds was quite clear, with no observable red tint. Cultivation-independent approaches, such as DGGE and T-RFLP, are required for the elucidation of the actual community structure of the solar saltern ponds.

Cultivation-dependent strategies have been found to produce a similar bacterial community both in moderately saline and hypersaline environments. The communities of moderately halophilic bacteria in thalassohaline (seawater-derived) hypersaline environments, including those associated with saltern ponds for the concentration of seawater, may resemble, to a large extent, the communities actually present in the seawater. This is not too surprising, considering the broad salt tolerance exhibited by marine bacteria. When only cultivation-dependent approaches were utilized, the γ-*Proteobacteria* and *Firmicutes* did, indeed, predominate, as evidenced in the previous studies of marine sediment by Gray and Herwing (1996) and Urakawa *et al.* (1999). However, the results of the study of Lee *et al.* demonstrated that the α-*Proteobacteria* are the dominant members of the coral-associated bacterial community, although the γ-*Proteobacteria* do exists in substantial numbers (Lee *et al.*, 1999). All of the strains isolated in our study were not extremely halotolerant, as most of them grew optimally at a 5% salt concentration, and also exhibited growth in a wide range of salt concentrations, from 5 - 20%. Thus, they should be considered to be moderate halophiles, adapted to the high salinity of the ponds. This presumably reflects their adaptation to periodic episodes of relatively high dilution.

Although cultivation-independent approaches are required for a thorough and accurate understanding of the community structure of solar saltern ponds, this study may also prove valuable for community analysis, in that living cells were obtained for the physiological investigations. Moreover, this study should be additionally subjected to comparative studies with bacterial communities obtained via cultivation-independent methods, as PCR-based approaches have biases which can distort community composition (Martin-Laurent *et al.*, 2001; Hur and Chun, 2004).

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