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Characterization of the Microbial Diversity in a Korean Solar Saltern by 16S rRNA Gene Analysis

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Abstract We studied the diversity of the halophilic archaea and bacteria in crystallizer ponds of a Korean solar saltern by analyzing 16S rRNA gene libraries. Although diverse halophilic archaeal lineages were detected, the majority (56%) were affiliated with the uncultured and cultured *Halorubrum* group. Halophilic archaea that have been frequently observed in solar saltern environments previously, such as *Halogeometricum*, *Halococcus*, *Haloarcula*, and *Haloferax*, were not detected in our samples. The majority of clones (53%) belonged to the *Cytophaga-Flavobacterium-Bacteroides* and α-, γ-, and δ-*Proteobacteria* groups, with 47% of the clones being affiliated with γ-*Proteobacteria*. We also identified new δ-*Proteobacteria*-related bacteria that have not been observed in hypersaline environments previously. Our data show that the diversity of the halophilic archaea and bacteria in our Korean saltern differs from that of solar salterns found in other geographic locations. We also showed by quantitative real-time PCR analysis that bacteria can form a significant component of the microbial community in solar salterns.

Key words: Halophilic archaea, halophilic bacteria, solar saltern, 16S rRNA, diversity

Diverse hypersaline environments such as solar salterns and salt rocks have been found to harbor halophilic microorganisms. Such halophilic microorganisms have been studied previously for various reasons. For example, they have been screened for novel biocatalysts and biomaterials that facilitate their adaptation to high salt conditions and that can be exploited for commercial applications [20, 25]. Moreover, they have been speculated to be candidates of extraterrestrial life in studies seeking to understand the development of life on earth [18].

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Halophilic organisms include members from both the domain *Archaea* and *Bacteria*. Some of these are the haloarchaea, which are the red-pigmented, extremely halophilic archaea that belong to the family *Halobacteriaceae* [11] within the phylum *Euryarchaeota* of the domain *Archaea*. The halophilic archaea are the most halophilic organisms that have been identified to date, and they dominate in hypersaline environments in which the salt concentration exceeds 250 g/l [29]. Bacteria are also frequently found in hypersaline environments, often at equivalent abundances as the halophilic archaea, but they have been less well studied [1].

Although halophiles have been isolated and characterized, many have not yet been cultivated, largely because of difficulties in determining suitable culture conditions. Such difficulties are responsible, for example, for the fact that a pure culture of a well-known haloarchaeon was established only recently [3]. Thus, it is likely that current culture-dependent methods are unsuitable for analyses that seek to estimate halophile diversity in a hypersaline environment. A better approach may instead be one based on 16S rRNA gene analyses without cultivation. This method does not require cultivation and it has been used previously in investigations of archaeal and bacterial diversity that have resulted in the discovery of many novel taxa [2, 8, 12, 21, 23, 28, 32, 34].

The western coastal area of the Korean Peninsula that is bounded by the Yellow Sea has been traditionally exploited as a source of solar saltern fields. A cultivation-dependent diversity analysis of these Korean saltern environments has resulted in the isolation of several bacterial strains [35]. However, the microbial diversity in these salterns has not yet been extensively investigated at the molecular level. In this study, we analyzed the diversity of the halophilic archaea and bacteria found in a Korean solar saltern by analyzing 16S rRNA gene libraries. We compared our observations with those of studies using solar salterns in

different geographic locations. We also analyzed the relative abundance of the bacteria and archaea in our saltern by using quantitative real-time PCR.

Analysis of the Diversity of the 16S rRNA Genes Obtained from Environmental Genomic DNA

Samples were collected in sterile plastic tubes from the crystallizer ponds of a solar saltern located in a coastal area of Daecheon (E 126°58', N 36°53') in Korea. Field measurements showed that the salinity of the saltern was 33.2%, with a pH of 7.4. Chemical analysis of these water samples indicated that Na^+ and Cl^- were the most abundant ions and conductance was 148,000 µS/cm. The samples were filtered by using a 0.2-µm pore size filter (Milipore, Bedford, MA, U.S.A.) and the filters were stored at −80°C until analysis. To extract genomic DNA, the frozen filters were cut into small pieces and subjected to an UltraClean soil DNA isolation kit (Mo Bio Laboratories).

The 16S rRNA genes were amplified from the resulting environmental genomic DNA by PCR employing *EF*-Taq (Solgent, Korea). The following thermal cycling parameters were used: 5 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 55°C, and 90 s at 72°C; and 10 min at 72°C. The bacterial 16S rRNA genes were amplified by using the eubac-27F (5'-AGAGTTTGATCMTGGCTCAG-3') and eubac-1492R (5'-TACGGYTACCTTGTTACGACTT-3') primers [15, 19], whereas the archaeal 16S rRNA genes were amplified by using the arch-20F (5'-TTCCGGTTGATCCYGCRG-3') and arch-958R (5'-TCCGGCGTTGAMTCCAATT-3') primers as previously described [7]. The PCR products were purified by using the AccuPrep PCR Purification Kit (Bioneer, Korea) according to the manufacturer's protocol. The purified PCR products were then ligated into the pGEM-T easy vector (Promega, U.S.A.) and transformed into *Escherichia coli* DH10B cells.

The clone inserts in the *E. coli* plasmids were amplified by PCR using the previously described prGTf (5'-TACG-ACTCACTATAGGGCGA-3') and prGTr (5'-CTCAAGC-TATGCATCCAACGC-3') primers [6]. For initial screening of 50 clones per library, we used amplified rDNA restriction analysis as described by Kim *et al.* [14] and So *et al.* [31]. PCR products of representative clones were then purified by using the AccuPrep PCR Purification Kit (Bioneer, Korea) and sequenced directly by using Bigdye terminator cycle sequencing ready reaction kits (Applied Biosystems, U.S.A.) and an ABI PRISM 3730xl DNA Analyzer (PE Applied Biosystems). All 16S rRNA gene sequences determined in this study were deposited in the GenBank database under the accession numbers DQ431075-DQ431112. The sequences were checked for possible chimeras by using the CHIMERA_CHECK program at the Ribosomal Database Project Web site (http://rdp8.cme.msu.edu). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database and multiple alignments were performed by the Clustal_X program [33]. The evolutionary distances were calculated by using the Kimura two-parameter model [16], and the phylogenetic trees were constructed by using the neighbour-joining method [30] employed by the MEGA 3 Program [17] with bootstrap values based on 1,000 replications [9].

Archaeal diversity. All archaeal clones that were sequenced were phylogenetically affiliated with halophilic archaea and were distributed into several phylogenetic groups. Some clones were related to cultured members of the *Halorubrum* and *Natronomonas* genera, which share 97% 16S rRNA sequence identity with our archaeal clones. Other clones were related to archaeal groups that have not yet been cultured, including those in the *Halorubrum* group (uncultured *Halorubrum* group, or UHG) and Clusters I–VI (Fig. 1). Our phylogenetic tree shows a tree topology that is similar to that determined previously by Kamekura [13].

The majority of the clones (about 56%) were related to *Halorubrum* (Fig. 1), which is a well-supported and broad phylogenetic group that contains clones from diverse hypersaline environments [2, 8, 21, 23, 28]. Only a few clones (A16A, A6, A16T, A3, and A13A) that fall into the *Halorubrum* group were related to haloarchaeal strains that have been cultured, namely, *H. laccusprofundi* or *H. xinjiangense*. These clones shared 96.6–98.6% 16S rRNA sequence identity with these species. Of the clones related to UHG clones, A2, A7T, A14, A7A, A12, and A8A were closely related to clone MAlk006.3, which was found in ancient salt deposits [21]. Moreover, clone A18, which also belongs to the *Halorubrum* group, is closely related to clone ss057u, which was isolated from a salt spring in Canada (91% sequence identity) [34].

With regard to the clones that were closely related to Clusters I–IV, the A10 clone, which falls into Cluster I, was only distantly related to cultured relatives of the *Halorubrum* group. Instead, it was closer to the environmental clone CSW6.14.5 that was isolated from an Australian saltern (95% sequence identity) [5]. In addition, A4 of Cluster II was close to the environmental sequence BbpA.3 found in an ancient salt deposit [21] (97% sequence identity) (Fig. 1), whereas A17 and A19A of Cluster III were closely related to the saltern clone ArcG01 with 95% and 93% 16S rRNA gene sequence identity, respectively [32]. Three clones, A13T, A11, and A1, were grouped into Cluster IV, which exclusively contains uncultivated phylotypes found in salt marshes [22], hypersaline Antarctic lakes [4], and solar salterns [23]. The clones that were not closely related to *Halorubrum* or Clusters I–IV include A9 and A19T, which were instead closely related to *Natronomonas pharaonis* DSM 2160 (97 and 99% sequence identity, respectively), as well as clone A15, which was close to the environmental clone ArcH05 that was isolated from a saltern in Israel (95% sequence identity) [32]. Moreover, A8T is distantly

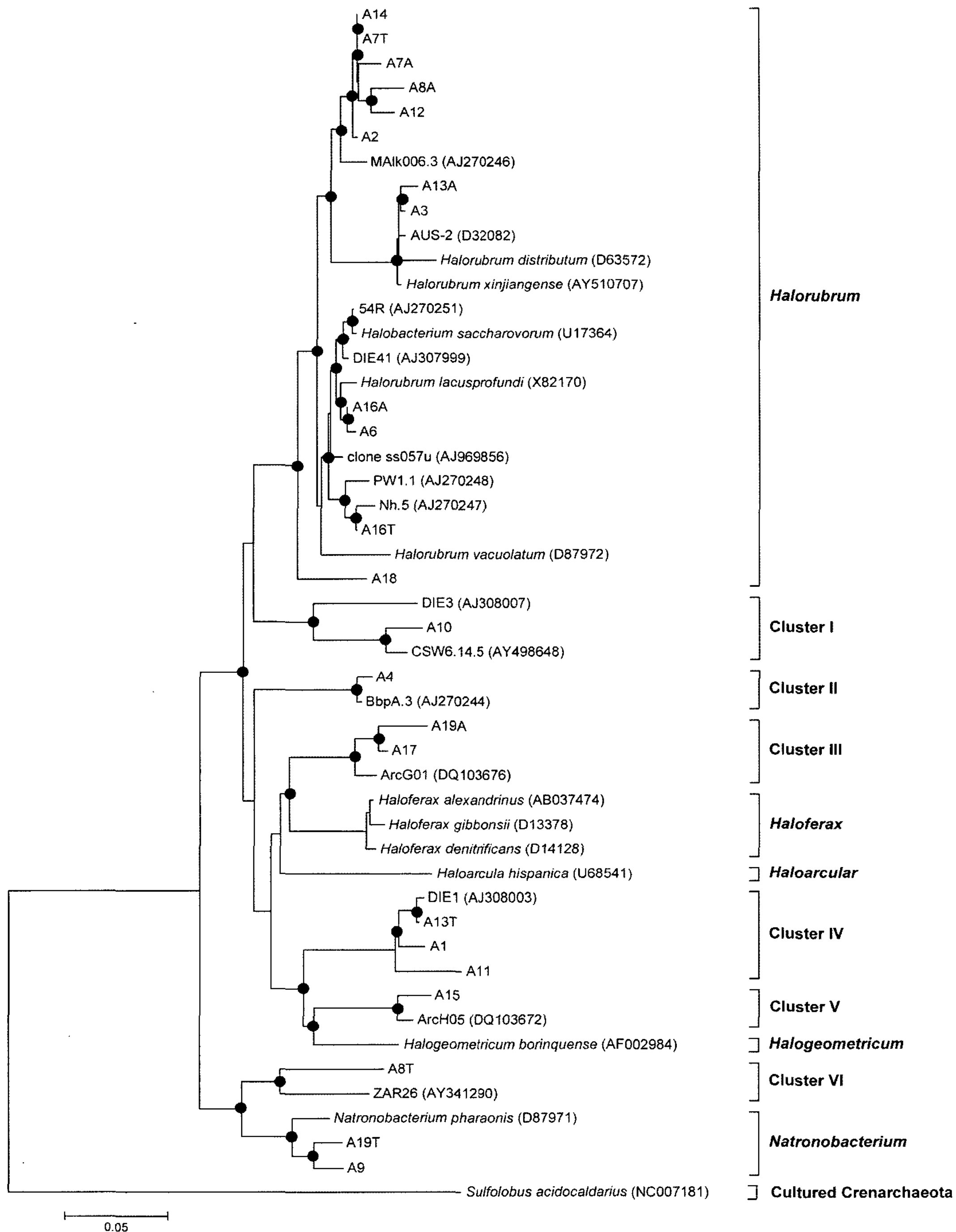

Fig. 1. Phylogenetic tree based on partial archaeal 16S rRNA sequences obtained from environmental genomic DNA isolated from a solar saltern.

The reference sequences were chosen to show the diversity of the sequences and to indicate the closest relatives to the sequences found in our study. The scale bar represents 5 substitutions per 100 nucleotide positions. Significant bootstrap values (≥50%; 1,000 replicates) are indicated by filled circles at branch points. As an outgroup, we used the 16S rRNA gene of the cultivated crenarchaeon *Sulfolobus acidocaldarius*.

related to clone ZAR26, which was obtained from a low-salt, sulfide-rich, and sulfur-rich spring (91% sequence identity) [8].

Our data thus show that many of the clones we isolated from our solar saltern were related to clones that have been isolated from hypersaline environments other than solar salterns. Apart from several clones related to *Natronobacterium* and *Halorubrum*, most of our clones were related to uncultured halophilic archaea found in diverse hypersaline environments. Moreover, halophilic archaea that have

Table 1. Characterization of the halophilic bacterial phylotypes found in our study.

Group	Sequences	Closest relative in BLAST search	Origin	Similarity (%)
Cytophaga-Flavobacterium-Bacteroides	e5	Clone HPB-88 (AY435199)	Hypersaline marine environment, Australia	91
	T18	Clone MPD-38 (AF348718)	Solar evaporation pond	97
α-*Proteobacteria*	T1	Clone SIMO-301 (AF547414)	Salt marsh tidal creek, Sapelo Island	99
	e1	Clone E4aF06 (DQ103605)	Hypersaline endoevaporitic	87
	T28	*Brevundimonas mediterranea* (AJ244706)	Mediterranean Sea, Germany	99
γ-*Proteobacteria*	T3	Clone A349 (AF477904)	Coastal solar saltern	97
	T9	Clone A349 (AF477904)	Coastal solar saltern	98
	e12	Clone A349 (AF477904)	Coastal solar saltern	99
	e13	Clone 48-UMH (AF477879)	Coastal solar saltern	98
	e9	Clone E8.3 (AJ517899)	Uranium-rich environments	93
	e14	*Pseudomonas halophila* (AB021383)	Great salt lake, USA	98
	e11	*Pseudomonas halophila* (AB021383)	Great salt lake, USA	100
δ-*Proteobacteria*	e6	Clone E6aH12 (DQ103652)	Hypersaline endoevaporitic	90
	e3	Unidentified bacterium wb1_H02 (AF317766)	Underwater	91
	e4	Clone T26-29 (AF332318)	Sakondani Coast, Japan	91

been frequently observed in solar saltern environments, such as *Halogeometricum*, *Halococcus*, and *Haloarcula*, [21, 23, 27] were not observed in our sample. This shows that geographically distant solar salterns differ with regard to their halophilic archaeal diversity.

Bacterial diversity. Although the archaeal diversity in hypersaline environments has been frequently studied, the bacterial diversity in such environments has been less extensively examined [1, 2, 32]. In our 16S rRNA gene library, we found that the major bacterial groups to which our clones were closely related were the *Cytophaga-Flavobacterium-Bacteroides* (CFB) and α-, γ-, δ-*Proteobacteria* groups (Table 1). In particular, about 47% of our bacterial clones were related to γ-*Proteobacteria*. Most of these were affiliated to γ-*Proteobacteria* clones found in coastal solar salterns and the Great Salt Lakes. However, the γ-*Proteobacteria*-related clone e9 was related to the uncultured bacterial clone E8.3 found in uranium-rich environments [10]. Moreover, the α-*Proteobacteria*-related clone T28 was affiliated to *Brevundimonas mediterranea*, which was isolated from the Mediterranean Sea. Finally, the δ-*Proteobacteria*-related clones e3 and e4 have not been observed in hypersaline environments previously and are only distantly related to any of the known bacterial phylotypes [12, 24].

Salterns from different geographic regions may differ in their bacterial diversity, as has also been observed with regard to their archaeal diversity [2, 32]. This is supported by our observations as none of our clones were related to *Salinibacter* [2] that form the majority of the bacterial clones (about 73%) found in a crystallizer pond of another solar saltern. In addition, we did not detect clones related to *Rhodobacteriales* belonging to α-*Proteobacteria* that have been detected in endoevaporite microbial communities [2, 32]. Moreover, we identified several clones (T28, e9, e3, and e4) that have not been found in hypersaline environments previously.

Relative Abundance of *Archaea* and *Bacteria*

To analyze the relative abundance of *Archaea* and *Bacteria* in our solar saltern, we determined the 16S rRNA gene copy number by real-time PCR. A dilution series of representative sequences of the archaeal clone A3 and the bacterial clone T3 served as the calibration standards. Universal primer sets for the *Archaea* and *Bacteria* domains designated as 320F (5'-ACTCCTACGGGAGGCAGCAG-3') and 528R (5'-ATTACCGCGGCTGCTGG-3') were used [26]. Real-time PCR (50 μl) was performed by using Thermo-fast 96 PCR plates (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and SYBRGreen (Molecular Probes, Eugene, OR, U.S.A.) on an iCycler IQ thermocycler (Bio-Rad Laboratories). Each measurement was performed three times. Based on the standard curve, the threshold cycle measured in a sample was converted to the copy number of the gene in the sample.

Analyses of the diversity of hypersaline environments whose sodium chloride concentrations are close to saturation have generally concentrated on the halophilic members of the *Archaea* domain. However, a recent analysis by Anton *et al.* [1] has revealed that bacteria may also be ecologically important members in these ecosystems, since about 5–25% of all the cells in a saltern were found to be bacteria by fluorescence *in situ* hybridization. To test this notion, we determined the relative abundance of the bacteria and archaea in our saltern by real-time PCR. The copy number of the bacterial 16S rRNA gene was about 50% of the archaeal 16S rRNA gene copy number. Although exact quantification would require determination of the 16S

rRNA gene copy numbers in archaeal and bacterial genomes, these data nevertheless suggest that bacteria probably constitute a significant component of the ecosystem in our solar saltern. Thus, our data support the notion forwarded by Anton *et al.* [1] that it is important to analyze bacterial diversity and determine its role if we are to fully understand the ecology of hypersaline environments.

In summary, we studied the diversity of the archaea and bacteria found in crystallizer ponds of a solar saltern in Korea by using a culture-independent molecular technique based on the 16S rRNA gene. This is the first report that has studied the diversity of the archaea and bacteria found in crystallizer ponds of a solar saltern in Korea by a culture-independent method. Our results indicate that the composition and diversity of the microbial community in solar salterns found on the coast of the Yellow Sea may differ from those in solar salterns located in other geographic regions.

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