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Phylogenetic Diversities and Community Structure of Members of the Extremely Halophilic *Archaea* (Order *Halobacteriales*) in Multiple Saline Sediment Habitats

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We investigated the phylogenetic diversity and community structure of members of the halophilic *Archaea* (order *Halobacteriales*) in five distinct sediment habitats that experience various levels of salinity and salinity fluctuations (sediments from Great Salt Plains and Zodletone Spring in Oklahoma, mangrove tree sediments in Puerto Rico, sediment underneath salt heaps in a salt-processing plant, and sediments from the Great Salt Lake northern arm) using *Halobacteriales*-specific 16S rRNA gene primers. Extremely diverse *Halobacteriales* communities were encountered in all habitats, with 27 (Zodletone) to 37 (mangrove) different genera identified per sample, out of the currently described 38 *Halobacteriales* genera. With the exception of Zodletone Spring, where the prevalent geochemical conditions are extremely inhospitable to *Halobacteriales* survival, habitats with fluctuating salinity levels were more diverse than permanently saline habitats. Sequences affiliated with the recently described genera *Halogranum*, *Halolamina*, *Haloplanus*, *Halosarcina*, and *Halorientalis*, in addition to the genera *Halorubrum*, *Haloferax*, and *Halobacterium*, were among the most abundant and ubiquitous genera, suggesting a wide distribution of these poorly studied genera in saline sediments. The *Halobacteriales* sediment communities analyzed in this study were more diverse than and completely distinct from communities from typical hypersaline water bodies. Finally, sequences unaffiliated with currently described genera represented a small fraction of the total *Halobacteriales* communities, ranging between 2.5% (Zodletone) to 7.0% (mangrove and Great Salt Lake). However, these novel sequences were characterized by remarkably high levels of alpha and beta diversities, suggesting the presence of an enormous, yet-untapped supply of novel *Halobacteriales* genera within the rare biosphere of various saline ecosystems.

Members of the halophilic *Archaea* (order *Halobacteriales*) are a physiologically and phylogenetically distinct group of microorganisms, characterized by their obligate halophilic lifestyle, aerobic heterotrophic metabolism, and characteristic red coloration (48). The development of red coloration in hypersaline brines is a phenomenon that was observed thousands of years ago (50). Studies in the early 20th century identified the cause of this coloration and described multiple halophilic archaeal isolates (50). The realization that these organisms are members of the *Archaea* came in 1978 (41). Since then, various *Halobacteriales* species have been used as model microorganisms for understanding physiological and structural adaptations to living in hypersaline conditions and for understanding basic cellular processes in *Archaea* (33).

The phylogenetic diversity and community structure of members of the *Halobacteriales* have been investigated in various thalassohaline (e.g., crystallizer ponds in solar salterns [3, 42, 45]) and athalasohaline (e.g., the Dead Sea and soda lakes [46]) water bodies. In such hypersaline habitats (>25% NaCl), members of the *Halobacteriales* often represent the dominant microbial fraction observed, and hence their diversity could readily be examined using general archaeal 16S rRNA gene primers. Collectively, these studies have indicated that while low overlap between communities in seemingly similar habitats (e.g., solar salterns) is often observed, *Halobacteriales* diversity in such habitats is often limited (3, 8, 45), with a single or few phylotypes often representing the majority of the *Halobacteriales* community within such ecosystems. Further, while 16S rRNA gene sequences belonging to putatively novel *Halobacteriales* genera have been identified in some of these studies (see, e.g., reference 45), the majority of halophilic *Archaea* identified in such habitats belong to various well-characterized *Halobacteriales* genera, e.g., *Halorubrum*, *Haloferax*, and *Haloquadratum* (3, 8, 45).

Nevertheless, recent culture-dependent and -independent studies have indicated that the scope of diversity within the order *Halobacteriales* is much broader than implied from the study of typical hypersaline water bodies. This is manifested by the detection and isolation of *Halobacteriales* in saline habitats where relatively lower salinity levels, steep geochemical gradients, and recurrent spatiotemporal fluctuations allow for the coexistence of members of the order *Halobacteriales* as a small fraction of the larger halophilic prokaryotic community. Examples of such habitats are naturally occurring and man-made saline soils, e.g., salt plains and alpine salt sediments (10, 54), soils adjacent to salt mines and salt-processing plants (44), traditional Asian salted and fermented seafood products (e.g., jeotgal) (57), deep-sea saline anoxic basins (1), and marine sponges (38). Further, multiple research groups have detected (24, 43) and subsequently isolated

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(25, 29, 35, 53, 58, 59) *Halobacteriales* strains from ecosystems with low salinities (1 to 3.5% NaCl), where they appear to possess exceptional survival capabilities and/or are capable of exploiting niches of relatively higher salinities created due to temporal and spatial variations in geochemical conditions in such ecosystems.

Surprisingly, in spite of the sustained interest in *Halobacteriales* diversity and ecology, evidence of the occurrence of multiple yet-uncultured *Halobacteriales* genera, and the phylogenetically coherent nature of the group, development of *Halobacteriales*-specific primers has not been widely applied in diversity surveys. To our knowledge, only two studies reported on the utilization of *Halobacteriales*-specific primers: (i) an investigation of the occurrence of *Halobacteriales* spp. in the human colon, where an archaeal 16S rRNA gene PCR product was used in a nested PCR to detect the presence of a single *Halobacteriales* sequence in human colonic mucosal biopsy specimens (51), and (ii) methodological report that provided a list of potential *Halobacteriales*-specific primers for possible utilization in community profiling using length heterogeneity PCR (LH-PCR) (39). The development and utilization of *Halobacteriales*-specific primers would especially be beneficial in investigating habitats where the *Halobacteriales* represent only a fraction of the archaeal population (24, 38, 57, 61). Further, such primers could be utilized in a high-throughput pyrosequencing approach to identify members of the community present in low abundance and hence could allow for more complete coverage of *Halobacteriales* communities in various ecosystems.

In this study, we designed *Halobacteriales*-specific 16S rRNA gene primers and utilized such primers to conduct a high-throughput pyrosequencing survey of five distinct permanently and transiently saline sediment habitats. We opted for sediment and soil samples rather than samples from water bodies due to the relative lack of information regarding *Halobacteriales* diversity in saline soils and sediments and the inherent environmental heterogeneity and hence potential higher diversity in such samples than in the fairly well studied hypersaline water bodies (48). Our results document the high level of diversity within all examined habitats, demonstrate the prevalence of several recently isolated and poorly characterized *Halobacteriales* genera in multiple habitats, indicate that *Halobacteriales* communities in sediments exhibit higher diversity than and a distinct microbial community structure compared to communities from hypersaline water bodies, and identify a remarkable level of novel genus-level diversity within all examined habitats.

MATERIALS AND METHODS

Samples. Sediment and soil samples were obtained from five different locations: (i) Topsoil crust from the Great Salt Plains in north-central Oklahoma (SPL sample); (ii) soil samples underneath saline crystals at a salt-manufacturing plant in Freedom, OK (CAR sample); (iii) top sediments from crusts at the banks of Zodletone Spring, a shallow, surficial, sulfide- and sulfur-rich spring in southwestern Oklahoma (ZDT sample); (iv) sediments from the base of a mangrove tree at the inlet of a small cove at Cabo Rojo, PR (MAN sample); and (v) sediment samples from Rozel Point within the northern arm of the Great Salt Lake in Utah (GSL sample). These sites experience various levels of salinity and salinity fluctuations, ranging from the permanently saline GSL and CAR samples and the predominantly saline SPL sample, which experience infrequent salinity fluctuations, to sites that experience frequent, continuous fluctuations in salinity levels on a daily or even hourly basis (MAN and ZDT).

The Great Salt Plains (SPL) sample was obtained from the top centimeters of the unvegetated salt crusts occurring approximately 0.6 m from a small (1.5 m in diameter) hypersaline water pond located on the western edge of the Salt Plains National Wildlife Refuge in north-central Oklahoma (latitude, +36.75; longitude, −98.15). The SPL are barren sandy mud flats covering 65 km^2 that are crusted with evaporite salt deposits derived from briny aquifers (10). The pH of the sample was 8.5, and the bulk sediment salinity, measured using a refractometer (model S-10; Atago Co. Ltd., Tokyo, Japan) by suspending and shaking sediments in an equal volume of water, was 8%. SPL sediments are permanently covered by salty surface deposits, except in the event of a rainfall in the area (the maximum annual rainfall is 115.3 cm, with 78.6 cm falling in 2010 and 11.2 cm falling in the month of sampling), which drastically dilutes salt deposits and lowers the salinity of that area. Salinity afterwards is naturally and gradually restored by evaporation. Samples were obtained on 21 July 2010 on a dry day, with the prior rainfall event (1.9 cm) occurring on 15 July 2010. Therefore, this predominantly, but not permanently, saline environment could be regarded as a poikiloenvironment, where the unpredictable and sporadic rainfall events cause unexpected and drastic changes in salinity (10).

Samples from Zodletone Spring (ZDT sample) were obtained in June 2010 from the top 2 cm of the banks of Zodletone Spring, a sulfide- and sulfur-rich spring in the Anadarko foothills in southwestern Oklahoma (latitude, +34.996; longitude, −98.689). Spring water has a fairly constant temperature of 22°C, a pH of 7, and a salinity of 1%. In spite of this low salinity, members of the *Halobacteriales* have been identified as a fraction of the archaeal community in a previous survey of archaeal diversity in the spring banks (24). Salinity and depth vertical profile measurements have indicated that as spring water with low salinity diffuses to the banks of the stream, evaporation results in a decrease in moisture and an increase in salinity at the top soil layers, resulting in the creation of a microenvironment with higher salinity (24). Subsequently, multiple novel genera and species have been isolated from that location (25, 58, 59), some of which possess an exceptional ability to survive at low salt concentrations (58). As such, Zodletone Spring represents a moderate environment, well suited for the growth of mesophiles and extremely inhospitable to halophiles and where *Halobacteriales* survive in a distinct, narrow microenvironment within the top layers of the spring bank, which is constantly being diluted through the flowing spring water to the banks. Samples analyzed in this study had a pH of 7 and a bulk salinity of 7.5%.

Mangrove samples (MAN samples) were collected from sediments underneath the base of a mangrove tree situated at a cove entrance in Cabo Rojo, PR, in June 2010 (latitude, +18.11; longitude, −67.18). Samples were collected during low tides. The collected sediment sample had a bulk salinity of 1.5% and a pH of 7.5 at the time of sampling, and the water samples overlying these sediments had a salinity of 7%. However, these values frequently change daily or even hourly, depending on the time of the day (and hence the temperature, level of sun exposure, and tide). Mangrove trees are known to be present in saline coastal tidal areas. Seawater is brought and trapped in high tide. When the tide recedes, evaporation of the seawater in the soil leads to increases in water and sediment salinities. The return of tide restores the salinity level back to that of seawater. Therefore, of all samples analyzed in this study, the microbial community in mangrove sediments experiences the most frequent oscillations in salinity. The presence of members of the order *Halobacteriales* on leaves of mangrove trees has previously been reported (55), but to our knowledge, no detailed culture-dependent or -independent analysis of *Halobacteriales* in mangrove tree sediments has previously been reported.

Salt-manufacturing plant (CAR) samples were obtained from a salt-manufacturing plant in the town of Freedom, located in Woods County in northwestern Oklahoma (latitude, +36.77; longitude, −99.11). Samples were obtained on 21 July 2010 by scraping the top 1 to 2 cm of salt crust-overlaid soil samples located at the edges of a small, slowly flowing water stream (1.5m wide) which flows from a man-made water pond past mined salt heaps in the plant's courtyard. The stream is always saturated with NaCl, and stream water exhibited the characteristic red coloration asso-

ciated with *Halobacteriales* blooms. Soil samples had a bulk salinity of 7%, and a pH of 7.5. The sampled location is permanently covered by salt crust year round. Rainfall events dissolve salt crystals from the salt heaps into the water stream, which in turn deposits the dissolved salt into the sampled area. Therefore, rainfall events do not dilute the salinity at the sampled location, and the sampled soils are thus permanently saline.

Great Salt Lake (GSL) samples were obtained from Rozel Point (latitude, +41.44; longitude, −112.67) at the northern edge of the northern arm of the Great Salt Lake in Utah in March 2008 and were kindly provided by Babu Fathepure (Oklahoma State University). Lake waters in the northern arm of the Great Salt Lake have had a fairly constant salinity of 25 to 28% for the past 15 years (5). The sampled sediments had a bulk salinity of 10.0% and a pH of 9.5.

Primer design. To design primers specific to the order *Halobacteriales*, 147 16S rRNA gene sequences representing all known *Halobacteriales* genera and species (as of July 2011) were aligned in Clustal X (37), and alignments were inspected to identify conserved regions within *Halobacteriales* 16S rRNA genes. Multiple primers were designed and checked for specificity and coverage using the RDP probe check function within the RDP Web interface (13), as well as by BLAST searches against the NCBI nr database (36). Degeneracy was introduced to expand coverage without sacrificing specificity when appropriate. Three primer pairs 682F-1199R, 287F-958R, and 287F-589R were chosen for experimental validation due to their high coverage (96.3%, 80.3%, and 97.8% of *Halobacteriales* sequences in the RDP database, respectively) and specificity (0, 0, and 6 nontarget sequences in the current collection of near-complete 16S rRNA prokaryotic sequences in RDP release 10, update 27, as of August 2011). Primer pairs 682F (5′-GGGTAGGAGTGAAATCCY-3′)-1199R (5′-YHC ATTCGGGGCATRCTG-3′), 287F (5′-AGGTAGACGGTGGGGTAAC-3′)-958R (5′-YCCGGCGTTGAMTCCAATT-3′), and 287F-589R (5′-RG CTACGRACGCTTTAGGC-3′) were experimentally evaluated using pure cultures of *Haloferax sulfurifontis* strain M6^T and *Haladaptatus paucihalophilus* strain DX253^T, as well as environmental DNA from Zodletone Spring. PCR products of the expected sizes were cloned using the TOPO-TA cloning kit (Invitrogen Corp., Carlsbad, CA), and 12 clones from each PCR were Sanger sequenced. In spite of its *in silico* specificity, primer pair 682F-1199R yielded only two *Halobacteriales*-affiliated sequences, while 10 sequences were distantly related (89 to 92%) to the archaeal order *Thermoplasmatales* (data not shown). On the other hand, primer pairs 287F-958R and 287F-589R yielded a diverse collection of exclusively *Halobacteriales*-affiliated sequences (data not shown). Primer pair 287F-589R was chosen for this study due to the suitability of amplicon size for pyrosequencing technology.

DNA extraction, PCR amplification, and pyrosequencing. DNA was extracted from all samples using the FastDNA spin kit for soil (MP Biomedicals, Solon, OH). The extracted DNA (1:10 dilution) was used as a template in PCRs that contained modified 287F and 589R primers. The forward primer was constructed by adding the 454 Roche adapter A to the 287F primer as previously described (31). The forward primer also contained a unique bar code sequence that was used to distinguish sediment samples from one another (31). The reverse primer was constructed by adding the 454 Roche adapter B to the 589R primer. PCR was performed in 50-μl reaction mixtures that contained 2 μl of the extracted DNA, 1× PCR buffer (Promega, Madison, WI), 2.5 mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphate (dNTP) mixture, 0.5 U of the GoTaq flexi DNA polymerase (Promega, Madison, WI), and 10 μM (each) forward and the reverse primers. PCR was carried out according to the following protocol: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. A final elongation step at 72°C for 10 min was included. All samples were PCR amplified in quadruplicate. The resulting PCR products of the expected size were gel purified using QIAquick gel extraction kit (Qiagen Corp., Valencia, CA). Equal amounts of all purified PCR products were pooled in a 1.7-ml microcentrifuge tube to give a total of 3 to 5 μg of DNA. These pooled PCR products were sequenced at the University of South Carolina Engencore Facility using Titanium technology.

Sequence analysis. Sequence quality control was handled in MOTHUR (60) as described previously (66). Briefly, sequences with an average quality score of below 25, sequences that did not have the exact primer sequence, sequences that contained an ambiguous base (N), sequences having a homopolymer stretch longer than 8 bases, and sequences shorter than 80 bp were considered of poor quality and removed from the data set.

High-quality reads from each sample were aligned against the SILVA alignment database available at the MOTHUR website as a template using a Needleman-Wunsch pairwise alignment algorithm. Aligned sequences were then filtered to remove empty columns in all sequences, and filtered alignments were used to generate an uncorrected pairwise distance matrix in MOTHUR. To assign sequences to operational taxonomic units (OTUs), a single-linkage preclustering methodology followed by an average-linkage clustering approach, as recently recommended to prevent overestimation of richness (34), was used. The computationally intensive average-linkage clustering algorithm was conducted on a high-performance computer containing 128 GB of shared addressable RAM and four hex-core Intel Xeon E7530 1.87GHz processors at the Oklahoma State High Performance Computing Center (http://hpc.it.okstate.edu/). Sequences were binned into OTUs at 3% and 6% cutoffs for computing diversity estimates at the species and genus levels, respectively. Rarefaction curve analysis and various species richness (e.g., Chao and ACE [11]) and diversity (e.g., Shannon [12]) estimates were obtained using MOTHUR. Coverage and diversity rankings of various data sets were determined as described previously (30, 40, 67).

For phylogenetic placement, all sequences were queried using the Blastall function of the downloaded NCBI stand-alone BLAST version 2.2.20 against a data set of 147 16S rRNA gene sequences representing the 132 and 38 validly published species and genera, respectively, within the order *Halobacteriales* (as of July 2011, including taxa in press in the *International Journal of Systematic and Evolutionary Microbiology*). A sequence was assigned to a specific genus if it was >94.0% similar to the reference 16S rRNA gene sequence belonging to that genus. While this cutoff is routinely used for sequence assignments at the genus level, we further examined its validity for our data set by examining sequence divergence values between all possible pairs of sequences within validly described *Halobacteriales* species (147 total) using the 16S rRNA gene fragment that would have theoretically been amplified using primer pair 287F-589R. Our results indicate that the majority of all possible pairs of sequences belonging to two different genera have less than 94% sequence similarity (20,318 out of 20.570 possible pairs [98.8%]) and that the majority of all possible pairs of sequences belonging to the same genus have more than 94% sequence similarity (487 out of 593 possible pairs [82.1%]). These results justify the utilization of 94% as a reasonable genus-level cutoff in this study.

We opted for classification using local Blastn searches against an annotated data set of known species and genera rather than automated classification schemes (e.g., using RDP [64], Greengenes [22], or SILVA [available through the MOTHUR package {60}] pipelines) for two main reasons: (i) updates to the lists of genera in these pipelines do not adequately keep up with the rapid pace of description of *Halobacteriales* taxa, with only 28 and 30 *Halobacteriales* genera listed in the RDP and the Greengenes databases, respectively (out of 38 as of August 2011), and (ii) multiple sequences that could be assigned to known *Halobacteriales* genera are often listed as "unclassified *Halobacteriales*" in the Greengenes database. Since the classification is based on identifying the phylogenetic affiliation of the nearest neighbor in the Greengenes database, such an approach would inflate the number of unclassifiable sequences in our data sets. The novelty of rare and abundant members in the different data sets sequenced was examined by plotting the percent divergence of each OTU representative from its closest relative against the number of sequences within this specific OTU as described previously (26).

TABLE 1 Number of sequences, number of observed OTUs at the species and the genus levels, alpha diversity indices, and diversity ranks for the 5 samples studied

Sample	No. of[a]: Sequences		Observed OTUs				Alpha diversity index[b]				Diversity rank[d]
			$OTU_{0.03}$		$OTU_{0.06}$						
	Total	Novel	Total	Novel	Total	Novel	Chao	ACE	Shannon	Coverage[c]	
Mangrove soil	32,677	2,301	2,987	672	678	206	5,924	8,755	5.23	94.8	1
Great Salt Plains	34,766	1,623	2,587	578	582	163	4,826	7,055	5.1	96	2
Great Salt Lake	30,213	2,105	1,971	454	448	146	3,718	5,249	4.56	96.5	3
Salt-processing plant	8,587	281	647	83	175	28	1,225	1,719	3.94	96	4
Zodletone Spring	5,658	142	350	43	102	24	768	1,050	3	96.3	5

[a] Values are for the total and putatively novel sequences and observed OTUs at both the species and the genus levels.
[b] Alpha diversity indices of the total samples.
[c] Coverage refers to Good's coverage.
[d] Numbers refer to the diversity rank of each of the communities, from the most diverse (1) to the least diverse (5). Ranks were obtained by comparing both rarefaction curves (created using the command rarefaction.single in MOTHUR) and diversity ranking curves using the right tail sum method as detailed in reference 67.

Comparing *Halobacteriales* communities across examined habitats. Multiple approaches were used to gauge beta diversity between all five data sets generated in this study. The relative abundances of *Halobacteriales* genera in the five environments studied were used to construct principal-component analysis (PCA) biplots using the R statistical package (56). Shared OTUs between different samples were identified by creating a joint distance matrix of all sequences within all data sets in MOTHUR and using this matrix to generate a shared OTU file (following preclustering and average-neighbor clustering) that shows the proportion of shared versus unique sequences between the data sets compared. This shared OTU file was used to construct Venn diagrams for graphical descriptions of shared OTUs between two, three, four, or five samples. The shared OTU file was also used to conduct pairwise comparisons between every possible pair of samples using both qualitative (those that use presence/absence data, e.g., that of Sørensen [62]) and quantitative (those that take OTU abundance or relative abundance into consideration, e.g., that of Bray and Curtis [7]) similarity indices. A similar shared OTU file was created for the putatively novel members of the community from all 5 environments studied. The novel shared OTU file was not used to construct Venn diagrams and calculate pairwise similarity indices (as explained above for the total communities). This shared file also was used as an input for constructing phylogenetic trees to highlight the phylogenetic positions of selected novel lineages. Three sequence representatives of the 20 most abundant shared OTUs ($n > 50$) were aligned with closely related database relatives in Clustal X (37) using default parameters. These OTUs were either unique to one community (6 out of the 20 OTUs) or were shared between 2 (8 OTUs), 3 (2 OTUs), 4 (3 OTUs), or 5 (1 OTU) environments. Evolutionary trees were constructed using PAUP (version 4.01b10; Sinauer Associates, Sunderland, MA), with the appropriate distance substitution model determined using jMODELTEST 0.1.1 (52).

Comparison of diversity of *Halobacteriales* in examined sediments to their diversity in typical hypersaline water bodies. We reexamined the community structure and species richness estimates obtained from four previously studied crystallizer ponds and compared them to estimates obtained in subsamples of our data sets with comparable number of sequences. We chose crystallizer ponds as representatives of hypersaline water bodies since they have always been regarded as the model thalassohaline environments for studying *Halobacteriales* ecology and also due to the availability of multiple 16S rRNA gene data sets from crystallizer ponds from multiple distinct geographical locations. In contrast to sediments, these ponds often have high, nonfluctuating levels of salinities ranging from 30 to 35% (49). Sequences from a crystallizer pond (32% salinity) located within a multipond solar saltern in Sfax, Tunisia (2), and three saltern crystallizer ponds from three distant geographical locations in Australia, each having a salinity of 34% (45), were obtained from GenBank. The number of sequences from these studies ranged between 40 and 78 (average, 54). To match the lengths of the pyrosequences obtained in the current study, each downloaded sequence was truncated using JALVIEW (65) at the forward and reverse primers (287F-589R) to simulate the pyrosequenced fragments in size. Moreover, since the number of sequences analyzed in these studies is much lower than that of those analyzed in the current pyrosequencing study, we randomly extracted five subsamples (of 54 sequences each) from each of the five environments studied. For each of these subsamples, as well as for each of the truncated sequences from the hypersaline data sets, phylogenetic diversity and diversity estimates were obtained as described above. Species richness estimates (observed number of species and Chao and Shannon indices) were compared between the group of permanently hypersaline samples and that of the predominantly saline or fluctuating-salinity samples using the Student *t* test. Values were considered significantly different if the *P* value associated with the *t* test was <0.005 (equivalent to an α value of 0.05 corrected for 10 pairwise comparisons using the Bonferroni correction). Relative abundances of *Halobacteriales* genera in the nine environments were used in a principal-component analysis to evaluate the differences in species composition between the environments compared, and results were shown in a three-dimensional (3D) scatter plot constructed using the R statistical package (56).

RESULTS

Diversity patterns in *Halobacteriales* communities. A total of 144,957 sequences were obtained from all five data sets. After implementation of quality control measures, 111,901 sequences were included in subsequent analysis. Details on the number of sequences, the numbers of $OTUs_{0.03}$ and $OTUs_{0.06}$, and various diversity estimates are shown in Table 1. Surprisingly, extremely diverse *Halobacteriales* communities were observed in all habitats, with between 350 (ZDT) and 2,987 (MAN) OTUs identified at the putative species level and 102 (ZDT) to 678 (MAN) OTUs identified at the putative genus level. Various diversity estimates, as well as sample size-independent diversity ranking approaches (67), indicated that mangrove sediments, which exhibit the most frequent salinity fluctuation events, harbor the most diverse *Halobacteriales* community, followed by the poikiloenvironment Great Salt Plains. The permanently saline Great Salt Lake and CAR saline sediments harbored lower diversity than mangrove and Great Salt Plains, and the Zodletone Spring *Halobacteriales* community was the least diverse (Table 1). Although Zodletone Spring is an environment with frequent salinity fluctuation, it is also ex-

tremely inhospitable to members of the *Halobacteriales*, since, as described in Materials and Methods, they can grow only within the top layers of the spring bank, a spatially confined environment that is constantly being diluted by the flowing of spring water to its banks.

Genus-level community patterns. In general, members of the *Halobacteriales* in all ecosystems examined showed an extremely high overall genus-level diversity, with sequences affiliated with all 38 currently recognized genera (as of August 2011, including genera in press the *International Journal of Systematic and Evolutionary Microbiology*) identified in at least one data set. The number of *Halobacteriales* genera identified within each environment ranged between 28 (in Zodletone) and 37 (in the Great Salt Plains sample), with sequences affiliated with 26 different genera being encountered in all five data sets.

Detailed analysis of the relative abundances of sequences affiliated with various *Halobacteriales* genera (Table 2) revealed a distinct pattern in all data sets, where only a few (between 3 and 7) different genera were present in relatively high abundance (e.g., more than 5%), followed by a longer list of genera that represented a minor fraction (e.g., 0.1 to 5%) of the community or were extremely rare (e.g., less than 0.01%). Surprisingly, members of several newly described genera were exceptionally abundant in multiple habitats: members of the genera *Halogranum*, *Halolamina*, *Halorientalis*, *Haloplanus*, and *Halosarcina*, in addition to members of the genera *Haloferax*, *Halorubrum*, and *Halobacterium*, were identified as abundant members (>5%) of the community in multiple environments. Sequences affiliated with these five recently described genera constituted 66.5% of sequences in the ZDT data set, 60.7% of sequences in the MAN data set, and 49.4%, and 41.1% of sequences in the SPL and the CAR samples, respectively. On the other hand, sequences affiliated with these five genera represented only 14.0% of sequences in the GSL sample.

(i) *Halogranum*. Sequences affiliated with members of the recently discovered genus *Halogranum* (15) were among the most abundant in four out of five data sets, representing over 1/2 of sequences in ZDT, as well as approximately 1/3, 1/5, and 1/10 of sequences in MAN, CAR, and SPL, respectively. While the type species, *Halogranum rubrum*, has been isolated from sediments of marine solar salterns on the coastline of eastern China (15), earlier isolates from relatively low-salinity habitats, e.g., Zodletone Spring (K. N. Savage et al., unpublished data) and Colne estuary in Essex, United Kingdom (referred to as *Halobacteriales* Gp2 in reference 53), have been reported. Collectively, these studies suggest that members of the genus *Halogranum* are among the most successful genera in withstanding events of transient low salinities and adjusting to salinity fluctuations. Physiological characterization of *Halogranum rubrum*, *H. gelatinilyticum*, and *H. amylolyticum* (15, 19), as well as strains isolated from Zodletone Spring (Savage et al., unpublished data), indicated that although optimal growth occurs at relatively high salinities (3.4 to 3.9 M NaCl), growth could also be observed at relatively lower salinities (e.g., 1.4 and 1.7 M NaCl for *Halogranum amylolyticum* and *Halogranum gelatinilyticum*, respectively). Although cells of all described *Halogranum* spp. immediately lyse in distilled water (15, 19), the prevalence and distribution of *Halogranum* described here suggest possession of a yet-unknown mechanism for resisting lower salt and salinity fluctuations.

(ii) *Haloplanus*, *Halolamina*, *Halorientalis*, and *Halosarcina*. In addition to the genus *Halogranum*, members of the recently described genera *Halolamina*, *Halorientalis*, *Haloplanus*, and *Halosarcina* represented a major fraction (>5%) of the *Halobacteriales* community in multiple habitats as well (Table 2). The first *Haloplanus* species discovered (*H. natans*) was isolated from experimental mesocosms where hypersaline Dead Sea waters were diluted with the less-saline Red Sea water (80:20 by volume) (4). Under such conditions, colonies of *Haloplanus* species could be obtained on plates alongside *Halorubrum* colonies. Subsequently, two more *Haloplanus* species were isolated from sediments obtained from an artificial marine saltern in China (14, 16). In the current study, sequences affiliated with the genus *Haloplanus* were abundant (>5%) at higher numbers in sediments from the Great Salt Plains and mangrove. Therefore, it appears that while members of the genus *Haloplanus* represent a small fraction in hypersaline, salt-saturated habitats, they are more adapted to saline environments with subsaturation levels of NaCl that experience

TABLE 2 Percentages of the 38 known *Halobacteriaceae* genera encountered in the samples studied

Genus	% in sample[a]				
	SPL	MAN	GSL	CAR	ZDT
Halolamina	14.1	6.1	1.5	2.0	5.8
Halomicrobium	10.9	2.5	2.7	1.3	1.7
Halorubrum	10.6	2.7	24.1	12.7	0
Halorientalis	9.7	7.3	7.6	3.6	1.6
Haloplanus	8.9	12.8	1.2	2.7	1.1
Halosarcina	8.6	2.8	0.67	10.9	2.0
Halogranum	8.1	31.7	3.0	21.9	56.0
Halorhabdus	4.1	1.2	10.3	0.20	0.05
Halobaculum	3.0	3.3	0.58	1.3	3.9
Halonotius	2.5	0.65	6.7	0.77	0.02
Halobellus	2.3	1.1	0.34	0.63	1.5
Halostagnicola	2.2	0.3	0.59	0.05	0
Halomarina	1.6	1.41	0.007	0.56	0.05
Halopelagius	1.2	0.43	0.05	19.4	0.53
Natronomonas	1.2	6.5	12.8	0.78	0.51
Halobacterium	0.94	0.85	7.7	5.1	3.7
Haloarcula	0.87	0.49	0.18	0.19	0.14
Natronorubrum	0.67	0.76	0.19	1.7	0.32
Haloferax	0.61	6.7	0.55	5.8	17.1
Natronolimnobius	0.56	0.23	2.3	0.15	0.09
Halobiforma	0.51	0.58	3.0	0.06	0
Halococcus	0.47	0.49	0.07	0.18	0.14
Haloterrigena	0.43	0.04	0.01	1.3	0.05
Natrialba	0.33	0.009	0.007	0	0
Salarchaeum	0.31	0.20	2.8	1.9	0.09
Halosimplex	0.24	0.05	0.10	0.01	0.02
Natronococcus	0.12	0.01	0.06	0	0
Natrinema	0.09	0.14	0.73	0.63	0.12
Haladaptatus	0.08	0.21	0.003	0.08	0.12
Haloquadratum	0.06	0.25	0.40	0	0
Haloarchaeobius	0.04	0.60	2.4	0.86	0.87
Halalkalicoccus	0.03	0.09	0.003	0	0.02
Natronoarchaeum	0.03	0.003	0.19	0.06	0.04
Halogeometricum	0.006	0.17	0	0	0
Halovivax	0.006	0.12	0	0	0
Halopiger	0.006	0.034	0.03	0	0
Natronobacterium	0	0.003	0	0	0
Halarchaeum	0	0	0.007	0	0
Novel	4.7	7.0	7.0	3.3	2.5

[a] CAR, CAR salt plant; GSL, Great Salt Lake; SPL, Great Salt Plains; MAN, mangrove tree sediment; ZDT, Zodletone Spring sediment.

various levels of salinity fluctuations. However, unlike members of the genus *Halogranum*, *Haloplanus* spp. do not appear to be well adapted to low-salinity habitats such as Zodletone Spring (Table 2). The genus *Halolamina* contains only one described species, which has recently been isolated from an artificial marine saltern in China (17). *Halolamina*-associated sequences were most abundant in sediments from the Great Salt Plains, mangrove, and Zodletone Spring, suggesting their adaptability to survive in environments with various degrees of salinity fluctuations (Table 2). Similar to the case for the genus *Halolamina*, only one species of the genus *Halorientalis* has been described from Chinese saline salterns (20). *Halorientalis*-affiliated sequences were abundant members (>5%) of the Great Salt Plains, mangrove, and Great Salt Lake sediments (Table 2). Finally, members of the genus *Halosarcina* were originally isolated from Zodletone Spring (59) and subsequently identified in Chinese salterns (21). *Halosarcina*-affiliated sequences, however, represented only 2% of the *Halobacteriales* community at Zodletone Spring (Table 2) but were present in relatively high abundance in SPL and CAR samples.

(iii) *Haloferax*, *Halorubrum*, and *Halobacterium*. In addition to the above-mentioned newly described genera, members of the genera *Haloferax*, *Halorubrum*, and *Halobacterium* have been observed as dominant members of the community in multiple habitats. Members of the genus *Haloferax* were present at >5% in CAR and MAN samples and at up to 17% in ZDT sample (Table 2). Members of the genus *Haloferax* are widely distributed in nature, ranging from salt-saturated habitats to atypical low-salinity environments, e.g., Zodletone Spring (24, 25). In addition, members of the genus *Haloferax* are often versatile, fast-growing microorganisms that are easily obtained in pure cultures (8) and have a wide range of substrate utilization and catabolic capabilities (27). They are relatively tolerant to low-salt conditions, and cells remain intact even at low (0.5 M) NaCl concentrations (23). This capability to survive in a wide range of saline ecosystems is evident by their abundance in the permanently saline (CAR) as well as the low-salinity (ZDT) samples in this study. Therefore, it appears that factors other than salinity levels determine the success and prevalence of *Haloferax* spp. in saline environments. Members of the genus *Halorubrum* were most abundant in the Great Salt Lake sample, where *Halorubrum*-affiliated sequences represented 24% of the entire *Halobacteriales* community, as well as in the SPL and CAR samples (Table 2). The abundance of *Halorubrum* species in a permanently saline environment and in a predominantly saline environment is in agreement with the observation that members of the genus *Halorubrum* are among the most prevalent genera at saturation and near-saturation salinities (47). Indeed, *Halorubrum* species appear to be prevalent in many salt-saturated crystallizer ponds (6, 8, 28, 32, 44, 45), which explains the prevalence of members of this genus in the Great Salt Lake (pH 9.5). On the other hand, members of the genus *Halorubrum* appear to be outcompeted in low- and fluctuating-salinity environments, as evident by their lower abundance in mangrove sediments and complete absence in Zodletone Spring *Halobacteriales* communities (Table 2). Finally, members of the genus *Halobacterium* are typically present in high-salinity environments, e.g., salterns, saline lakes, and the Dead Sea, and were present at high abundance in permanently saline habitats (CAR and GSL) in this study (Table 2).

(iv) Habitat-specific genera: *Halorhabdus*, *Natronomonas*, and *Halopelagius*. In addition to the widely distributed genera described above, few genera were present in high abundance in only one sample. Within the GSL, members of the genera *Natronomonas* and *Halorhabdus* were in high abundance compared to in other habitats (Table 2). The alkaliphilic nature of the majority, but not all (9), of the *Natronomonas* species could explain the preference of the genus *Natronomonas* for the slightly alkaline Great Salt Lake habitat. The genus *Halorhabdus* has previously also been identified in the southern arm of the Great Salt Lake (63), in addition to in multiple hypersaline anoxic basins at the Red Sea (1), but the ecological and physiological reasons for its preference for such habitats are not well understood. Finally, sequences affiliated with the genus *Halopelagius* represented 20% of the community in CAR but a minor fraction of the community in all other examined habitats (Table 2). Only one *Halopelagius* species is known from sediments of Chinese artificial marine salterns (18). The ecological and physiological reasons behind such an observation remain unclear.

Community comparisons using PCA biplots. The relative abundances of genera encountered in all 5 data sets (with abundances of >1% in at least 3 of the data sets) were used in a principal-component analysis (PCA) to construct a PCA biplot (Fig. 1). The directions of the genus arrows in the biplot are indicative of the respective maximal abundances of the genera; e.g., *Halogranum* abundance is highest in the ZDT data set, and *Halopelagius* abundance is highest in the CAR data set. The lengths of the genus arrows are proportional to the differential abundances of the genera: genera with large differences in their relative abundances in different data sets (e.g., *Halopelagius*, *Halogranum*, *Haloferax*, and *Halorubrum*) have longer arrows than genera with similar relative abundance values in different data sets (e.g., *Halobaculum*, and *Halorientalis*). The results highlight the high level of similarity observed between MAN and ZDT, the two habitats with highly fluctuating salinities, mainly due to shared presence of the genera *Haloferax* and *Halogranum*. The results also highlight the similarity between the highly diverse mangrove and SPL due to shared presence of the genera *Halolamina* and *Haloplanus* and between the slightly alkaline SPL and alkaline GSL habitats due to the shared abundance of the genera *Halorhabdus*, *Halorientalis*, *Halomicrobium*, and *Natronomonas*. The results also highlight the uniqueness of the CAR sample, mainly due to the high abundance of the genus *Halopelagius*. The results of pairwise beta diversity and five-way Venn diagrams (see Fig. S1, Table S1, and text in the supplemental material) further validated the cooccurrence patterns observed using genus-level affiliation PCA biplots (Fig. 1) and provided quantitative estimates of pairwise beta diversity between various habitats.

Putatively novel *Halobacteriales* genera. Sequences that were unaffiliated with any of the identified *Halobacteriales* genera formed a relatively small fraction (from 2.5% in Zodletone to 7.0% in mangrove and Great Salt Lake) of the total number of sequences identified within each data set (Table 2). Within each environment, unclassified sequences clustered into a surprisingly large number of putative novel species ($OTU_{0.03}$), ranging from 43 in ZDT to 672 in mangrove, and putative novel genera ($OTU_{0.06}$), ranging between 24 (ZDT) and 206 (MAN) (Table 1). In general, the majority of novel OTUs in all data sets were present in low abundance, with the most abundant novel $OTU_{0.03}$ in each data set representing from 0.37% (SPL) to 1.66% (ZDT) of the entire community and the most abundant putatively novel genus representing 0.41% (SPL) to 1.8% (GSL) of the entire community. Indeed, within all five data sets, plotting the percent sequence diver-

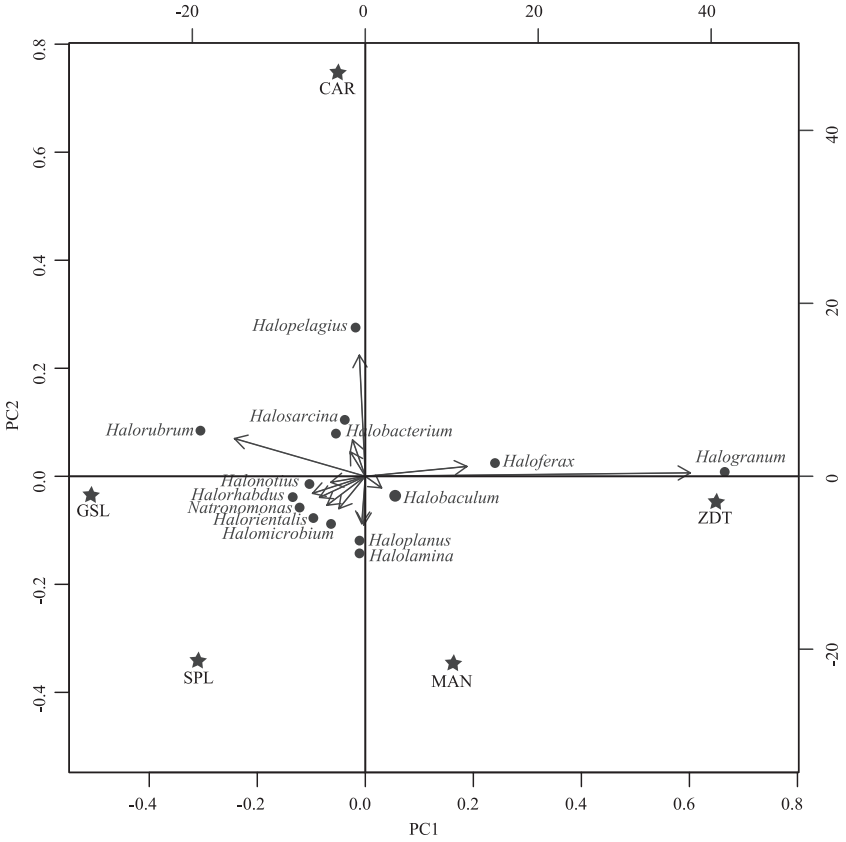

FIG 1 Principal-component analysis biplot of distribution of genera in the 5 studied sites. Samples are represented by stars, and genera are represented by arrows. CAR, salt-manufacturing plant; GSL, Great Salt Lake; SPL, Great Salt Plains; MAN, mangrove tree sediment; ZDT, Zodletone Spring sediment. Only genera that were represented in all 5 samples and with an abundance of >1% in at least 3 of the samples were used in the analysis. Two exceptions were the genera *Halopelagius*, which was added due to its exceptionally high abundance in the CAR sample, and *Halonotius*, which was added due to its relatively high abundance in the Great Salt Lake sample. The arrow directions follow the maximal abundance, and their lengths are proportional to the maximal rate of change between samples.

gence between each $OTU_{0.03}$ in our data set and its closest database relative (on the *y* axis) against the abundance, i.e., number of sequence in that $OTU_{0.03}$ (on the *x* axis) indicates that most of the novel OTUs (i.e., those with more than 6% sequence divergence from their closest relative) are rare, e.g., with fewer than 10 sequences in each OTU (see Fig. S2 in the supplemental material). Thus, it appears that within the relatively small fraction of novel *Halobacteriales* genera identified, a remarkable number of previously undocumented lineages are present, mostly within the rare biosphere of all examined environments.

Similar to the remarkable level of alpha diversity within novel lineages in all five examined habitats, a comparative analysis of the putatively novel sequences across all five environments showed a remarkably high level of beta diversity (i.e., low similarity) between all habitats. Five-way Venn diagrams (Fig. 2) showed that an extremely small number of OTUs have a shared composition: only 1 OTU has sequences from all five environments, and only 5 and 19 OTUs have sequences from four and three environments, respectively (Fig. 2A). Further, within each environment, the percentage of novel sequences that were unique to a specific environment (Fig. 2B to F) was much higher than the percentage of unique sequences belonging in the entire data set (see Fig. S1B to F in the supplemental material): for example, in Zodletone Spring sediment, 19.7% of all novel sequences were unique to Zodletone Spring, while only 4.4% of the total number of sequences in the data were unique to the spring (Fig. 2F). Similarly, the proportions of unique novel sequences versus unique sequences in the entire data set in other communities were 41.8 and 16.6%, 46.8 and18.6%, 58.3 and 40%, and 22.4 and 21.2% in the SPL, MAN, GSL, and CAR data sets, respectively (Fig. 2B to E). Finally, pairwise beta diversity indices between the novel communities of all environments were much lower (i.e., there was a higher level of beta diversity) than those comparing the entire data sets (see Table S1 in the supplemental material).

The 20 most abundant ($n > 50$) putatively novel, shared genera are presented in Table S2 in the supplemental material, and detailed phylogenetic analysis (Fig. 3) confirmed that these genera are unaffiliated with any of the currently described *Halobacteriales* genera. Collectively, these patterns suggest that novel, yet-uncultured *Halobacteriales* genera in saline sediments are not composed of few lineages that are universal and ubiquitous in all environments and that appear to resist isolation using the current approaches. Rather, given the observed high beta diversity between novel lineages identified in all examined environments, it appears that an extremely large number of novel, mostly rare *Halobacteriales* genera are present within the various saline sediments.

Comparing *Halobacteriales* communities in saline sediments and typical hypersaline water bodies. The high level of phylogenetic diversity observed in all five habitats is in stark con-

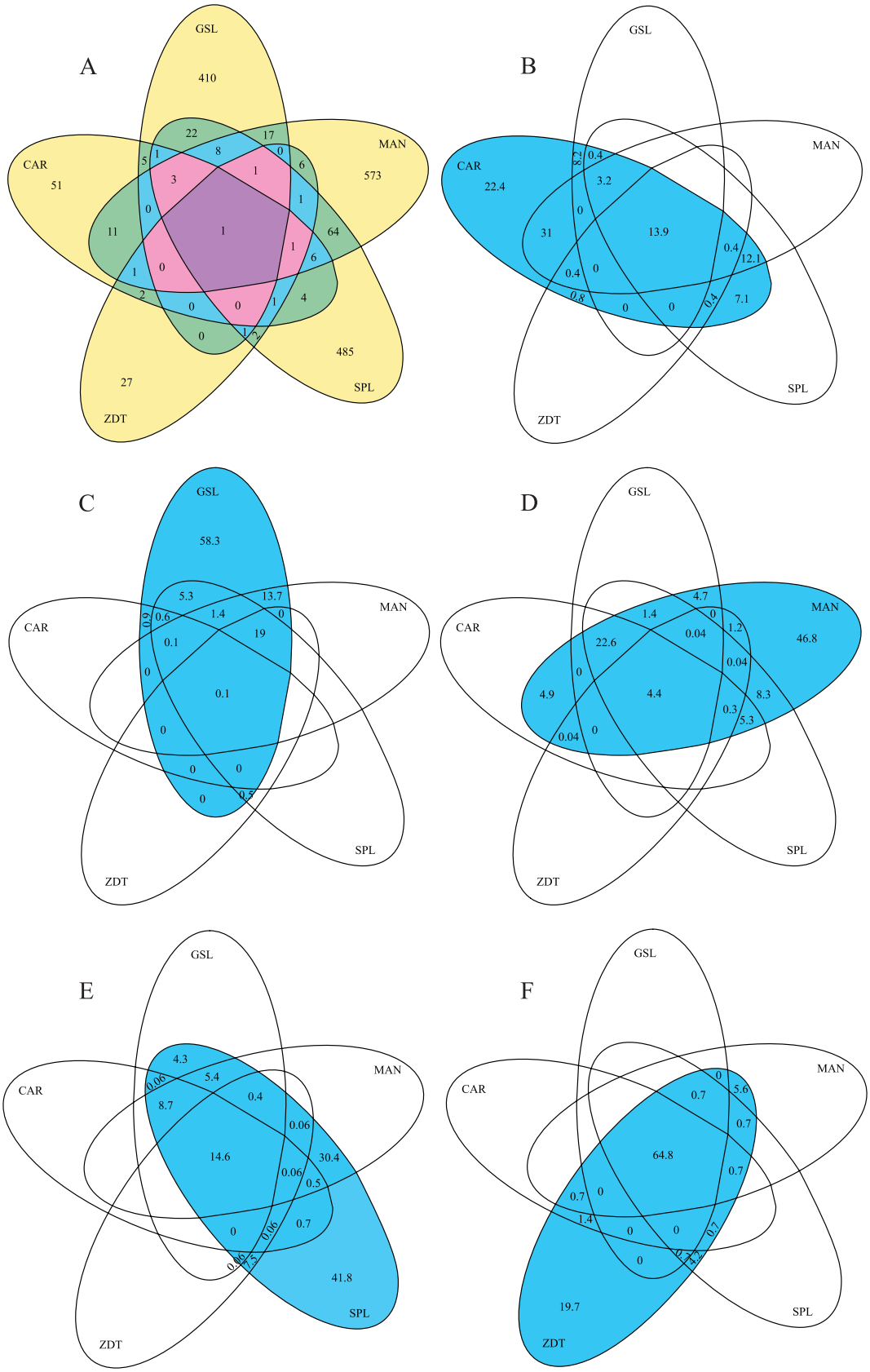

FIG 2 Shared communities between the putatively novel sequences in the 5 communities studied. (A) Color-coded 5-way Venn diagram for the OTUs shared between all data sets. CAR, CAR salt plant; GSL, Great Salt Lake; SPL, Great Salt Plains; MAN, mangrove tree sediment; ZDT, Zodletone Spring sediment. Unshared OTUs are shown in yellow, OTUs shared between only 2 communities are shown in green, OTUs shared between only 3 communities are shown in blue, OTUs shared between only 4 communities are shown in pink, and OTUs shared between all 5 communities are shown in purple. (B to F) Percentages of the total number of putatively novel sequences in each sample that are unique or shared with the other communities for CAR (B), GSL (C), MAN (D), SPL (D), and ZDT (F) samples.

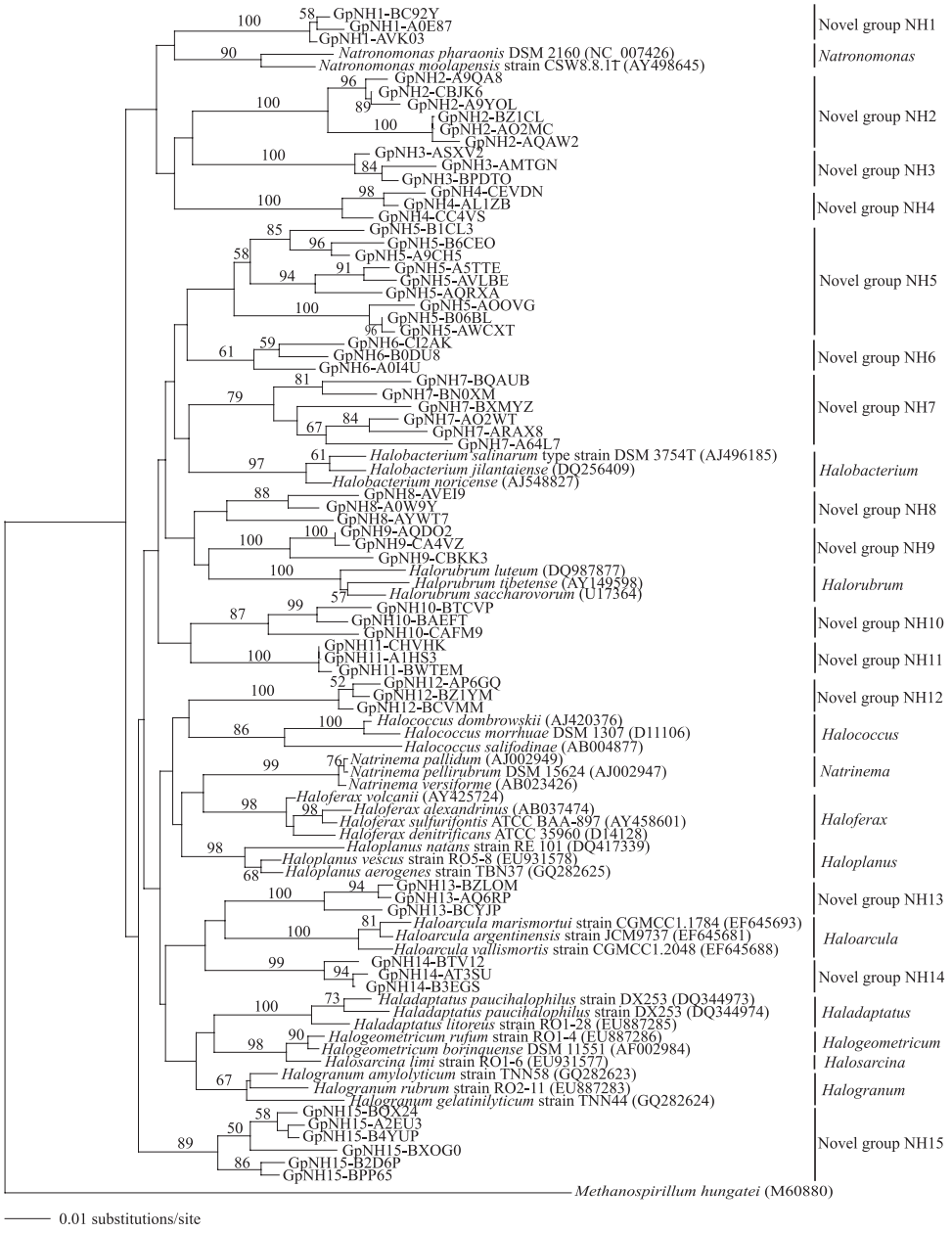

FIG 3 Distance phylogenetic tree based on the 16S rRNA gene sequences affiliated with the 20 most abundant ($n > 50$) putatively novel OTUs from all 5 communities studied. The tree was obtained under a three-parameter model with unequal base frequencies, a proportion of invariable sites of 0.001, and a variable site γ shape parameter of 0.408. *Methanospirillum hungatei* was used as the outgroup. Bootstrap values, in percent, are based on 1,000 replicates and are shown for branches with >50% bootstrap support. Novel groups are labeled NH1 to NH15. Accession numbers for pure culture isolates are shown in parentheses.

trast to the limited phylogenetic diversity observed in prior examination of hypersaline water bodies (3, 6, 42, 45, 46). To determine whether the higher level of diversity observed in the current study is truly a reflection of the ecological diversity of *Halobacteriales* in saline sediment habitats versus hypersaline water bodies and not due to sample size difference or the size of the 16S rRNA gene fragment used in the analysis, we reexamined the phylogenetic diversity and species richness estimates obtained from four different data sets (2, 45) as described above. The results from these environments were compared to phylogenetic diversity and species richness estimates obtained from 5 random subsamples of comparable size from each of the 5 environments of the current study. Alpha diversity estimates indicated that hypersaline environments show a much lower diversity than the current studied environments, and these differences were statistically significant ($P < 0.005$) (Table 3). Further, the genus-level community compositions of the hypersaline environments (see Table S3 in the supplemental material) were clearly different from those obtained from saline sediments in this study, with members of the genera *Haloquadratum* and *Halonotius*, in addition to *Halorubrum*, being

TABLE 3 Comparison of 4 previously studied hypersaline environments to random subsamples of the currently studied communities

Group	Environment	No. of observed OTUs	Chao index	Shannon index
Hypersaline environments[a]	Tunisia	13	13.6	1.93
	Bajool	6	6	1.41
	Cryst7	13	27	1.91
	LDS1	9	12	1.7
Sediment subsamples[b]	CAR	24.6	51.4	2.86
	GSL	30.2	66.3	3.17
	SPL	36.6	81.9	3.44
	MAN	34.6	86.1	3.34
	ZDT	18.2	55.2	2.21
P value[c]		0.0009	0.0001	0.0011

[a] Previously studied hypersaline environments. Tunisia refers to the hypersaline (32% salinity) crystallizer pond in a solar saltern located in Sfax, Tunisia, studied by Baati et al. (3). Bajool, Cryst7, and LDS1 are 3 saltern crystallizer ponds from 3 distant geographical locations in Australia, named as in reference 45. All sequences were truncated to simulate the length of the pyrosequences generated in the current study as described in the text.

[b] CAR, CAR salt plant; GSL, Great Salt Lake; SPL, Great Salt Plains; MAN, mangrove tree sediment; ZDT, Zodletone Spring sediment. For each site, 5 subsamples were drawn and analyzed, and the results shown are the averages of 5 values.

[c] Student *t* test *P* value for assessing significant difference.

the most abundant genera in these crystallizer salterns. The community structures of such salterns (represented by the relative abundances of genera) were compared to the five data sets generated from saline sediments in this study using principal-component analysis. The results (Fig. 4) indicate that the 2 groups of environments are completely divergent, with all the hypersaline environments clustering close together at one end of the plot and all sediment environments except GSL clustering together on the other side. Hence, the observed higher diversity and distinct patterns are a true reflection of the *Halobacteriales* community and are not due to methodological and analytical differences.

DISCUSSION

In this study, we designed and utilized specific primers targeting members of the archaeal order *Halobacteriales* to examine their abundance, phylogenetic diversity, and community structure in five different saline soil and sediment habitats. Our results indicate the following. (i) An extremely diverse *Halobacteriales* community exists within all habitats examined, with the highest level of diversity observed in two habitats (mangrove and Great Salt Plains) that experience various levels of salinity fluctuations. (ii) Saline sediment communities are more diverse than hypersaline water body communities and are characterized by the affiliation of a large fraction of their communities with some of the recently described genera that have a limited number of validly described species and documented isolation habitats, e.g., *Halogranum*, *Halolamina*, *Halorientalis*, *Halosarcina*, and *Haloplanus*. (iii) Sequences that could not be binned to currently existing genera represented a relatively small, yet extremely diverse, fraction of the *Halobacteriales* community within each environment.

Extensive *Halobacteriales* diversity in saline sediments. The utilization of *Halobacteriales*-specific primers allowed for the targeted identification of members of this lineage in various saline environments. This is especially important in saline habitats where the prevailing salinity levels, fine-scale spatial variations in salinity levels, and frequent salinity fluctuations allow for the coexistence of various groups of halotolerant, halophilic, and nonhalophilic microorganisms. Detailed understanding of the phylogenetic diversity and community composition of *Halobacteriales* in such ecosystems is limited, since they often represent a fraction of the total archaeal and prokaryotic population in such environments. The results of the current study indicate that members of the *Halobacteriales* are extremely diverse in such communities, as evident by the observed high number of putative novel genera and species (Table 1) and the high number of *Halobacteriales* genera identified within all environments tested (Table 2). The highest level of phylogenetic diversity was observed in mangrove sediments (Table 2), which experience the highest frequency of temporal salinity and environmental fluctuations on a daily or even hourly basis, followed by the sediments from the poikiloenvironment of the Great Salt Plains. Thus, it appears that environments with salinity fluctuations allow for the coexistence of multiple taxa of *Halobacteriales* and that this constant fluctuation results in a highly dynamic environment where constant promotion and demotion, but not elimination, of various halophilic taxa result in higher diversity than in permanently saline habitats.

Distinct community structure of *Halobacteriales* in saline sediments. The diversity of the microbial communities described in this study is in contrast to the documented relatively limited genus- and species-level diversity observed in prior studies of hypersaline water bodies such as crystallizer salterns and the Dead Sea (3, 6, 42, 45, 46). In such studies, usually an extremely low number of OTUs (usually 3 to 7) are identified in these *Halobacteriales*-dominated environments. Our comparative analysis (Table 3 and Fig. 4) clearly indicates that such differences are a true representation of differences in *Halobacteriales* community structure and are not due to differences in sampling depth, se-

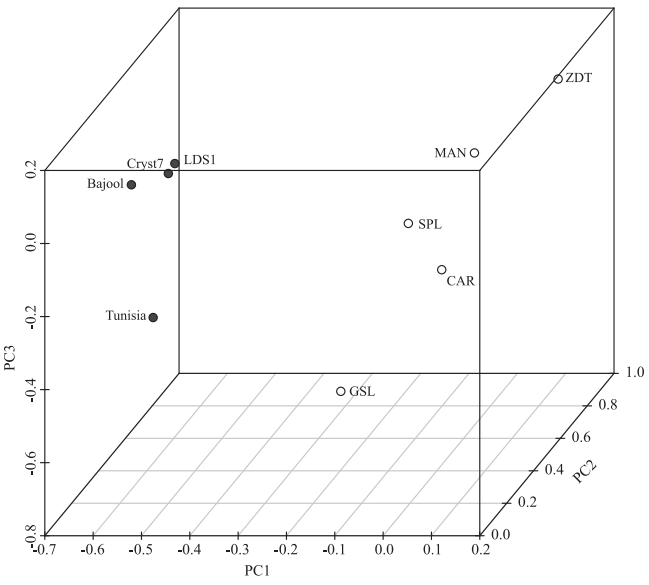

FIG 4 Principal-component analysis of distribution of genera in the 4 previously studied hypersaline samples (filled circles) and subsamples of the currently studied sites (open circles). The first 3 axes (shown) contributed to 87.9% of the total variance, distributed as 48.1% for the first axis, 30.2% for the second axis, and 9.6% for the third axis.

quencing technology, or the 16S rRNA gene fragment utilized in our analysis.

Not only did the habitats analyzed in this study show higher levels of phylogenetic diversity than typical hypersaline water bodies (Table 3), but they also exhibited drastically different community compositions (Fig. 4), especially in the MAN, SPL, ZDT, and CAR samples. Prior studies have identified members of the *Halorubrum* and *Haloquadratum* and, in some samples, *Halonotius*, *Natrinema*, *and Haloferax* as dominant members of crystallizer salterns (8, 45, 47), while in our study, members of the recently described genera *Halogranum*, *Halolamina*, *Halosarcina Halorientalis*, and *Haloplanus* represented a major fraction of the *Halobacteriales* community in multiple habitats (e.g., 60.8, 66.4, 41.1, and 49.4% in MAN, ZDT, CAR, and SPL, respectively). This attests to the relevance and significance of recent efforts for the isolation and characterization of novel *Halobacteriales* genera in various habitats beyond typical hypersaline water bodies (14–21, 59). Unfortunately, aside from standard information presented in species description papers, little information is available regarding the physiological capabilities, adaptive mechanisms, and genomic characteristics of these genera. Many of these novel genera (*Halogranum*, *Halolamina*, *Halorientalis*, and *Halopelagius*) were first identified in studies on the *Halobacteriales* communities in various solar salterns along China's eastern coast. It is telling that most, but not all, of these strains have been isolated from saline soils and sediments rather than from brine pools (14–16, 18, 19, 21).

These differences in community structure between sediments and hypersaline water bodies, coupled to the recent efforts to isolate *Halobacteriales* from saline sediments, are significant since they suggest that the *Halobacteriales* communities in hypersaline habitats (e.g., salterns and saline lakes) represent only a small fraction of the *Halobacteriales* phylogenetic diversity on Earth. This is important since the majority of saline environments on Earth are not hypersaline habitats but are areas of low and moderate salinities. Most of these environments also experience various degrees of fluctuation in salinity and other environmental parameters. Therefore, we reason that the majority of *Halobacteriales* phylogenetic diversity in nature is present not in typical hypersaline habitats, where they have often been isolated and examined, but in relatively lower-salinity habitats and that the community in hypersaline habitats represents the fraction of the *Halobacteriales* community that is best adapted and most competitive for growth in hypersaline settings. It follows that if the *Halobacteriales* community in hypersaline habitats represents a fraction of the overall *Halobacteriales* communities, then novel phylogenetic, physiological, and metabolic properties could indeed be identified upon further examination of the larger pool of *Halobacteriales* diversity present in nature.

Novel genus-level diversity within the *Halobacteriales*. One of the more surprising finding of this study is the relatively low abundance yet extremely high levels of alpha and beta diversity of sequences unaffiliated with any of the 38 currently recognized archaeal genera. The low percentage of novel sequences within all data sets examined in this study is due to the recent success in isolating and describing novel genera from multiple soil and sediment habitats (14–21, 59). It is also a function of implementation of a local Blast approach against a curated data set of all validly described *Halobacteriales* sequences as detailed in Materials and Methods. Indeed, implementing the RDP and Silva classification schemes with our data sets gave proportions of 60.2, 55.3, 82.4, 72.5, and 76.5% of the total community of CAR, GSL, MAN, SPL, and ZDT, respectively, with RDP and 59.7, 45.5, 75.8, 75.2, and 69.7% of the total community of CAR, GSL, MAN, SPL, and ZDT, respectively, with Silva. This suggests the relevance of building a custom, annotated data set to compare against when dealing with lineages with a manageable number of described genera and species. In addition to documenting the presence of a large number of putatively novel *Halobacteriales* genera in each environment (Table 1), the results also highlight that little overlap has been observed between members of such novel species and genera in different environments (see Table S1 in the supplemental material). If this pattern of high beta diversity was valid and widespread in all saline ecosystems, then these results would suggest the presence of an enormous, yet-untapped supply of novel *Halobacteriales* genera in nature.

In conclusion, this study greatly expands the breadth of phylogenetic diversity within the order *Halobacteriales*, provides the first detailed and targeted *Halobacteriales* community structure analysis in five different habitats, provides new tools for targeting *Halobacteriales* in habitats with various degrees of complexity, and highlights the high genus-level diversity within each environment as well as the remarkable alpha and beta diversity within novel, and mostly rare, members of the *Halobacteriales* community.

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