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Patterns and Determinants of Halophilic *Archaea* (Class *Halobacteria*) Diversity in Tunisian Endorheic Salt Lakes and Sebkhet Systems

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We examined the diversity and community structure of members of the halophilic *Archaea* (class *Halobacteria*) in samples from central and southern Tunisian endorheic salt lakes and sebkhet (also known as sebkha) systems using targeted 16S rRNA gene diversity survey and quantitative PCR (qPCR) approaches. Twenty-three different samples from four distinct locations exhibiting a wide range of salinities (2% to 37%) and physical characteristics (water, salt crust, sediment, and biofilm) were examined. A total of 4,759 operational taxonomic units at the 0.03 (species-level) cutoff ($OTU_{0.03}$s) belonging to 45 currently recognized genera were identified, with 8 to 43 genera (average, 30) identified per sample. In spite of the large number of genera detected per sample, only a limited number (i.e., 2 to 16) usually constituted the majority (≥80%) of encountered sequences. *Halobacteria* diversity showed a strong negative correlation to salinity (Pearson correlation coefficient = −0.92), and community structure analysis identified salinity, rather than the location or physical characteristics of the sample, as the most important factor shaping the *Halobacteria* community structure. The relative abundance of genera capable of biosynthesis of the compatible solute(s) trehalose or 2-sulfotrehalose decreased with increasing salinities (Pearson correlation coefficient = −0.80). Indeed, qPCR analysis demonstrated that the *Halobacteria otsB* (trehalose-6-phosphatase)/16S rRNA gene ratio decreases with increasing salinities (Pearson correlation coefficient = −0.87). The results highlight patterns and determinants of *Halobacteria* diversity at a previously unexplored ecosystem and indicate that genera lacking trehalose biosynthetic capabilities are more adapted to growth in and colonization of hypersaline (>25% salt) ecosystems than trehalose producers.

The class *Halobacteria* represents a physiologically and phylogenetically distinct lineage within the archaeal phylum *Euryarchaeota*. Members of the *Halobacteria* are encountered in a wide range of environments where their absolute requirement for salt is satisfied. Within various hypersaline (>25% salt), thalassohaline (e.g., crystallizer ponds in solar salterns), and athalassohaline (e.g., the Dead Sea, hypersaline lakes, and soda lakes) water bodies, members of the *Halobacteria* represent the majority of the cellular biomass (1–6). However, in environments with relatively lower salinity and/or fluctuating salinities, e.g., saline soils (salt plains and alpine salt sediments, soils adjacent to salt-processing plants), traditional Asian salted and fermented seafood products (e.g., jeotgal), and marine sponges, they usually coexist as a smaller fraction of the more diverse prokaryotic community inhabiting these settings (7–13). These habitats with moderate or low salinity and/or fluctuating salinity have been the source of species of many recently described novel *Halobacteria* taxa (14–18) and are partially responsible for the rapid expansion of recognized *Halobacteria* spp. during the last decade (19, 20).

Patterns of *Halobacteria* community structure have mostly been examined in a few model hypersaline habitats with relatively limited *Halobacteria* diversity. These studies documented the dominance of specific *Halobacteria* genera in high-pH soda lakes (genera *Natronococcus*, *Natronobacterium*, *Natronomonas*, *Natrialba*, *Natronolimnobius*, *Natronorubrum*, *Halorubrum*, *Halalkalicoccus*, *and Halobiforma*) (21, 22) and in Mg^{2+}-rich water bodies (genera *Halosarcina*, *Natronococcus*, *Halorhabdus*, and *Natronomonas*) (23, 24), as well as in solar salterns and crystallizer ponds (genera *Halorubrum*, *Haloquadratum*, *Halonotius*, and *Haloplanus*) (1–3, 25). However, with the exception of these few model ecosystems, patterns and determinants of *Halobacteria* community structure remain poorly understood.

Given the current recognition of the wide range of *Halobacteria* phylogenetic diversity (26) and the novel habitats in which *Halobacteria* spp. are encountered (12, 14, 27–33), extrapolation of diversity and community structure studies to these atypical, nonhypersaline habitats is warranted. Such studies would expand our knowledge regarding overall diversity and ecological distribution within the *Halobacteria* and aid in deciphering the importance of various factors, e.g., salinity, physical characteristics, and geographical location, on shaping their diversity and community structure patterns. The relatively lower number of *Halobacteria* cells in such habitats often hinders the use of archaeal domain-wide 16S rRNA gene primers for their targeting, a common procedure in surveying *Halobacteria* diversity in hypersaline settings. To overcome this problem, we have recently designed, validated, and utilized *Halobacteria*-specific 16S rRNA gene primers for targeted high-throughput pyrosequencing. Further, we developed a pipeline for accurate phylogenetic assignment of obtained se-

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TABLE 1 Sample description, numbers of sequences and OTUs, and diversity indices of datasets analyzed

Sample collection site	Sample			Data set[a]											
	Name	Description	Salinity (%)	No. of sequences	No. of OTUs		Rarefaction rank		Good's coverage index		Simpson index		Shannon index		Avg Bray-Curtis index	
					0.03	0.06	0.03	0.06	0.03	0.06	0.03	0.06	0.03	0.06	0.03	0.06
Chott El-Djerid	S1	Salt crust	37	617	53	25	2	1.5	0.94	0.98	0.07	0.15	3.20	2.34	0.22	0.36
	S2	Salt crust	37	322	12	8	1	1.5	0.96	0.98	0.2	0.24	1.74	1.57	0.18	0.26
	S3	Salt crust	37	3,956	668	132	6	4	0.88	0.99	0.02	0.11	5.28	3.22	0.14	0.26
	S4	Sediment	37	455	74	34	7.5	7	0.88	0.96	0.06	0.09	3.52	2.77	0.19	0.30
	S5	Sediment	13.1	1,845	56	23	13	13	0.97	1.00	0.10	0.13	3.06	2.47	0.20	0.33
	S6	Sediment	13.4	1,927	317	139	11.5	10	0.84	0.94	0.02	0.05	4.77	3.57	0.23	0.37
	S7	Sediment	10.5	1,766	143	39	15	14	0.93	0.99	0.03	0.08	3.93	2.88	0.22	0.37
	S10	Water	30	743	324	111	4	6	0.66	0.92	0.01	0.04	5.21	3.76	0.18	0.33
	S11	Salt crust	37	1,363	297	86	5	4	0.85	0.98	0.02	0.06	4.93	3.41	0.25	0.38
	S12	Salt crust	37	6,543	414	164	3	4	0.92	0.97	0.06	0.13	3.98	2.72	0.15	0.22
Sebkhet Douz	S13	Water	9.4	8,284	515	129	11.5	15	0.93	0.99	0.02	0.06	4.78	3.30	0.12	0.20
	S14	Sediment	6.7	522	62	31	18.5	18.5	0.89	0.96	0.09	0.12	3.18	2.57	0.11	0.18
	S15	Sediment	14.1	1,959	355	91	7.5	10	0.87	0.98	0.02	0.10	4.89	3.20	0.16	0.31
	S16	Biofilm	2.9	108	12	7	22.5	23	0.91	0.99	0.21	0.26	1.84	1.51	0.08	0.10
	S17	Sediment	2.7	695	55	24	22.5	20	0.96	0.99	0.06	0.10	3.36	2.59	0.13	0.23
	S18	Sediment	13.7	4,520	1,512	283	9.5	10	0.76	0.97	0.01	0.11	6.33	3.44	0.14	0.27
	S19	Sediment	2.2	1,532	303	107	20	21	0.85	0.96	0.02	0.05	4.82	3.57	0.17	0.30
Sebkhet El-Melah	S40	Sediment	7.6	402	132	51	14	17	0.75	0.94	0.04	0.10	4.10	2.97	0.06	0.1
	S42	Sediment	10.8	5,287	485	103	9.5	12	0.92	0.99	0.03	0.10	4.56	2.80	0.06	0.1
Chott El-Fejej	S26	Sediment	6.6	588	177	66	21	18.5	0.81	0.95	0.03	0.09	4.41	3.16	0.22	0.35
	S27	Sediment	5.5	325	50	27	17	22	0.89	0.97	0.06	0.10	3.25	2.68	0.17	0.31
	S28	Sediment	6.3	243	110	39	18.5	16	0.66	0.94	0.02	0.08	4.31	3.00	0.19	0.32
	S29	Sediment	12.7	3,133	735	228	16	8	0.82	0.95	0.02	0.12	5.41	3.30	0.09	0.15

[a] Rarefaction curves were used at both 97% (species level [0.03]) and 94% (genus level [0.06]) to rank the diversity of the samples. Samples whose rarefaction curves lie at the top are considered more diverse than samples whose rarefaction curves lie at the bottom. Samples were given diversity rankings ranging from the least diverse (rank 1) to the most diverse (rank 23). Good's coverage index, the Shannon index of sample diversity, and the Simpson index of sample evenness are shown at both the species (0.03) and genus (0.06) levels. The Bray-Curtis index of β diversity between samples was calculated for all possible pairwise comparisons for samples from each site. The average Bray-Curtis index is shown at both the species (0.03) and genus (0.06) levels.

quences to the genus level by comparison to a curated database of validly described *Halobacteria* species using BLASTN (12). Within saline and hypersaline ecosystems, the level of and spatiotemporal fluctuations in salinity obviously play an important role in selection of taxa (34), although the impact of other factors, e.g., pH, temperature, physical characteristics, availability of dissolved O_2, redox potential, and ionic composition (35), could not be discounted.

To survive in high-salinity environments, cells maintain an intracellular osmotic pressure that is equal to or higher than that of the surrounding environment to prevent osmotically induced cell lysis (36). The most prevalent mechanism for osmoadaptation is "salting in," where cells accumulate molar concentrations of potassium ions to counter the high extracellular osmotic pressure. This strategy appears to be universally adapted by all members of the *Halobacteria* (37, 38). In addition to salting in, some members of the *Halobacteria* maintain high intracellular osmotic pressure by synthesis and/or uptake of highly soluble organic solutes that do not interfere with intracellular enzymatic activities and cellular processes. We have recently demonstrated that multiple genera within the *Halobacteria* biosynthesize and accumulate molar levels of trehalose (or 2-sulfotrehalose) as an osmoadaptive compatible solute (37). Currently, the impact of the possession (or lack thereof) of such a capability within members of the *Halobacteria* on their ecological fitness, habitat preferences, and, consequently, the overall *Halobacteria* community structure within a specific saline or hypersaline habitat is unclear.

Here, we sought to examine the diversity and community structure of members of the class *Halobacteria* in samples from central and southern Tunisian endorheic salt lakes and sebkhet (also known as sebkha) systems using targeted 16S rRNA gene diversity survey and quantitative PCR (qPCR) approaches. We further investigated whether the possession of trehalose biosynthetic capacity is an ecologically relevant trait that impacts fitness and niche colonization process within the *Halobacteria*. Our results suggest that the possession of trehalose biosynthetic capacity, or lack thereof, is an ecologically relevant trait, with genera possessing the machinery for trehalose biosynthesis as an osmoadaptive strategy appearing to be less suited for survival and propagation at higher salinities.

MATERIALS AND METHODS

Location and sampling. A total of 23 samples from 4 different saline systems were obtained (Table 1; see also Fig. S1 in the supplemental material). Briefly, these systems are as follows: (i) Chott El-Djerid (33°56.977′N, 8°25.279′E), a large endorheic salt lake located in south-

TABLE 2 Primers used in this study

Name	Sequence (5′–3′)	Gene amplified	Use(s)	Reference or source
287F	AGGTAGACGGTGGGGTAAC	*Halobacteria*-specific 16S rRNA gene	Pyrosequencing and qPCR	12
589R	RGCTACGRACGCTTTAGGC			
OtsB-F	GAYTTCGACGGBWCCCT	*Halobacteria*-specific *otsB* (trehalose-6-phosphatase) gene	qPCR	This study
OtsB-R	GGBAAYCACGGNYTSGA			

western Tunisia (10 samples); (ii) Chott El-Fejej (33°49.96′N, 9°2.025′E), a long narrow inlet to Chott El-Djerid (4 samples); (iii) Sebkhet El-Melah (33°23.655′N, 10°55.745′E), a salt flat southwest of Zarzis (2 samples); and (iv) Sebkhet Douz (33°27.469′N, 9°0.465′E), a salt flat located in south central Tunisia (7 samples). Temperature and pH were recorded on site, and ~50 g was sampled (using sterile spatulas) into sterile Falcon tubes, placed on ice, and transported to the laboratory, where the samples were kept frozen (−20°C) until DNA extraction. Salinities were measured using a hand-held SW series VistaVision refractometer (VWR, Radnor, PA) as previously described (39) and ranged from very low (2% to 3%, 3 samples from 1 site) to low (5% to 6.6%, 4 samples from 2 sites), medium-low (7.6% to 9.5%, 2 samples from 2 sites), medium-high (10.5% to 12.7%, 3 samples from 3 sites), high (13% to 14%, 4 samples from 2 sites), very high (30%, 2 samples from 1 site), and saturated (37%, 5 samples from 1 site). The physical characteristics of the samples differed between sediment (usually located below a layer of salt crust; $n = 15$, from 4 sites), saline water ($n = 2$, from 2 sites), and salt crust ($n = 5$, from 1 site) samples and 1 biofilm sample.

DNA extraction, PCR amplification, sequencing, and analysis. DNA was extracted using a PowerSoil DNA extraction kit (MoBio, Carlsbad, CA) following the manufacturer's instructions and quantified using a Qubit fluorometer (Life Technologies, Grand Island, NY). For 16S rRNA gene amplification and pyrosequencing, the extracted DNA was used as a template in PCRs that contained the *Halobacteria*-specific 287F and 589R primers (Table 2). The forward primer was constructed by adding 454 Roche FLX adaptor A (GCCTCCCTCGCGCCATCAG) to the 287F primer as previously described (12). The forward primer also contained a unique bar code (octamer) sequence for multiplexing (12). The reverse primer was constructed by adding 454 Roche FLX adaptor B (GCCTTG CCAGCCCGCTCAGT) to the 589R primer. PCR analysis was performed in 50-μl reaction mixtures that contained 2 μl of the extracted DNA, 1× PCR buffer (Promega, Madison, WI), 2.5 mM $MgSO_4$, a 0.2 mM deoxyribonucleoside triphosphate (dNTP) mixture, 0.5 U of GoTaq flexi DNA polymerase (Promega, Madison, WI), and a 10 μM concentration of each of the forward and 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 run in at least triplicate, and the resulting PCR products of the expected size were gel purified using a QIAquick gel extraction kit (Qiagen Corp., Valencia, CA) and pooled to give a total of 3 to 5 μg of DNA per sample. Pyrosequencing was performed on a Roche 454-Junior sequencer at the Oklahoma State University Biochemistry and Molecular Biology core facility.

Sequence quality filtering, OTU identification, and phylogenetic assignments. Sequence quality control was handled in the program mothur (40) as described previously (12). Briefly, sequences with an average quality score 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. Filtered alignments were used to generate an uncorrected pairwise distance matrix, followed by binning the sequences into operational taxonomic units (OTUs) at 3% and 6% cutoffs, corresponding to the species and genus levels, respectively. Rarefaction curves were computed in mothur using a resampling-without-replacement approach. Good's coverage indices were also calculated in mothur.

For phylogenetic placement, all sequences were queried using the BLASTN function of the downloaded NCBI standalone BLAST program (version 2.2.26) against a data set of 207 16S rRNA gene sequences representing the validly published species within the class *Halobacteria* (as of October 2014). A sequence was assigned to a specific genus if it was at least 94.0% similar to the reference 16S rRNA gene sequence belonging to that genus. Sequences with percent similarity of <94% to any known validly described genus were considered novel. Percentages of abundances of genera in each sample were used to construct a heat map for genera representation using the phyloseq package in R (41).

NMDS. To examine patterns of genus-level co-occurrence and compare community structures and memberships between different data sets, sequences from all samples within a single site (range, 4,289 to 19,537; average, 11,783 ± 7,905) were pooled and binned into OTUs at the putative genus (6%) level. Membership patterns within these OTUs were used to compute pairwise diversity estimates. Bray-Curtis indices (at the putative genus level) were calculated for all possible sample pairs. The indices were then employed in constructing nonmetric multidimensional scaling (NMDS) plots using the command nmds in mothur. The obtained axes for all samples across all sites studied were represented on the same scatter plot, and the proximities of sample points to each other in ordination space were used as an indication of the similarity in community structure.

Deducing the proportion of trehalose-producing genera using 16S rRNA gene pyrosequencing data sets. Our previous study (37) has provided experimental and genomic evidence that multiple *Halobacteria* genera are capable of trehalose or 2-sulfotrehalose biosynthesis as an osmoadaptive strategy and that the *otsAB* operon (encoding trehalose-6-phosphate synthase/trehalose-6-phosphatase) mediates trehalose biosynthesis in the *Halobacteria*. Further, analysis of the occurrence patterns of *otsAB* genes demonstrates a distinct phylogenetic pattern, where the *otsAB* operon was identified in all members of *Halobacteria* clade I (as defined in references 42 and 43), as well as in the genera *Halococcus*, *Haladaptatus*, *Halalkalicoccus*, and *Halosimplex*. On the other hand, *otsAB* was absent in all members of *Halobacteria* clade II (42, 43) and in all members of *Halorhabdus-Halomicrobium-Haloarcula* clade III (as defined in reference 44), as well as within the genera *Halobacterium* and *Natronomonas*. This distinct pattern allows estimation of the proportion of *otsAB*-bearing versus *otsAB*-lacking genera within a specific sample using 16S rRNA genus-level assignments. A list of *otsAB*-harboring versus *otsAB*-lacking genera is presented in Table S1 in the supplemental material. Using the genus-level assignments obtained for each sample and the *otsAB* distribution patterns described above, we calculated the relative abundances of *otsAB*-harboring genera in all data sets examined.

Nevertheless, the presence or absence of the *otsAB* operon could not be deduced for 18 genera that were not evaluated previously in prior studies and that do not have representatives with sequenced genomes. For those genera, a phylogenetics prediction system based on 16S rRNA sequence phylogeny was implemented. Genera within this group that phylogenetically belong to clade I, or are closely related to any of the genera *Halococcus*, *Haladaptatus*, and *Halalkalicoccus*, were predicted to harbor the *otsAB*

system. These include the genera *Haloarchaeobius*, *Halorubellus*, *Halorussus*, *Natronoarchaeum*, *Salinarchaeum*, and *Salinirubrum*. Those genera phylogenetically belonging to clade II or to clade III or that are closely related to *Halobacterium*, or *Natronomonas*, were predicted to lack the *otsAB* system. These include the genera *Halapricum*, *Haloarchaeum*, *Halobellus*, *Halolamina*, *Halomarina*, *Halomicroarcula*, *Halonotius*, *Halopelagius*, *Halopenitus*, *Halorientalis*, *Halovenus*, and *Salarchaeum*. These two groups of genera were labeled "predicted *otsAB*-harboring" genera and "predicted *otsAB*-lacking" genera, respectively (see Table S1 in the supplemental material). Using this system, all *Halobacteria* genera were classified into *otsAB*-harboring genera (17 genera), predicted *otsAB*-harboring genera (6 genera), *otsAB*-lacking genera (10 genera), and predicted *otsAB*-lacking genera (12 genera) (see Table S1).

Quantification of total *Halobacteria* community and *otsAB*-harboring community. The total *Halobacteria* community within each sample was quantified using a qPCR protocol targeting the *Halobacteria* 16S rRNA gene. The same primer pair (287F and 589R) used in pyrosequencing-based diversity survey was utilized in qPCR.

The abundance of *Halobacteria* community capable of synthesizing trehalose was assessed by quantifying *otsB* gene copy numbers. Specific *otsB* primers were designed in Primrose (45) on the basis of all available *Halobacteria otsB* gene sequences ($n = 48$) (June 2014). The specificity of the primer pair was initially evaluated *in silico* by comparison to the nr database using BLASTN (46) with the exclusion of class *Halobacteria*. Specificity of the primer pair was further experimentally verified in two samples (S1 and S19) by PCR amplification, cloning of the PCR product, and sequencing of 12 clones randomly selected from each sample. Primer sequences are shown in Table 2.

qPCR was conducted using a MyIQ thermocycler (Bio-Rad Laboratories, Hercules, CA) and SybrGreenER qPCR mix (Life Technologies, Carlsbad, CA). The same amplification protocol was used to quantify both 16S rRNA and *otsB* genes. The 25-μl reaction mixtures contained 2 μl of DNA template, 0.5 μM (each) forward and reverse primers, and 10 μl of the qPCR mix. The reactions were heated at 50°C for 2 min, followed by heating at 95°C for 8.5 min. This was followed by 65 cycles, with one cycle consisting of 15 s at 95°C, 60 s at 52°C, and 30 s at 72°C. *Haladaptatus paucihalophilus* strain DX253^T genomic DNA was used as a positive control, as well as to construct a standard curve to deduce the gene copy number/mg DNA. For each sample, the threshold cycle (C_T) values obtained for the 16S rRNA gene and the *otsB* gene were used to calculate the corresponding gene copy number as well as the fraction of the *otsAB* system-harboring *Halobacteria* community (calculated as the ratio of the *otsB*/16S rRNA gene copy numbers).

RESULTS

Sampling results. Twenty-three distinct samples were analyzed (Table 1; see also Fig. S1 in the supplemental material). These samples belong to 4 different ecosystems in central and southern Tunisia, display different physical characteristics (ranging between salt crystals, hypersaline water, sediments below salt crusts, and biofilm samples), and range in salinities from 2.2% to 37% (Table 1). While some of the samples yielded only a relatively low number of sequences, the calculated genus-level coverage for all samples was always >92% (average, 96.8%) (Table 1).

***Halobacteria* community and genus-level assignments.** A total of 45 *Halobacteria* genera were identified in the entire data set, attesting to the indiscriminant performance of the *Halobacteria*-specific primers used. No non-*Halobacteria*-affiliated sequences were identified in all data sets. The number of genera within each sample ranged between 8 and 43 (average, 30), indicating the high level of *Halobacteria* phylogenetic diversity within each sample (Fig. 1; see also Table S2 in the supplemental material). Sequences unaffiliated with currently recognized *Halobacteria* genera represented only 14% of all sequences. In spite of the large number of genera identified per sample, a general pattern was observed in which a low number of genera always represented the majority of sequences encountered followed by a long tail of less-abundant genera. For example, within each sample, sequences belonging to the three most abundant genera represented 38% to 92% of the community and those belonging to the five most abundant genera represented 51% to 97.8%.

Depending on their occurrence and relative abundance, *Halobacteria* genera observed in this study could be broadly classified into the following groups (see Table S3 in the supplemental material).

(i) Consistently abundant genera (group 1). These genera (*Halorientalis*, *Halorubrum*, and *Halogranum*) represented >10% of the community in a few samples ($n > 4$) and represented >5% of the community in the majority of the samples (see Table S3 in the supplemental material).

(ii) Moderately abundant genera (group 2). These genera (*Haloferax*, *Halonotius*, *Halobiforma*, *Haloquadratum*, and *Halolamina*) represented >10% of the community in a few samples ($n < 4$) and represented 1% to 5% of the community in the majority of samples (see Table S3 in the supplemental material).

(iii) Genera with occasional moderate abundance (group 3). These genera (*Natronomonas*, *Halobellus*, *Halomicrobium*, *Haloplanus*, *Halorussus*, *Halorhabdus*, *Halostagnicola*, and *Halorubellus*) represented >5% of the community in 1 to 2 samples and represented 1% to 5% of the community in the majority of samples (see Table S3 in the supplemental material).

(iv) Consistently low-abundance genera (group 4). These genera (*Halobacterium*, *Haloterrigena*, *Halovenus*, *Halopelagius*, and *Halapricum*) represented 1% to 5% of the community in the majority of samples (see Table S3 in the supplemental material).

(v) Rare genera with occasional low abundance (group 5). These genera (*Natronorubrum*, *Salarchaeum*, *Halovivax*, *Haladaptatus*, *Halobaculum*, *Natronoarchaeum*, *Natrononococcus*, *Salinarchaeum*, *Salinirubrum*, *Natrinema*, *Natronolimnobius*, *Halomarina*, *Halosimplex*, and *Halomicroarcula*) represented 1% to 5% of the community in just a few samples ($n = 2$ to 8) and represented <1% in the remaining samples (see Table S3 in the supplemental material).

(vi) Consistently rare genera (group 6). These genera (*Natrialba*, *Halalkalicoccus*, *Halococcus*, *Haloarchaeobius*, *Halopiger*, *Natronobacterium*, and *Haloarchaeum*) always represented <1% of the total community and were encountered in only a few samples ($n = 1$ to 8) (see Table S3 in the supplemental material).

Diversity estimates and patterns. Various diversity estimates (Shannon diversity index and Simpson evenness index for taxonomic alpha diversity; Bray-Curtis and rarefaction curve ranking for beta diversity) were computed (Table 1). Rarefaction curve-based ranking was chosen for comparative diversity purposes since this approach overcomes bias originating from variations in the size of data sets (47). The computed diversity ranks were clearly negatively correlated with salinity in the entire data set at both the genus (Pearson correlation coefficient = −0.92) and species (Pearson correlation coefficient = −0.90) levels (Fig. 2). This was also true when the diversity-salinity relationship was examined for an individual site (Pearson correlation coefficients between −0.55 and −0.97 at the species level and between −0.94 and −0.98 at the genus level; see Fig. S2 in the supplemental material) or for a specific physical condition (e.g., sediment samples; see Fig. S3).

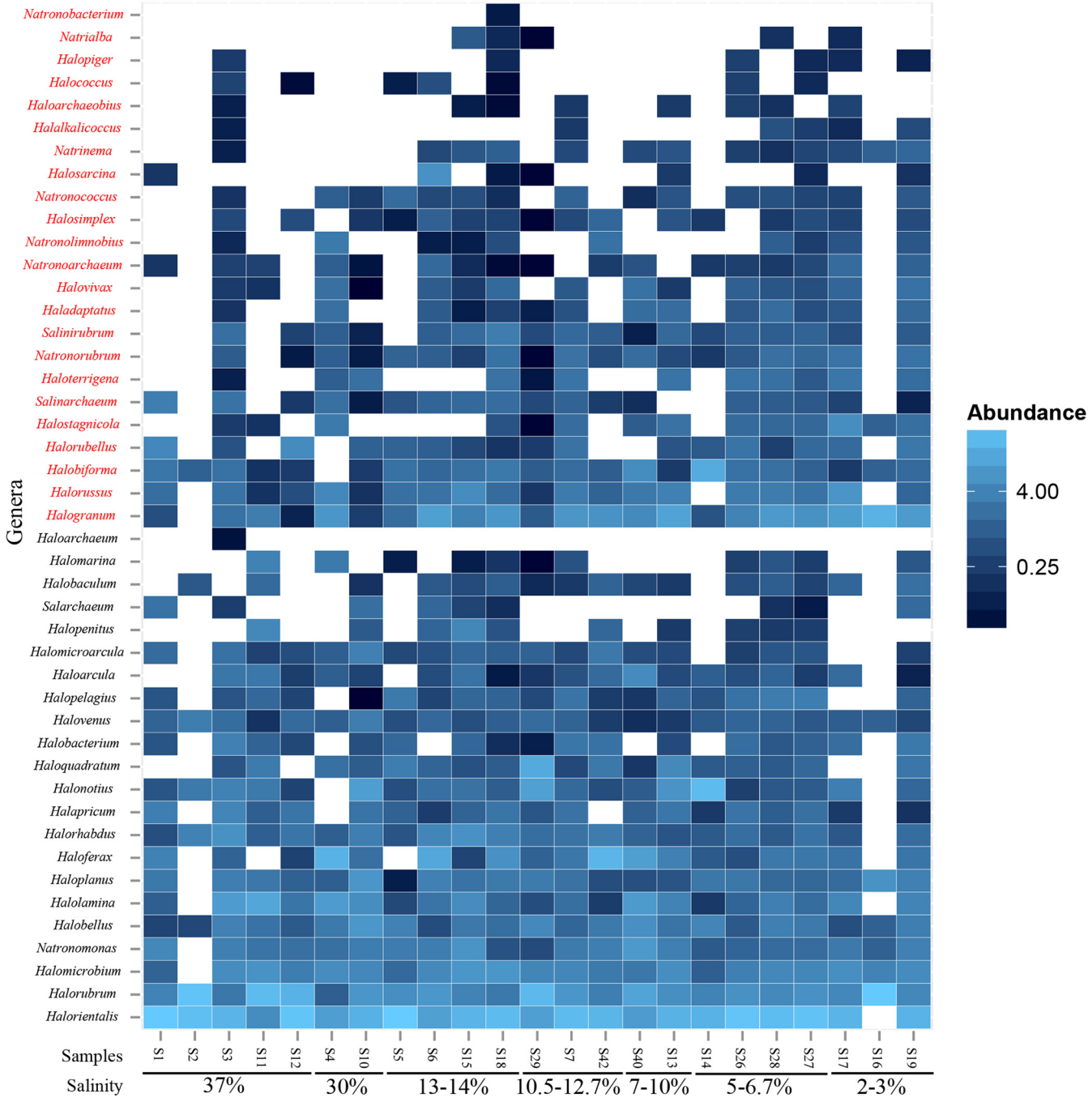

FIG 1 Heat map of percent abundance of *Halobacteria* genera in analyzed samples. Salinity is shown for different samples. Genera in red are those known or predicted to harbor the *otsAB* system for trehalose biosynthesis.

Community structure analysis. Nonmetric multidimensional scaling (NMDS) was used to identify community structure and co-occurrence patterns between different samples. The results (Fig. 3; see also Fig. S4 in the supplemental material) suggest that salinity plays an important role in shaping the community structure. Samples with very low salinity (2% to 3%) ($n = 3$ of 3 in this salinity range) clustered together, as did samples with moderately low salinity (5.5% to 9.4%) ($n = 6$ of 6 in this salinity range) and samples with moderately high salinity (10.5% to 14%) ($n = 6$ of 7 in this salinity range), as well as samples with high to saturated salinity (>30%) (two distinct clusters of $n = 4$ and $n = 3$). On the other hand, neither the physical characteristics of the sample (see Fig. S3A) nor the geographical location (see Fig. S3B) played a clear role in shaping community structure.

Salinity-abundance correlation for individual *Halobacteria* genera. The results described above clearly suggest that salinity plays an important role in shaping the microbial community structure of the *Halobacteria*. To zoom in on genus-level specific

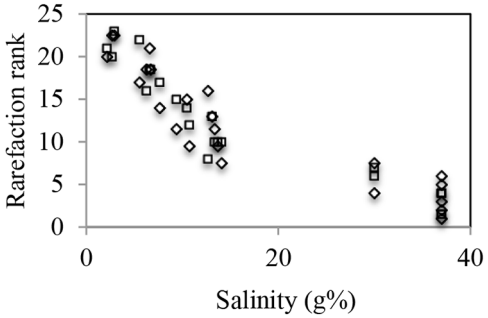

FIG 2 Salinity-diversity relationship. Sample salinity (*x* axis) was correlated to the diversity ranking of each sample (from 1 [least diverse] to 23 [most diverse]) (*y* axis), computed at both 97% (□) and 94% (◇) sequence similarity cutoffs.

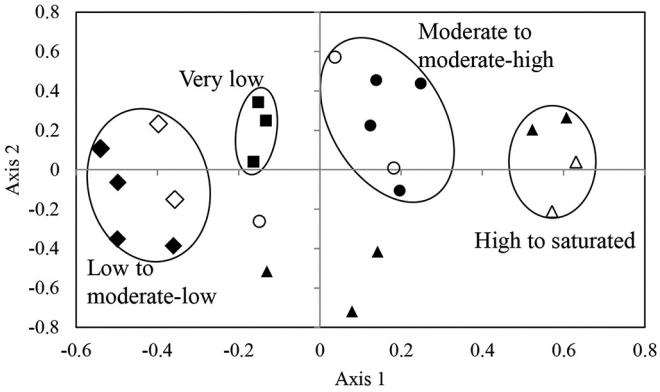

FIG 3 Nonmetric multidimensional scaling based on pairwise Bray-Curtis dissimilarity indices. Each symbol represents one sample, and sample symbols reflect their salinities. ■, very low salinities (2% to 3%); ◆, low salinities (5.5% to 6.7%); ◇, moderate-low salinities (7.6% to 9.4%); ○, moderate salinities (10.5% to 12.7%); ●, moderate-high salinities (13% to 14%); △, high salinity (30%); ▲, saturated salinity (37% [salt crusts]).

preferences and determine the genera that appear most sensitive and responsible for the observed community structure shifts (Fig. 3), we examined salinity-relative abundance correlations for genera present above an empirical occurrence cutoff defined as a relative abundance of 1% or more in at least 5 samples ($n = 28$ genera). In general, three distinct patterns were observed (Fig. 4; see also Table S4 in the supplemental material).

(i) Genera whose percentages of abundance increased with the increase in sample salinity (Pearson correlation coefficient, 0.62 to 0.9). This group included the genera *Haloferax*, *Halobellus*, *Halorhabdus*, *Halapricum*, *Halovenus*, and *Halomicroarcula*.

(ii) Genera whose percentages of abundance decreased with the increase in sample salinity (Pearson correlation coefficient, −0.65 to −0.97). This group included the genera *Halogranum*, *Halobiforma*, *Halorussus*, *Halostagnicola*, *Haloterrigena*, *Halovivax*, *Haladaptatus*, *and Natronorubrum*.

(iii) Genera with no clear effect of salinity fluctuations on their relative abundance. This group included the genera *Halorientalis*, *Halorubrum*, *Halonotius*, *Haloquadratum*, *Halolamina*, *Halomicrobium*, *Natronomonas*, *Haloplanus*, *Haloarcula*, *Halobacterium*, *Halorubellus*, *Salinarchaeum*, *Salinirubrum*, and *Halopelagius*.

The ecological significance of trehalose biosynthesis capacity. A close examination of salinity-abundance profiles of various genera (Fig. 4; see also Table S4 in the supplemental material) indicated that all genera whose percentages of abundance increase with salinity ($n = 6$) lack the *otsAB* operon and are hence incapable of utilizing trehalose biosynthesis as an osmoadaptive strategy. On the other hand, all genera whose percentages of abundance decrease with salinity ($n = 8$) belong to genera known for their capability to produce trehalose as a compatible solute (see Table S1 for a list of *otsAB* distributions within the *Halobacteria*).

To further examine this hypothesis, we identified in each data set the overall percentage of sequences belonging to genera shown to possess the *otsAB* operon. The relative proportions of such genera (listed in Table S1 in the supplemental material) ranged between 1.1% and 38.97% (Fig. 5; see also Fig. S5 in the supplemental material) and showed a strong negative correlation with salinity (Pearson correlation coefficient = −0.83).

Finally, we used qPCR to quantify the *otsB* gene copy number per sample and the normalized *Halobacteria otsB*/16S rRNA gene ratio. The latter was used as a quantitative index of the relative abundance of trehalose-producing *Halobacteria* as a fraction of the overall community within a specific sample. A progressive decrease in this ratio was observed in samples with higher salinity (Fig. 6). Therefore, multiple lines of evidence (salinity-relative abundance curves of individual genera, proportions of sequences belonging to *otsAB*-harboring genera, and qPCR-based quantifications of *Halobacteria otsB*/16S rRNA ratios in various samples) strongly indicate that genera lacking the *otsAB* operon are more adapted to growth in and colonization of hypersaline (>25%) environments than trehalose producers.

DISCUSSION

In this study, we examined the diversity and community structure of the halophilic *Archaea* (class *Halobacteria*) in samples from central and southern Tunisian endorheic salt lakes and sebkhet systems. Our results suggest that (i) a high level of genus-level phylogenetic diversity exists within samples, with distinct genera consistently representing the majority of sequences in all data sets (Fig. 1; see also Table S2 in the supplemental material); (ii) *Halobacteria* diversity estimates within samples exhibited strong negative correlation with salinity (Fig. 2; see also Fig. S2 and S3); (iii) salinity was the most important factor shaping the observed microbial community structure, rather than geographical location or sample physical characteristics (Fig. 3; see also Fig. S4); and (iv) genera possessing the machinery for trehalose biosynthesis as an osmoadaptive strategy appear to be less suited for survival and propagation at higher salinities (Fig. 5 and 6; see also Fig. S5).

In general, extremely high genus-level diversity was observed in all samples (8 to 43 genera; average, 30), with sequences affiliated with all 45 currently described *Halobacteria* genera detected in the entire data set. The highest levels of diversity were observed in sediment and water samples of relatively lower salinity, providing additional evidence for the emerging view that habitats of lower and fluctuating salinity are reservoirs for novel *Halobacteria* diversity (7–18). The use of 16S rRNA gene primers targeting members of the *Halobacteria* class for diversity and quantification studies is crucial for the targeted exploration of *Halobacteria* diversity in such habitats, since the prevailing levels of salinity and putative frequent salinity fluctuation are conducive to the coexistence of additional halotolerant and nonhalophilic microorganisms.

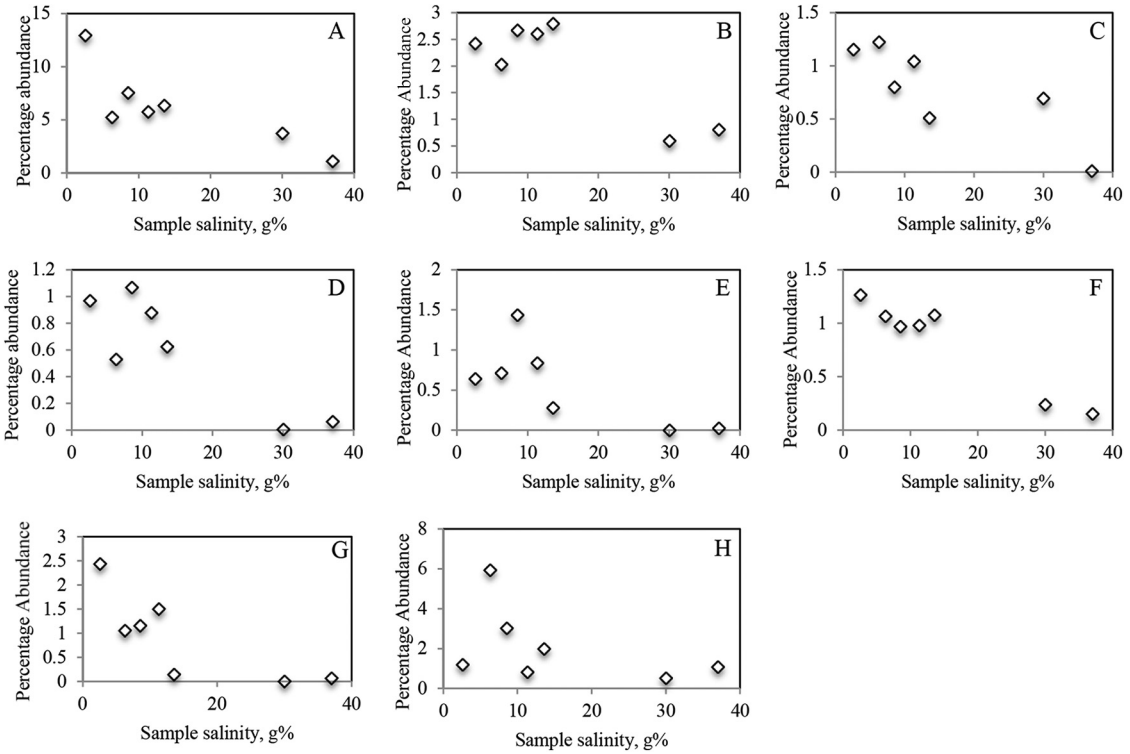

FIG 4 Percentages of abundance of individual *Halobacteria* genera capable of trehalose biosynthesis as a function of sample salinity. Values shown are averages for samples within the same salinity range as follows: very low (2% to 3%, 3 samples from 1 site), low (5% to 6.6%, 4 samples from 2 sites), medium-low (7.6% to 9.5%, 2 samples from 2 sites), medium-high (10.5% to 12.7%, 3 samples from 3 sites), high (13% to 14%, 4 samples from 2 sites), very high (30%, 2 samples from 1 site), and saturated (37%, 5 samples from 1 site). Results are shown only for *otsAB*-harboring genera present with >1% abundance in at least 5 samples. (A) *Halogranum*. (B) *Halobiforma*. (C) *Halorussus*. (D) *Halostagnicola*. (E) *Haloterrigena*. (F) *Halovivax*. (G) *Haladaptatus*. (H) *Natronorubrum*.

Within all samples, a distinct community structure pattern was observed in which a few genera (groups 1 and 2 in Table S3 in the supplemental material) were present in relatively high abundance, followed by a longer tail of less-abundant and rare genera. *Halorubrum*, *Halogranum*, and *Halorientalis* genera were the three most abundant and consistently encountered genera within all data sets. *Halorubrum* is a well-described and ubiquitous genus within the *Halobacteria*, members of which have been isolated and detected (using 16S rRNA diversity surveys) in a wide range of saline environments (2, 9, 25, 48–50). *Halorientalis* and *Halogranum*, on the other hand, are two recently identified genera, with few cultured representatives. *Halogranum* species were first isolated from marine solar salterns in eastern China (51, 52). Recently, *Halogranum* species were also isolated from evaporitic salt

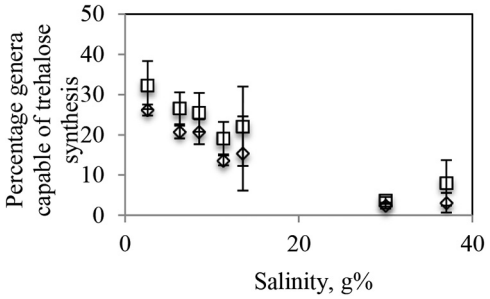

FIG 5 Effect of sample salinity on the total abundance of trehalose-producing genera. The sum of percent abundances of genera possessing an *otsAB* system determined on the basis of either experimental or genomic evidence (○) or predicted on the basis of phylogenetic affiliations (◇) is plotted on the *y* axis versus salinity on the *x* axis. Values shown are averages ± standard deviations for samples within the same salinity range as follows: very low (2% to 3%, 3 samples from 1 site), low (5% to 6.6%, 4 samples from 2 sites), medium-low (7.6% to 9.5%, 2 samples from 2 sites), medium-high (10.5% to 12.7%, 3 samples from 3 sites), high (13% to 14%, 4 samples from 2 sites), very high (30%, 2 samples from 1 site), and saturated (37%, 5 samples from 1 site). Individual sample data are shown in Fig. S3 in the supplemental material.

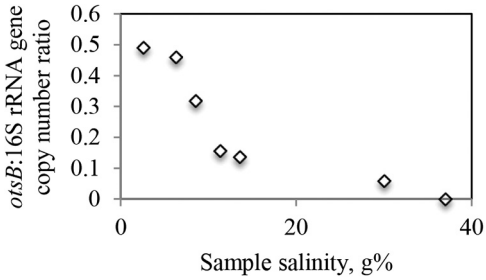

FIG 6 Quantification of *otsB*-harboring cells as a fraction of the total *Halobacteria* community. The ratio of the number of copies of the *otsB* gene to the *Halobacteria* 16S rRNA gene copy number is plotted as a function of salinity. Values shown are averages ± standard deviations for samples within the same salinity range as follows: very low (2% to 3%, 3 samples from 1 site), low (5% to 6.6%, 4 samples from 2 sites), medium-low (7.6% to 9.5%, 2 samples from 2 sites), medium-high (10.5% to 12.7%, 3 samples from 3 sites), high (13% to 14%, 4 samples from 2 sites), very high (30%, 2 samples from 1 site), and saturated (37%, 5 samples from 1 site).

crystals collected along the seashore of Namhae, South Korea (53), as well as from Zodletone Spring in southwestern Oklahoma (unpublished data). Physiological studies and sample origins suggest the genus capability to survive in environments with various salinities. Similarly, *Halorientalis* species were first isolated from marine solar salterns in eastern China (54) and then recently from a salt lake in Iran (55). All *Halorientalis* species isolated so far seem to require at least 2.5 M (14.6%) NaCl. The current study demonstrated the dominance of this genus not only in hypersaline environments but also in environments with fluctuating and low salinities. Interestingly, detection of *Halogranum* species and *Halorientalis* species in culture-independent studies is currently curtailed by a methodological oddity. Most curated 16S rRNA gene databases (RPD, Greengenes, and GenBank) do not acknowledge these two validly published names (as well as many other additional recently described genera) as part of their *Halobacteria* taxonomic outline. Therefore, we suspect that, in many cases, sequences affiliated with these two genera are usually deemed "unclassified" in culture-independent diversity surveys. Hence, our current approach for *Halobacteria* identification, which depends on using 16S rRNA sequences retrieved from all described species within the class *Halobacteria* as a BLAST database, circumvents this problem.

Interestingly, although some genera appeared to be predominant in all samples regardless of salinities, physical conditions, or locations (see groups 1 and 2 in Table S3 in the supplemental material), we observed distinct shifts in community structure between samples (Fig. 3). We argue that this is a reflection of the fact that, beyond the few highly abundant genera that seem to be salinity indifferent, a highly dynamic community of salinity-sensitive genera with moderate to low abundance (see groups 3, 4, and 5 in Table S3) exists and is responsible for the observed differences in community structure between samples. Indeed, in classifying genera belonging to each of the groups in Table S3 according to their response to salinity (salinity indifferent versus salinity sensitive), we saw that 67% of the group showing consistently high abundance (group 1 in Table S3) and 60% of the group showing moderately high abundance (group 2 in Table S3) are salinity-indifferent genera. Similarly, 67% of the group showing consistently low abundance (group 4 in Table S3) and 60% of the group showing occasionally low abundance (group 5 in Table S3) are salinity-sensitive genera.

Perhaps the most interesting observation in this study is the identified strong negative correlation between salinity and possession of genes for trehalose biosynthesis. *otsAB*-harboring genera showed a distinct negative correlation between abundance and salinity in genus-level salinity-abundance correlations (Fig. 5 and 6; see also Fig. S5 and Table S4 in the supplemental material). Further, while the combined percentages of abundance of all sequences affiliated with *otsAB*-harboring genera never exceeded 40% in any data set, a strong negative correlation to salinity was shown (Fig. 5). Finally, quantification of *Halobacteria otsB*/16S rRNA gene ratio (Fig. 6) confirmed the notion that the percentages of abundance of genera capable of trehalose biosynthesis and accumulation decrease with increases in salt concentrations.

These results demonstrate that the recently recognized divergence between trehalose producers and nonproducers is ecologically relevant. The most prevalent mechanism for osmoadaptation in the *Halobacteria* is salting in, where cells accumulate molar concentrations of potassium ions intracellularly to counter the high extracellular osmotic pressure. The salting-in strategy has long been demonstrated in model *Halobacteria* isolates such as *Halobacterium salinarum*, *Haloarcula marismortui*, *Haloferax volcanii*, *Haloferax mediterranei*, *Haloferax gibbonsii*, *Halorubrum saccharovorum*, and *Halorubrum trapanicum* (56–62) and, more recently, in a large number ($n = 18$) of *Halobacteria* taxa (37). Further, a pathway for the dependence on the H^+/K^+ symporter of the Trk family for potassium uptake has recently been proposed on the basis of an extensive genomic survey of 80 different *Halobacteria* genomes (63). In addition to the salting-in strategy, recent studies have led to an increasing appreciation of the role played by another osmoadaptive mechanism, compatible solute accumulation, as a supplemental strategy in species of numerous taxa within the *Halobacteria* (37, 64–66). We have recently demonstrated that the biosynthesis and accumulation of molar levels of trehalose (or 2-sulfotrehalose) occur in multiple genera within the *Halobacteria* and that the genes mediating the process (the *otsAB* operon) are present in 61/80 examined genomes (37). Interestingly, trehalose biosynthesis capability within the *Halobacteria* appears to follow a phylogenetic pattern, where all genera within a major clade either possess or lack trehalose biosynthetic capability. We argue that this pattern is a reflection of the benefits/costs of utilizing this system for osmoadaptation under different environmental conditions. In habitats of low and fluctuating salinity, a compatible solute strategy provides much-needed flexibility in responding to the salinity fluctuations frequently encountered, thus justifying the energetic costs associated with the production of molar quantities of this divalent sugar. In permanently hypersaline habitats, the energetic cost associated with this process leads to a growth rate lower than that seen with non-trehalose producers, decreased ecological fitness, and eventual out-competition from such ecosystems. It is telling that *otsAB*-harboring genera are rarely identified in typical hypersaline water bodies (1, 2, 25, 67, 68), attesting to the ecological advantages imparted by the loss of this gene system (or of the compatible solute osmoadaptive strategy as a whole) to allow niche colonization and dominance in hypersaline habitats.

ACKNOWLEDGMENTS

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