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ORIGINAL ARTICLE

Trehalose/2-sulfotrehalose biosynthesis and glycine-betaine uptake are widely spread mechanisms for osmoadaptation in the *Halobacteriales*

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We investigated the mechanisms of osmoadaptation in the order *Halobacteriales*, with special emphasis on *Haladaptatus paucihalophilus*, known for its ability to survive in low salinities. *H. paucihalophilus* genome contained genes for trehalose synthesis (trehalose-6-phosphate synthase/trehalose-6-phosphatase (OtsAB pathway) and trehalose glycosyl-transferring synthase pathway), as well as for glycine betaine uptake (BCCT family of secondary transporters and QAT family of ABC transporters). *H. paucihalophilus* cells synthesized and accumulated ~1.97–3.72 μmol per mg protein of trehalose in a defined medium, with its levels decreasing with increasing salinities. When exogenously supplied, glycine betaine accumulated intracellularly with its levels increasing at higher salinities. RT-PCR analysis strongly suggested that *H. paucihalophilus* utilizes the OtsAB pathway for trehalose synthesis. Out of 83 *Halobacteriales* genomes publicly available, genes encoding the OtsAB pathway and glycine betaine BCCT family transporters were identified in 38 and 60 genomes, respectively. Trehalose (or its sulfonated derivative) production and glycine betaine uptake, or lack thereof, were experimentally verified in 17 different *Halobacteriales* species. Phylogenetic analysis suggested that trehalose synthesis is an ancestral trait within the *Halobacteriales*, with its absence in specific lineages reflecting the occurrence of gene loss events during *Halobacteriales* evolution. Analysis of multiple culture-independent survey data sets demonstrated the preference of trehalose-producing genera to saline and low salinity habitats, and the dominance of genera lacking trehalose production capabilities in permanently hypersaline habitats. This study demonstrates that, contrary to current assumptions, compatible solutes production and uptake represent a common mechanism of osmoadaptation within the *Halobacteriales*.

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Introduction

Members of the halophilic archaea (order *Halobacteriales*) are characterized by an absolute salt requirement and conspicuous growth in hypersaline habitats where they often represent the majority of microbial biomass. To survive in high salinity environments, the *Halobacteriales* maintain an intracellular osmotic pressure that is equal to or higher than that of the surrounding environment (Oren, 2013). In general, maintenance of high intracellular osmotic pressure by halophilic and halotolerant microorganisms could be achieved either by the uptake and accumulation of high concentrations of inorganic ions such as K^+ (salting-in strategy) or by synthesis and/or uptake of highly soluble organic solutes that do not interfere with intracellular enzymatic activities and cellular processes (compatible solute strategy) (Grant, 2004).

Within the *Halobacteriales*, utilization of salting-in strategy for osmoadaptation has been demonstrated in multiple model species such as

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Halobacterium salinarum, *Haloarcula marismortui*, *Haloferax volcanii*, *Haloferax mediterranei*, *Haloferax gibbonsii*, *Halorubrum saccharovorum and Halorubrum trapanicum* (Christian and Waltho, 1962; Ginzburg *et al.*, 1970; Lanyi and Silverman, 1972; Matheson *et al.*, 1976; Pérez-Fillol and Rodríguez-Valera, 1986; Mojica *et al.*, 1997; Oren *et al.*, 2002a). These studies reported high levels of intracellular K^+ (1.9–5.5 M) in all examined species. Intracellular K^+ levels were shown to be dependent on the extracellular salt concentrations, with its levels increasing with the increase of the total salinity of the growth media (Pérez-Fillol and Rodríguez-Valera, 1986). This salting-in strategy for osmoadaptation is more energetically favorable when compared with the alternative compatible solute accumulation strategy, as the latter requires the synthesis and accumulation of increasing amounts of organic solutes at higher salinities. However, the permanently high levels of intracellular K^+ trigger multiple structural and metabolic adaptations that limit the metabolic flexibility and ecological range of microorganisms utilizing this strategy for osmoadaptation (Oren, 2013).

In addition to their prevalence in hypersaline habitats, members of the *Halobacteriales* are also consistently observed in saline (10–17% NaCl) environments as a fraction of a more complex halophilic and halotolerant prokaryotic and eukaryotic population (Bowman *et al.*, 2000; Perreault *et al.*, 2007; Stivaletta *et al.*, 2011; Youssef *et al.*, 2012; Xiao *et al.*, 2013). Further, members of the *Halobacteriales* have also been identified as a minor constituent of the microbial population in low-salinity environments including sea water (Ventosa *et al.*, 1984), seawater aquarium (Inoue *et al.*, 2011), anaerobic springs (Elshahed *et al.*, 2004), low salinity salterns (Mani *et al.*, 2012), coastal salt marshes (Munson *et al.*, 1997, Purdy *et al.*, 2004), and hydrothermal (Takai *et al.*, 2001) and geothermal (Ellis *et al.*, 2008) vents, where pockets of salinity occasionally develop. Therefore, in such habitats, the *Halobacteriales* must cope with extended periods of low salinities and/or constant fluctuations in salinity levels, conditions that are not conducive to microorganisms solely dependent on a salting-in strategy for osmoadaptation.

Prior investigations of osmoadaptive strategies within the *Halobacteriales* have been conducted on isolates obtained from permanently hypersaline environments, for example, *Halobacterium salinarum* strain NRC 34002, *Haloferax mediterranei* and *Haloarcula marismortui* (isolated from the dead sea (Oren *et al.*, 1990)). Here, we decided to investigate the potential for alternative mechanisms of osmoadaptation in multiple *Halobacteriales* genera, with special emphasis on *Haladaptatus paucihalophilus*, known for its capability to grow in low and fluctuating salinities and survive extended incubations in distilled water (Savage *et al.*, 2007). While few prior studies on the production (Nicolaus *et al.*, 1988; Desmarais *et al.*, 1997; Goh *et al.,* 2011) and uptake (Kokoeva *et al.*, 2002) of compatible solutes in the *Halobacteriales* have been published, detailed understanding of the extent of distribution, genetic basis and ecological significance of the process is lacking. We demonstrate that multiple *Halobacteriales* genera are capable of trehalose or 2-sulfotrehalose production, and that the uptake of glycine betaine is a widely utilized process for osmoadaptation in the *Halobacteriales*. These results suggest that the osmoadaptive strategies of the *Halobacteriales* are more complex than previously implied from prior studies on model *Halobacteriales* strains.

Materials and methods

Archaeal strains and growth media

H. paucihalophilus strain DX253^T (DSM18195) was isolated (Savage *et al.*, 2007) and maintained in our laboratory. All other strains were either kindly provided by Dr Marc T Facciotti (University of California Davis) or purchased from the German Collection of Microorganisms (DSMZ, Braunschweig, Germany). *H. paucihalophilus* was grown in a defined media similar in composition to DSMZ 1125 but with no yeast extract and with pyruvate rather than sucrose as a carbon source. This was done to ensure that any compatible solutes detected in cell extracts are synthesized *de novo*. All other strains were cultured in their DSMZ media at three different salt concentrations (Supplementary Table S1).

Genomic sequencing

The genome of *H. paucihalophilus* strain DX253^T was sequenced using the service of a commercial sequencing provider (Engencore, now Selah Genomics, Columbia, SC, USA) using 454 FLX technology. The genome was assembled using Newbler assembler (Branford, CT, USA) into 32 Contigs (N50 = 267, 250 bp, N90 = 1 00 497 bp). Gene calling, annotation and metabolic construction were conducted using the Department of Energy Integrated Microbial Genomes (IMG) platform (Markowitz *et al.*, 2012).

Analytical methods

Identification of and quantification of K^+ and compatible solutes in cell-free extracts. Details on the methods for identification and quantifications of ions and compatible solutes are presented as Supplementary Text. In brief, the presence and identity of compatible solutes in ethanolic cell-free extracts were determined using ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR) and high performance liquid chromatography (HPLC). Quantification of various compatible solutes and ions

was done in perchloric acid cell-free extracts (Supplementary Text). Trehalose was determined enzymatically using using a trehalase assay kit (Megazyme, Wicklow, Ireland). To differentiate trehalose from 2-sulfotrehalose, which is also detectable using this kit, we devised a fast method to detect the presence of a 2′-O-sulfonate group based on the action of abalone snail sulfatase (Sigma-Aldrich, St Louis, MO, USA), shown to be specific for cleaving the 2′-O-sulfonate group of trehalose (Uzawa *et al*., 2004), followed by quantification of the released inorganic sulfate turbidimetrically (Lundquist *et al*., 1980) (Supplementary Text). Glycine betaine was quantified using a colorimetric assay for quaternary ammonium compounds, as described before (Grieve a*n*d Grattan, 1983). Intracellular K^+ levels were quantified in cell-free extracts of various *Halobacteriales* species spectophotmetrically (Takagi *et al*., 1981). Intracellular free amino acids in cell-free extracts were assayed for free amino acids using the services of the Protein Chemistry Lab of Texas A&M University (Supplementary Text)

RNA extraction and gene expression. Cell pellets from late exponential phase cultures were solubilized in TRI Reagent Solution (Ambion, Carlsbad, CA, USA) and processed according to the manufacturer's instruction. Contaminating DNA was removed from all RNA preparations using RQ1 DNase (Promega, Madison, WI, USA). DNA-free RNA was then precipitated using Lithium Chloride Precipitation Solution (Ambion) according to the manufacturer's instructions to remove any contaminating small RNAs. Total RNA reverse transcription (cDNA construction) was conducted on 1 μg of RNA preparations using Superscript III first-strand synthesis kit (Invitrogen, Carlsbad, CA, USA) with random hexamers (Invitrogen). Reverse transcription qPCR (RT-qPCR) was conducted on the cDNA obtained using primers shown in Supplementary Table S2. Levels of expression of different genes were compared with glyceraldehyde-3-phosphate dehydrogenase gene expression (Supplementary Text).

Phylogenetic analysis. RNA polymerase *β*-subunit (RpoB′) protein sequences from publicly available *Halobacteriales* genomes ($n=82$) were aligned in ClustalX (Larkin *et al*., 2007) and used to construct a maximum likelihood tree in Mega (Tamura *et al*., 2011) using a WAG model with Γ distribution of rates among sites as previously suggested (Minegishi *et al*., 2010). *Halobacteriales* OtsA (trehalose-6-phosphate synthase) and OtsB (trehalose-6-phosphatase) protein sequences, as well as those from reference bacterial and archaeal taxa were also aligned using ClustalX, and the best protein substitution model was predicted in Mega (Tamura *et al*., 2011). The predicted model was applied in maximum likelihood tree construction and support values were obtained using 100 bootstrapping events.

Ecological distribution of otsAB-harboring genera. We examined the correlation between the presence/absence of trehalose synthesis *otsAB* genes in a genome and the respective salt minimum, maximum and optimum concentrations using χ^2 Contingency tables for correlation (Plackett, 1983) (see Supplementary Text for details). To study the correlation between *otsAB* presence and the ecological distribution of various genera, we re-examined the relative abundance of various *Halobacteriales* genera in 13 different environments with salinities ranging from permanently hypersaline ($n=9$), saline and fluctuating salinity habitats (where salinity is usually $<250\,g\,l^{-1}$ and exhibits fluctuation; $n=3$), and mostly non-saline with only occasional development of temporal and spatial saline pockets ($n=1$, Zodletone spring). Relative abundances of *Halobacteriales* genera with and without the OtsAB system in their genome were compared between various groups of environments using Student's *t*-test. Values were considered significantly different if the *P*-value associated with the *t*-test was <0.0038 (equivalent to an α value of 0.05 corrected for 13 pairwise comparisons using the Bonferroni correction). The relative abundances values obtained were also used in a principal-component analysis to evaluate the differences in genera composition between the environments using the R statistical package (R Development Core Team, 2011).

GenBank accession number. The genome of *H. paucihalophilus* has been deposited in Genbank under the accession number AEMG00000000.

Results

General features and osmoadaptive capabilities inferred from H. paucihalophilus genome

The general features of *H. paucihalophilus* genome are shown in Supplementary Table S3. The genome and predicted proteome displayed several features characteristic of the *Halobacteriales* such as high GC content (61.8%), a higher proportion of acidic residues (Glu + Asp = 15.95%), lower proportion of basic residues (Lys + Arg = 8.79%), lower proportion of cysteine residues (0.73%) and lower proportion of large hydrophobic residues (Ile + Leu + Met + Phe = 18.98%). The predicted proteome also showed a low average isoelectric point (pI = 5.12), with the pI of cytoplasmic proteins (average pI = 4.69) significantly lower (Student's *t*-test *P*-value = $2.4E^{-205}$) than the pI of membrane proteins (average pI = 6.88) (Supplementary Table S3, S4).

We queried the *H. paucihalophilus* genome for genes putatively involved in osmoadaptation. As expected, the genome contained all genes necessary

for Na^+ extrusion, K^+ uptake and chloride homeostasis (Supplementary Text). More importantly, the genome contained genes necessary for the synthesis of trehalose. Out of the five mechanisms known for trehalose biosynthesis (Avonce *et al.*, 2006), two were identified in the genome: the trehalose-6-phosphate synthase/trehalose-6-phosphatase (OtsA/B) pathway and the trehalose glycosyl-transferring synthase pathway (TreT) (Table 1). The genome contained two copies of the trehalose-6-phosphate synthase gene (otsA), one copy of trehalose-6-phosphatase (otsB) clustered with one of the otsA genes and one copy of trehalose glycosyl-transferring synthase TreT (Table 2). No genes for the synthesis of other compatible solutes such as glycine betaine or ectoine were identified. However, the genome contained genes necessary for the uptake of glycine betaine as evident by the presence of genes encoding the two major betaine transporter families: The Betaine/Carnitine/Choline Transporter (BCCT) Family of secondary transporters (TC 2.A.15), and the Quaternary Amine Uptake Transporter (QAT) Family of ABC transporters (TC 3.A.1.12) (Table 1).

Trehalose biosynthesis and glycine betaine uptake by H. paucihalophilus

Trehalose was identified using ^{1}H-NMR in cell-free extracts of *H. paucihalophilus* cells grown on a defined yeast extract-free medium with pyruvate as the sole carbon source (Figure 1). Trehalose was differentiated from 2-sulfotrehalose previously identified in the cell-free extracts of several *Halobacteriales* spp. (Desmarais *et al.*, 1997) by differences in the ^{1}H chemical shifts at the C-2 position. The trehalose spectrum shows similar resonances for C-2 and C-2′ of ~3.63–3.64 p.p.m. (Figure 1, blue), whereas 2-sulfotrehalose shows a downfield shift for one of the C-2 positions, due to the presence of the sulfontate group, to 4.35 p.p.m. (Desmarais *et al.*, 1997). Further, the absence of 2-sulfotrehalose was confirmed by the lack of free inorganic sulfate accumulation after treatment of cell-free extracts with C-2-specific abalone snail sulfatase, using *Natronococcus occultus*, *Natrialba magadii* and *Natronobacterium gregoryi* as positive controls. No evidence for the accumulation of other polyols (for example, glycerol), zwitterions (for example, glycine betaine) or free amino acids was observed using ^{1}H-NMR scans or HPLC. Intracellular trehalose concentrations in *H. paucihalophilus* cells were highest when grown at lower salinities, and progressively decreased in cultures grown at higher salinities (from 3.72 μmol per mg protein of trehalose when grown at 75 g l^{-1} salinity to 1.97 μmol per mg protein of trehalose when grown at 275 g l^{-1} salinity) (Figure 2a). Intracellular K^+ levels in *H. paucihalophilus* were ~17 times that measured in *E. Coli* K12 cells grown in LB media (Figure 2c), but only a fraction (0.55 times at 125 g l^{-1} salinity to 0.28 times at 275 g l^{-1} salinity) of the intracellular K^+ concentration measured in *Halobacterium salinarum* strain NRC-1. Interestingly intracellular K^+ levels in *H. paucihalophilus* were relatively stable at all salinities examined, as opposed to the salinity-dependent increase in intracellular K^+ concentrations observed in *Halobacterium salinarum* (Figure 2a; Pérez-Fillol and Rodríguez-Valera (1986)).

No glycine betaine was observed in cell-free extracts of *H. paucihalophilus* grown in a defined media (Figure 2a). However, when exogenously supplied (10 g l^{-1}), cells of *H. paucihalophilus* accumulated large amounts of glycine betaine intracellularly in a salinity-dependent pattern (0.9 μmol per mg protein of betaine when grown at 75 g l^{-1} salinity to 3.21 μmol per mg protein of betaine when grown at 275 g l^{-1} salinity; Figure 2b). Overall patterns of intracellular K^+ and trehalose accumulation in the presence of glycine betaine (Figure 2b) were comparable to those observed in defined media (Figure 2a). Although trehalose levels were slightly lower in presence of glycine betaine, these differences were only significant at higher salinities (175 and 225 g l^{-1}, Student's *t*-test *P*-value = 0.017 and 0.007, respectively).

Table 1 Genes for trehalose synthesis and betaine uptake in *Haladaptatus paucihalophilus* strain DX253 genome

Genes	*GenBank accession numbers*
Trehalose biosynthesis	
From UDP-glucose (OtsAB system)	
Trehalose-6-phosphate synthase (otsA)	EFW91797, EFW92049
Trehalose-6-phosphatase (otsB)	EFW92050
From ADP-glucose (TreT)	
Trehalose glucosyltransferase (EC: 2.4.1.245)	EFW90639
Betaine uptake	
Secondary transporters	
Betaine/carnitine/choline transporter (BCCT)	EFW91498, EFW90175
ATP-binding cassette transporter, proline/glycine betaine transport systems (ABC)	
ATPase component	EFW90134
Permease components	EFW90133, EFW90135
Substrate-binding component	EFW90132

Transcriptional analysis of trehalose synthesis genes in H. paucihalophilus

We used RT-qPCR to quantify gene expression levels of all four genes putatively involved in trehalose biosynthesis in *H. paucihalophilus*. Expression levels of the complete OtsAB system genes (*otsA2* and *otsB* genes) were 3–4 orders of magnitude higher than that of *otsA1* or *treT* genes (Table 2). Further, the pattern of gene expression of the complete OtsAB system mirrored the pattern of intracellular trehalose accumulation observed at various salinities, where gene expression was

Table 2 Transcription levels of various trehalose-biosynthesis genes in *Haladaptatus paucihalophilus* strain DX253 genome as compared with glyceraldehyde-3-phosphate dehydrogenase transcription level

Salt conc (%)	*Copies relative to glyceraldehyde-3-P dehydrogenase gene*[a]			
	otsA (EFW91797)	*otsA (EFW92049)*	*otsB (EFW92050)*	*Glucosyltransferase (EFW90639)*
7.5	1.51 ± 0.01	4687.7 ± 92.41	2015.8 ± 49.52	0.66 ± 0.13
12.5	0.45 ± 0.003	1848.2 ± 58.89	998.47 ± 40.22	1.9 ± 0.39
17.5	ND	150.87 ± 6.87	81.87 ± 1.42	0.25 ± 0.01
22.5	0.005 ± 0.001	3.54 ± 1.14	1.66 ± 0.54	0.15 ± 0.06
27.5	0.003 ± 0.0004	2.84 ± 0.46	0.74 ± 0.01	0.004 ± 0.001

Abbreviations: ND, not detected; otsA, trehalose-6-phosphate synthase; otsB, trehalose-6-phosphatase.
[a]Numbers are averages ± standard deviations from triplicate cultures at each salt concentration.

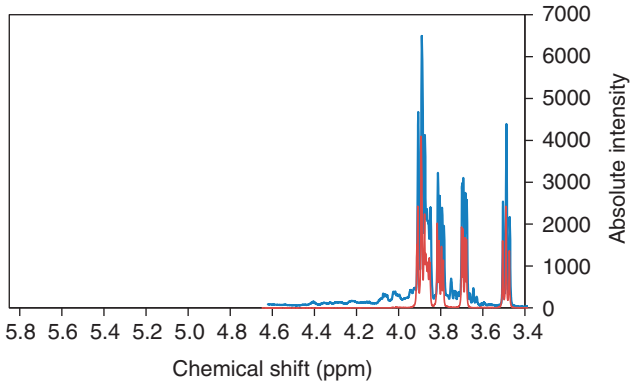

Figure 1 ^{1}H-NMR spectrum of *H. paucihalophilus* ethanolic extracts (blue) compared to standard trehalose (red).

highest at lower salinity (2016–4688 times that of glyceraldehyde-3-phosphate expression at 75 g l^{-1} salinity) and progressively decreased at higher salinities (to 0.74–2.84 times that of glyceraldehyde-3-phosphate expression at 275 g l^{-1} salinity) (Table 3). These results strongly suggest the involvement of the complete OtsAB system in trehalose synthesis in *H. paucihalophilus*.

Genes mediating trehalose synthesis and betaine uptake are widely distributed in Halobacteriales genomes

Out of 83 genomes belonging to 28 different *Halobacteriales* genera examined, a complete OtsAB system was identified in 39 different species comprising 17 different genera (*Haladaptatus*, *Halalkalicoccus*, *Halosimplex*, *Halococcus*, *Halovivax*, *Halobiforma*, *Haloterrigena*, *Halobacterium*, *Halogranum*, *Halosarcina*, *Halopiger*, *Natrialba*, *Natronobacterium*, *Natronococcus*, *Natronorubrum*, *Natronolimnobius* and *Natrinema*; Table 3). The presence or absence of a complete OtsAB system was a defining characteristic in all genera: within any *Halobacteriales* genus; all species either possessed or lacked a complete OtsAB system. Additional systems of trehalose production (trehalose glycosyl-transferring synthase (TreT), trehalose synthase (TreS) and trehalose phosphorylase (TreP) but not maltooligosyl-trehalose synthase/maltooligosyl-trehalose trehalohydrolase (TreYZ)) were sporadically distributed in few of the sequenced genomes (Table 3).

Genes for glycine betaine synthesis from glycine were not identified in any of the *Halobacteriales* genomes examined. However, genes encoding representatives of the BCCT family of betaine transporter were identified in 60 *Halobacteriales* genomes and genes encoding the complete ABC betaine transporter were identified in 13 genomes (Table 3). Three distinct patterns for the distribution of glycine betaine uptake genes were identified: (1) genera or species with no evidences for betaine transport (for example, members of the genera *Halobiforma*, *Halostagnicola*, *Haloplanus*, *Halobaculum*, and *Halomicrobium* and multiple species within the genera *Haloferax*, *Halorubrum*, *Halorhabdus*, *Halobacterium*, *Natronomonas*, *Natrialba* and *Natrinema*; (2) genera or species with only the BCCT transporters (for example, genera *Halobacterium*, *Halalkalicoccus*, *Haloterrigena*, *Haloarcula*, *Halosimplex*, *Halovivax*, *Haloquadratum*, *Halogeometricum*, *Halogranum*, *Halopiger*, *Natronobacterium* and *Natronolimnobius*, and multiple species within the genera *Halococcus*, *Halorhabdus*, *Haloferax*, *Halorubrum*, *Natronomnas*, *Natrialba*, *Natronorubrum*, *Natronococcus* and *Natrinema*) and (3) genera or species with representatives of both families of transporters (for example, genera *Haladaptatus* and *Halosarcina* and various species within the genera *Halococcus*, *Haloferax*, *Natronorubrum*, *Natronococcus* and *Natrinema*) (Table 3).

Experimental evaluation of trehalose/2-sulfotrehalose production and glycine uptake within the Halobacteriales

We quantified the intracellular levels of trehalose and glycine betaine in 17 different *Halobacteriales* species grown at different salinities that are close to their low, optimum and high salt concentrations (Table 3 and Supplementary Table S5). Trehalose production was observed in the examined *otsAB*-harboring species *Halovivax ruber*, *Halococcus saccharolyticus*, *Halosarcina pallida*, *Halogranum*

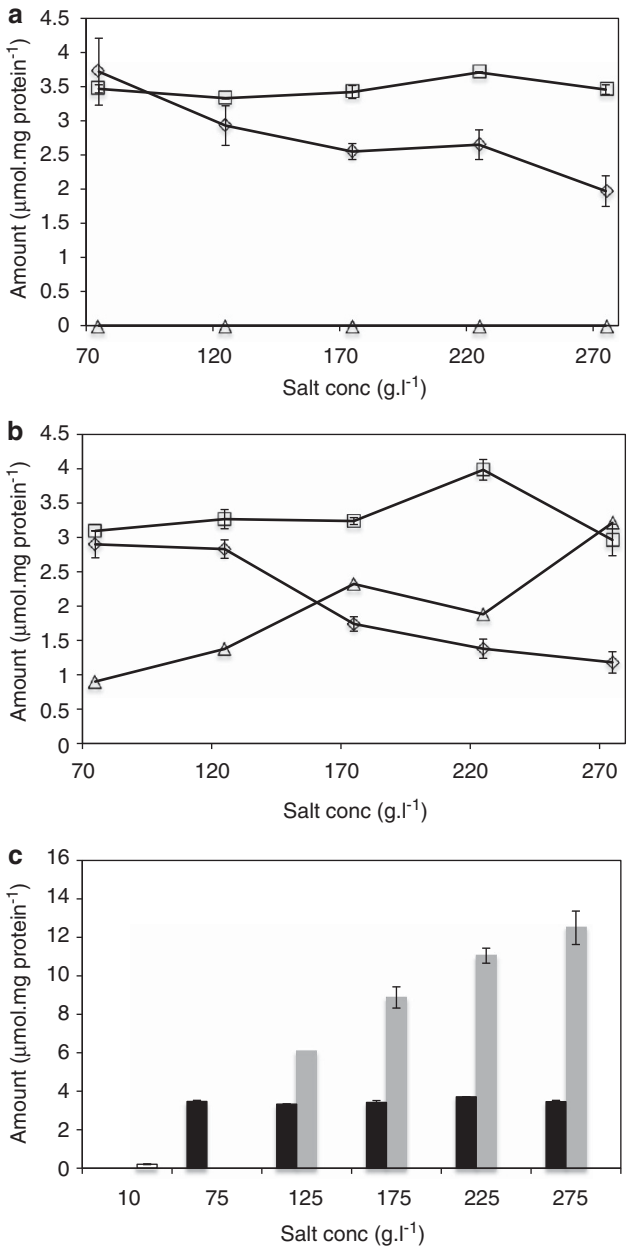

Figure 2 Trehalose (-<>-), glycine betaine (-Δ-) and intracellular potassium levels (□) in *H. paucihalophilus* grown in defined medium (**a**) and in the presence of 1% betaine (**b**). Comparison of *H. paucihalophilus* intracellular potassium levels (■) to those in *Halobacterium salinarum* strain NRC-1 (▒) and *E. coli* (□) is shown in (**c**). Intracellular K+ are levels are determined only at 10 g l^{-1} for *E. coli* due to its inability to grow at higher salinities.

rubrum, *Haloterrigena turkmenica* and *Natronorubrum tibetense*, whereas 2-sulfotrehalose production was observed in the examined *otsAB*-harboring species *Natrialba magadii*, *Natronococcus occultus*, *Natronobacterium gregoryi*, *Natrinema pellirubrum* and *Halalkalicoccus jeotgali*. No trehalose was produced in examined species lacking the OtsAB system (*Haloferax mediterranei*, *Halorhabdus utahenesis*, *Halogeometricum borinquense*, *Halostagnicola larsenii*, *Halorubrum lacusprofundi* and *Halobacterium salinarum* NRC-1) (Table 3). Similar to patterns observed in *H. paucihalophilus*, trehalose or 2-sulfotrehalose concentrations were highest at lower salinities (with the exception of *Halovivax ruber*, *Halosarcina pallida* and *Natrinema pellirubrum*) (Supplementary Table S5).

Several patterns of glycine betaine accumulation were observed amongst the examined species. Strains with no genomic evidences of both betaine uptake systems did not accumulate betaine (for example, *Halostagnicola larsenii*). Strains with genomic evidence of both families of betaine uptake accumulated betaine (for example, *Halococcus saccharolyticus*, *Halosarcina pallida* and *Haloterrigena turkmenica*). Within strains harboring only the BCCT family of betaine uptake transporters, some species did not accumulate betaine (*Halalkalicoccus jeotgali*, *Halovivax ruber*, *Natrialba magadii* and *Natronobacterium gregoryii*), whereas others did (*Halorhabdus utahenesis*, *Haloferax medeterranei*, *Halogeometricum borinquense*, *Halorubrum lacusprofundi*, *Natrinema pellirubrum*, *Natronococcus occultus* and *Halogranum rubrum*) (Table 3). Intracellular glycine betaine levels in all glycine betaine-accumulating species examined were salinity-dependent (Supplementary Table S5). While these results do not exclusively establish the role of either system in glycine betaine uptake, they suggest that (1) the BCCT system can function in some strains as the sole mediator of glycine betaine uptake and (2) the presence of a BCCT family representative in a genome does not necessarily translate into betaine uptake capabilities.

Intracellular K^+ levels in trehalose- and 2-sulfotrehalose-producing strains ranged between 2.1 and 9.6 µmol per mg protein and, in general, were fairly constant at various levels of salinities examined (Supplementary Table S5, with the exception of *Halosarcina pallida*). On the other hand, intracellular K^+ levels in genera incapable of trehalose or 2-sulfotrehalose production were significantly higher (4.19–29.7 µmol per mg protein) (Student's *t*-test *P*-value = 1.57E^{-6}), and in general, increased with the increase of media salinity (Supplementary Table S5).

Phylogeny and distribution of Halobacteriales OtsAB system genes

Halobacteriales OtsA- and OtsB-predicted protein sequences showed high level of homology (average sequence identities of 67.59% and 60.76% for OtsA and OtsB, respectively). Within OtsA and OtsB protein trees, *Halobacteriales* proteins formed a coherent, deep-branching cluster that was only distantly related to sequences from other bacterial and archaeal phyla (Figures 3a and b). Further, *Halobacteriales* OtsA and OtsB phylogenies mirrored those inferred from reference 16S rRNA

Table 3 Genomic and experimental evidence for trehalose biosynthesis and glycine betaine transport in halophilic archaea with sequenced genomes

Genus	*Species sequenced*	*Genomic evidence*				*Experimental evidence*[a]	
		Trehalose biosynthesis[b]		*Glycine betaine uptake*[c]		*Tre/STre*	*GB*
		OtsAB	*Other*[d]	*BCCT*	*ABC*		
Haladaptatus	***paucihalophilus***	Y	Y (TreT)	Y	Y	Y (Tre)	Y
Halococcus	*hamelinensis*	Y	N	Y	Y		
	morrhuae	Y	N	Y	Y		
	saccharolyticus	Y	N	Y	Y	Y (Tre)	Y
	salifodinae	Y	N	Y	N		
	thailandensis	Y	N	Y	Y		
Halogranum	***salarium***	Y	N	Y	N	Y (Tre)	Y
Halosarcina	***pallida***	Y	Y (TreS)	Y	Y	Y (Tre)	Y
Haloterrigena	*limicola*	Y	N	Y	N		
	salina	Y	N	Y	N		
	thermotolerans	Y	N	Y	N		
	turkmenica	Y	N	Y	N	Y (Tre)	Y
Halovivax	*asiaticus*	Y	N	Y	N		
	ruber	Y	Y (TreP)	Y	N	Y (Tre)	N
Natronorubrum	*bangense*	Y	Y (TreP)	Y	Y		
	sulfidifaciens	Y	Y (TreT)	Y	N		
	tibetense	Y	N	Y	N	Y (Tre)	Y
Halobiforma	*lacisalsi*	Y	N	N	N		
	nitratireducens	Y	N	N	N		
Halopiger	*xanaduensis*	Y	Y (TreP)	Y	N		
Halosimplex	*carlsbadense*	Y	N	Y	N		
Halalkalicoccus	***jeotgali***	Y	N	Y	N	Y (STre)	N
Natrialba	*aegyptiaca*	Y	N	Y	N		
	asiatica	Y	N	Y	N		
	chahannaoensis	Y	N	N	N		
	hulunbeirensis	Y	N	Y	N		
	magadii	Y	Y (TreP)	Y	N	Y (STre)	N
	taiwanensis	Y	N	Y	N		
Natrinema	*altunense*	Y	N	N	N		
	gari	Y	N	N	N		
	pallidum	Y	N	N	N		
	pellirubrum	Y	N	Y	N	Y (STre)	Y
	versiforme	Y	N	Y	Y		
Natronobacterium	***gregoryi***	Y	Y (TreT)	Y	N	Y (STre)	N
Natronococcus	*amylolyticus*	Y	Y (TreP, TreT)	Y	Y		
	jeotgali	Y	Y (TreP)	Y	N		
	occultus	Y	Y (TreT)	Y	N	Y (STre)	Y
Natronolimnobius	*innermongolicus*	Y	Y (TreP)	Y	N		
Halobacterium	***salinarum str. NRC-***1	N	N	Y	N	N	Y
	Salinarum str. R1	N	N	Y	N		
Haloferax	*alexandrines*	N	N	Y	N		
	denitrificans	N	N	Y	Y		
	elongans	N	N	N	N		
	gibbonsii	N	N	Y	Y		
	larsenii	N	N	N	N		
	lucentense	N	N	Y	N		
	mediterranei	N	N	Y	N	N	Y
	prahovense	N	N	Y	N		
	sulfurifontis	N	N	Y	N		
	volcanii	N	N	Y	N		

Table 3 (Continued)

Genus	*Species sequenced*	*Genomic evidence*				*Experimental evidence*[a]	
		Trehalose biosynthesis[b]		*Glycine betaine uptake*[c]		*Tre/STre*	*GB*
		OtsAB	*Other*[d]	*BCCT*	*ABC*		
Haladaptatus	***paucihalophilus***	Y	Y (TreT)	Y	Y	Y (Tre)	Y
Halogeometricum	*borinquense*	N	N	Y	N	N	Y
Halorhabdus	*Tiamatea*	N	Y (TreS)	N	N		
	utahensis	N	N	Y	N	N	Y
Halorubrum	*aidingense*	N	N	Y	N		
	arcis	N	N	N	N		
	californiense	N	N	N	N		
	coriense	N	N	Y	N		
	distributum	N	N	N	N		
	ezzemoulense	N	N	N	N		
	kocurii	N	Y (TreS)	Y	N		
	lacusprofundi	N	Y (TreS)	Y	N	N	Y
	lipolyticum	N	Y (TreS)	N	N		
	litoreum	N	N	N	N		
	saccharovorum	N	N	Y	N		
	tebenquichense	N	N	Y	N		
	terrestre	N	N	N	N		
Halostagnicola	***larsenii***	N	N	N	N	N	N
Haloarcula	*amylolytica,*	N	N	Y	N		
	argentinensis	N	N	Y	N		
	californiae	N	N	Y	N		
	hispanica	N	N	Y	N		
	japonica	N	N	Y	N		
	marismortui	N	N	Y	N		
	sinaiiensis	N	N	Y	N		
	vallismortis	N	N	Y	N		
Halobaculum	*gomorrense*	N	N	N	N		
Halomicrobium	*Katesii*	N	N	N	N		
	mukohataei	N	N	N	N		
Haloplanus	*natans*	N	N	N	N		
Haloquadratum	*walsbyi*	N	N	Y	N		
Natronomonas	*Moolapensis*	N	N	N	N		
	pharaonsis	N	N	Y	N		

Species in which trehalose or 2 sulfotrehalose production and glycine betaine uptake was examined are in bold. Intracellular trehalose and glycine betaine values are given in Supplementary Table S5.
[a]Experimental evidence for either trehalose (Tre)/2-sulfotrehalose (STre) production or glycine betaine (GB) uptake in the species tested.
[b]Genomic evidence for trehalose biosynthesis in all sequenced species shown in column 2. Ots refers to the complete trehalose-6-phosphate synthase/trehalose-6-phosphatase (OtsAB) system. In some cases such as in *Haloarcula* genomes, an incomplete system was identified with just the *otsA* gene.
[c]Genomic evidence for glycine betaine uptake in all sequenced species. BCCT refers to Betaine/Carnitine/Choline Transporter (BCCT) Family of secondary transporters (TC 2.A.15), whereas ABC refers to the Quaternary Amine Uptake Transporter (QAT) Family of ABC transporters (TC 3.A.1.12).
[d]Other systems identified in some genomes. TreS system comprises trehalose synthase enzyme (EC 5.4.99.16) belonging to the glycosyltransferase family 13 (GT13). TreT system comprises the enzyme trehalose glycosyl-transferring synthase (EC 2.4.1.245) that catalyzes the transfer of a glucose moiety from ADP-glucose to a glucose molecule in an α1–α1 linkage, thereby producing trehalose. TreP system comprises the enzyme trehalose phosphorylase (EC 2.4.1.231) catalyzing the reversible hydrolysis of trehalose into glucose and glucose-1-phosphate.

and RpoB′ trees (Figure 4) (Walsh *et al.*, 2004; Enache *et al.*, 2007; Minegishi *et al.*, 2010). Collectively, these patterns suggest that trehalose production is an ancient trait within the *Halobacteriales* rather than a recent acquisition through horizontal gene transfer.

We further examined the occurrence pattern of the *ostAB* genes within the *Halobacteriales* by constructing an RpoB′ tree of all species with sequenced genomes (Figure 4). We identified a distinct distribution pattern of the *ostAB* genes at the supra-genus level. This pattern suggests the occurrence of

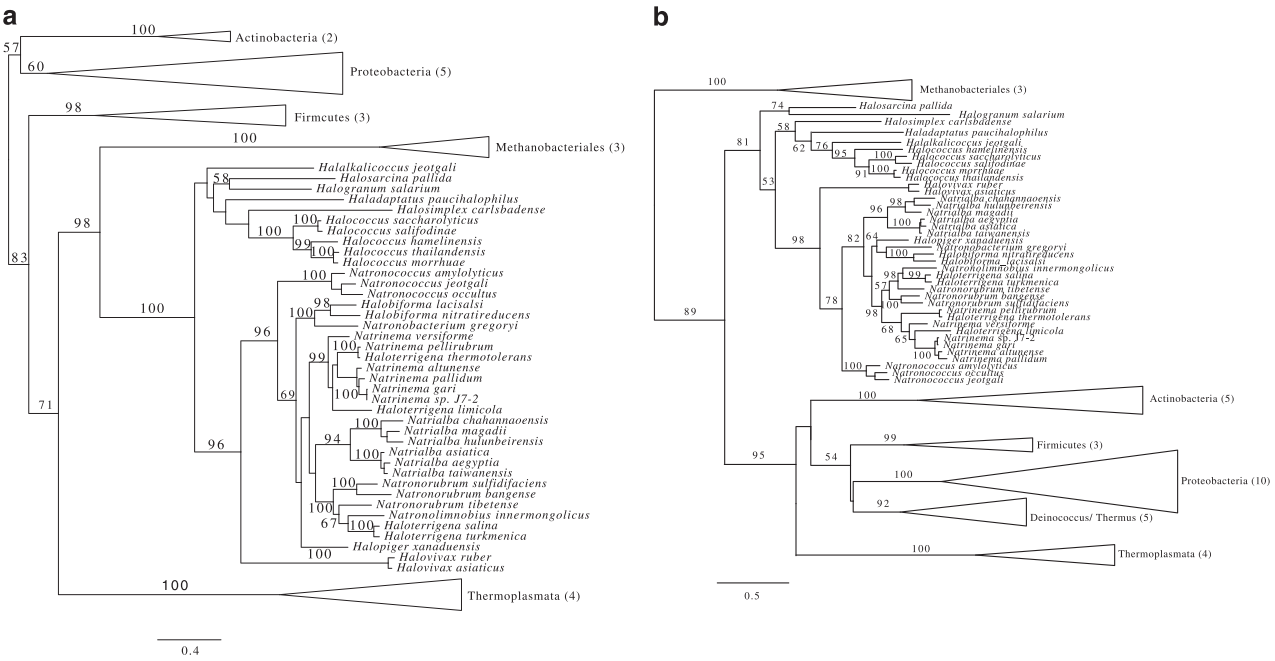

Figure 3 Maximum likelihood phylogenetic tree inferred from: (**a**) trehalose-6-phosphate synthase (OtsA) and (**b**) trehalose-6-phosphatase (OtsB) protein sequence alignments for members of Halobacteriaceae as well as other bacterial and archaeal species. The optimal model used as predicted by Mega was WAG + Γ with γ shape parameter = 1.03, and proportion of invariable sites ($I = 0.016$). Bootstraps values (100 replicates) are shown for the nodes with >50 bootstrap support. The tree was rooted using OtsA/B protein sequence of *Schizosaccharomyces japonicum*. Numbers in parenthesis represent the number of taxa comprising each wedge.

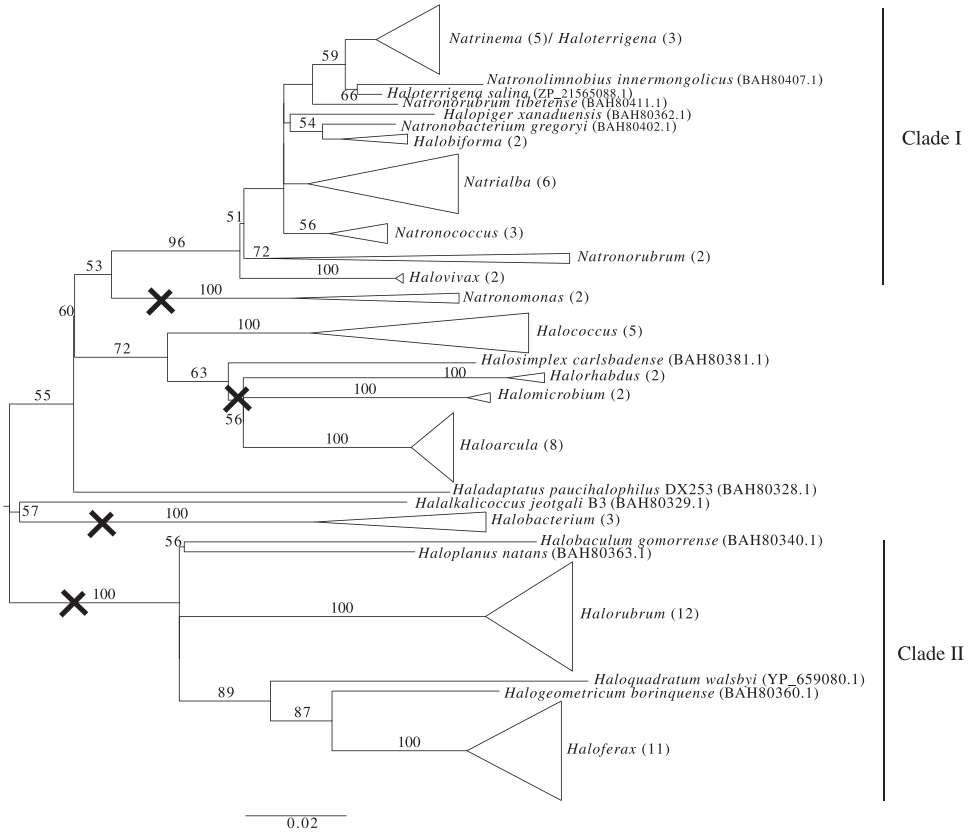

Figure 4 Maximum likelihood phylogenetic tree inferred from RNA polymerase β-subunit (RpoB′) protein sequence alignment for members of the *Halobacteriales*. The optimal model used was WAG + Γ as suggested by Minegishi *et al.* (2010). Bootstraps values (100 replicates) are shown for the nodes with more than 50 bootstrap support. × indicates putative incidences of gene loss events during *Halobacteriales* evolution. Numbers in parenthesis represent the number of taxa comprising each wedge.

several gene loss events during *Halobacteriales* evolution. *otsAB* genes were present in all members of *Halobacteriales* Clade I (as defined by Walsh *et al.* (2004), Enache *et al.* (2007) and Minegishi *et al.* (2010), including the genera *Halovivax*, *Natronobacterium*, *Halobiforma*, *Natronorubrum*, *Haloterrigena*, *Halopiger*, *Natronolimnobius*, *Natrinema*, *Natrialba* and *Natronococcus*), as well as the genera *Halococcus*, *Haladaptatus*, *Halalkalicoccus* and *Halosimplex*. On the other hand, *otsAB* genes were absent in all members of Halobacteriales clade II (as defined by Walsh *et al.* (2004), Enache *et al.* (2007), Minegishi *et al.* (2010), including the genera *Haloferax*, *Halorubrum*, *Halogeometricum*, *Haloquadratum*, *Haloplanus* and *Halobaculum*), the *Halorhabdus–Halomicrobium–Haloarcula* clade, as well as within the genera *Halobacterium* and *Natronomonas*.

Ecological distribution of Halobacteriales genera with and without OtsAB system

Comparison of the minimum, optimum and maximum salt requirements between various *Halobacteriales* species revealed that *otsAB*-harboring genera have lower salt optima than those lacking the system (3.2 M NaCl for OtsAB harboring genera versus 3.8 M NaCl for OtsAB lacking genera, $P = 0.0372$, albeit weak, Cramer's V value $= 0.37$). No similar significant correlation was identified between *otsAB* occurrence and salt minima or maxima of various *Halobacteriales* species.

We quantified the relative abundances of *Halobacteriales* genera with and without OtsAB system in relation to the total *Halobacteriales* community with published genomes (referred to as 'sequenced *Halobacteriales* community' thereafter) in previously published data sets from 13 different environments with salinities ranging from permanently hypersaline ($n = 9$), saline and fluctuating salinity habitats (where salinity is usually $< 250\,g\,l^{-1}$, exhibits fluctuation and the *Halobacteriales* community is usually a fraction of a more diverse prokaryotic community) ($n = 3$) and mostly non-saline with only occasional development of temporal and spatial pockets ($n = 1$, Zodletone spring). OtsAB-lacking genera represented the majority of the sequenced *Halobacteriales* community in permanently hypersaline habitats such as the Dead Sea and Great Salt lake (87.9% and 85.7% of sequenced *Halobacteriales* communities, respectively) and different crystallizer ponds examined (100% of sequenced *Halobacteriales* community, Table 4). On the other hand, the saline and fluctuating salinity habitats examined had a population of both OtsAB-harboring (44.5–55.1% of the sequenced *Halobacteriales* community) and OtsAB-lacking genera. Finally, Zodletone spring, an environment that is mostly non-saline with only occasional development of temporal and spatial pockets, has the largest proportion of OtsAB-harboring genera (67.6% of the sequenced *Halobacteriales* community). Principal component analysis conducted using the relative abundances of *Halobacteriales* genera with and without OtsAB system in relation to the sequenced *Halobacteriales* community indicated that the two groups of environments (hypersaline versus saline or fluctuating salinity) are divergent with respect to the abundances of OtsAB-lacking and OtsAB-harboring genera (Figure 5).

Discussion

In this study, we demonstrate that the following: (1) trehalose and 2-sulfotrehalose biosynthetic capabilities are widely spread in multiple *Halobacteriales* genera; (2) *ots*AB genes in *H. paucihalophilus* are highly expressed and their expression patterns broadly correspond to trehalose production patterns at various salinities; (3) a phylogenetic distinction exists between *otsAB*-harboring versus *otsAB*-lacking *Halobacteriales* taxa at the genus and supragenus levels; (4) the predominance of *otsAB*-lacking genera in permanently hypersaline habitats and (5) the occurrence of glycine betaine uptake as an additional osmoadaptive mechanism in the *Halobacteriales*.

Osmoadaptive landscape in trehalose- and 2-sulfotrehalose-synthesizing Halobacteriales

Quantification of trehalose and 2-sulfotrehalose in 17 different species (Figure 2, Supplementary Table S5) demonstrates that, in general, while these compounds are produced at all examined salinities, their concentrations are highest at lower media salinities. These production patterns are consistent with our understanding of the energetic costs of compatible solutes biosynthesis (Oren, 1999), with prior studies that documented trehalose importance as a compatible solute at lower salinities (for example in *Chromohalobacter israelenis* (Regeva *et al.*, 1990)) and the fact that within specific lineages such as Cyanobacteria, the utilization of trehalose as an osmoprotectant appears to be more prevalent in strains with relatively lower salt tolerance (Hagemann, 2011).

The observed trehalose/2-sulfotrehalose production patterns, coupled to the near-constant levels of K^+ observed at various salinities in the majority of species examined, argues for the need to employ additional osmoadaptive mechanisms when these genera encounter higher salinities. We argue that glycine betaine uptake represents an important mechanism for osmoadaptation in trehalose- and 2 sulfotrehalose-producing genera at higher salinities. Genes for glycine betaine uptake were identified in 36 out of 39 *otsAB*-harboring genomes, and glycine betaine uptake was experimentally verified in 7 out of 11 trehalose/2 sulfotrehalose-producing

Table 4 Distribution of *otsAB*-harboring and *otsAB*-lacking Halobacteriales genera in environments of varying salinities

Environment	*No. of sequences*	*% sequenced genera*[a]	*otsAB-harboring genera*		*otsAB-lacking genera*		*Reference*
			%	*Dominant genera*[b]	*%*	*Dominant genera*[b]	
Hypersaline							
Crystallizer pond, Tunisia	80	73.1	0		100	*Haloquadratum*, *Halorubrum*, *Halorhabdus*	Baati *et al*., 2008
Crystallizer pond, AU (Bajool)	40	80	0		100	*Haloquadratum*, *Halorubrum*, *Haloplanus*	Oh *et al*., 2010
Crystallizer pond, AU (Cryst7)	41	65.9	0		100	*Haloquadratum*, *Halorubrum*, *Natronomonas*	Oh *et al*., 2010
Crystallizer pond, AU (LDS1)	40	52.5	0		100	*Haloquadratum*, *Halorubrum*	Oh *et al*., 2010
Crystallizer pond, AU	57	83.7	0		100	*Haloquadratum*, *Halorubrum*	Burns *et al*., 2004
Solar saltern, CA	32	88	0		100	*Haloarcula*, *Halobacterium*, *Halorubrum*	Bidle *et al*., 2005
Tyrell hypersaline lake	15008	55.7	0		100	*Haloquadratum*, *Halorhabdus*, *Halobaculum*	Podell *et al*., 2013
Dead sea	1759	100	12.1	*Haloterrigena*	87.9	*Haloquadratum*, *Halorubrum*, *Halorhabdus*, *Halobacterium*, *Halomicrobium*, *Haloarcula*, *Halogeometricum*, *Natronomonas*	Bodaker *et al*., 2009
Great salt lake	30213	71.4	14.3	*Halobiforma*, *Halogranum*	85.7	*Halorubrum*, *Halorhabdus*, *Natronomonas*, *Halobacterium*	Youssef *et al*., 2012
Low/fluctuating Salinity							
Mangrove soil	32677	74.9	49.6	*Halogranum*	50.4	*Haloplanus*, *Haloferax*, *Natronomonas*	Youssef *et al*., 2012
Salt-processing plant	8587	66.9	55.1	*Halogranum*, *Halosarcina*	44.9	*Halorubrum*, *Halobacterium*, *Haloferax*	Youssef *et al*., 2012
Saline-saturated soil, 13% salinity	130	69.9	44.5	*Natronorubrum*	55.5	*Halorubrum*	Walsh *et al*., 2005
Low-salinity habitats							
Zodletone spring	5658	87	67.6	*Halogranum*	32.4	*Haloferax*, *Halobacterium*, *Halobaculum*	Youssef *et al*., 2012

[a]% of sequences belonging to genera with sequenced genomes within the entire data set.
[b]Identity of genera with >5% abundance.

species (Table 3). The production of additional yet-unidentified compatible solutes cannot be ruled out, although genomic and experimental analysis failed to identify the production of any of the commonly produced compatible solutes (Roberts, 2005) within examined species.

2-sulfotrehalose, rather than trehalose, was identified as the osmoadaptive agent in multiple *Halobacteriales* species (Supplementary Table S5). It appears that 2-sulfotrehalose producers exhibit a distinct phylogenetic affiliation and pH preference when compared with trehalose producers. 2-sulfotrehalose producers identified belonged to *Halobacteriales* Clade II, with the notable exception of *Haloalkalicoccus jeotgali*. Further, the majority of 2-sulfotrehalose producers identified in this as well as in a prior study (Desmarais *et al*., 1997) are haloalkaliphiles, with the exception of the two neutrophilic species *Natrinema pellirubrum* and *Halalkaicoccus jeotgali*. The rationale for the modification of a non-reducing neutral sugar to an anionic derivative, and whether such modification

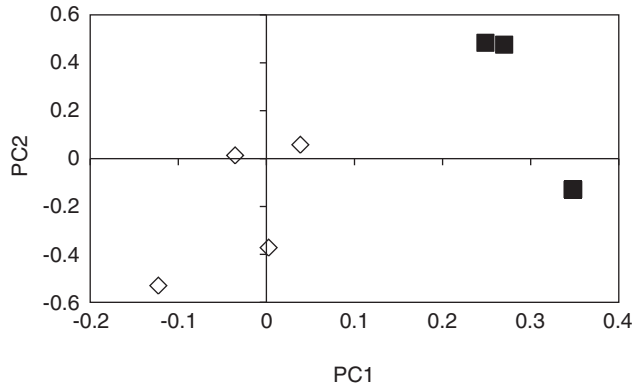

Figure 5 Principal component analysis of the percentages of OtsAB-harboring and OtsAB-lacking genera in permanently hypersaline environments (closed squared) versus environments of moderate and fluctuating salinity (open diamonds).

confers any advantage in alkalisaline conditions is not yet entirely clear. It is interesting to note that orthologs for the well described and only known enzyme specific for trehalose sulfonation (Mougous *et al.*, 2004) were not identified in *Halobacteriales* genomes examined, and hence an alternative, yet unknown, mechanism must be involved in the process.

Overall, trehalose- and 2-sulfotrehalose-producing lineages had lower intracellular K^+ levels when compared with genera incapable of trehalose/2-sulfotrehalose production. However, these levels are significantly higher than those observed in moderately halophilic heterotrophs and Cyanobacteria that are dependent on compatible solute production for osmoadaptation (Ventosa and Oren, 1996; Hagemann, 2011). Therefore, these relatively higher intracellular K^+ levels in trehalose- and 2-sulfotrehalose-producing *Halobacteriales* lineages imply that K^+ accumulation still has an integral role in osmoadaptation within these lineages, and that trehalose/2-sulfotrehalose-producing *Halobacteriales* should not be regarded as solely dependent on compatible solute production and uptake mechanisms for osmoadaptation. In addition, trehalose/2-sulfotrehalose-producing *Halobacteriales*, similar to other *Halobacteriales* lineages, have an acidic proteome with a high concentration of Asp and Glu residues ($\sim$17%) and a low predicted pI (4.76 ± 0.12). Hence, the high levels of intracellular K^+ in trehalose/2-sulfotrehalose-producing *Halobacteriales* also function to stabilize the acidic proteome via interactions with the negatively charged acidic protein surface required for increasing the protein hydration shell and preventing unfolding or salting out (Oren and Mana, 2002).

Osmoadaptive landscape in genera lacking OtsAB system

On the other hand, 11 out of 28 genera with sequenced genomes examined lack the genes necessary for trehalose biosynthesis, an observation that was experimentally verified in 6 representative strains. Within this group, a salting-in strategy depending on the accumulation of high levels of intracellular K^+ in a salinity-dependent manner appears to be the primary mechanism for osmoadaptation. As such, our results are in agreement with prior studies on osmoadaptive mechanisms in the *Halobacteriales* (Christian and Waltho, 1962; Ginzburg *et al.*, 1970; Lanyi and Silverman, 1972; Matheson *et al.*, 1976; Pérez-Fillol and Rodríguez-Valera, 1986; Mojica *et al.*, 1997; Oren *et al.,* 2002b), all of which have been conducted on strains that lack trehalose production capabilities (*Halobacterium salinarum*, *Haloarcula marismortui*, *Haloferax volcanii*, *Haloferax mediterranei*, *Haloferax gibbonsii, Halorubrum saccharovorum* and *Halorubrum trapanicum*). However, in addition to the well-established role of intracellular K^+ accumulation, this study demonstrates that uptake of the compatible solute glycine betaine is a common process in *otsAB*-lacking genera. Several bacterial and eukaryotic ineages capable of synthesizing glycine betaine could thrive in hypersaline ($>$25% NaCl) habitats, for example *Cyanobacteria* (Oren, 2010) and *Dunaliella* species (Oren, 2005), and hence this mechanism could have environmental relevance in specific habitats. Although glycine betaine uptake has been reported in *Halobacterium salinarum* sp. strain NRC-1 (Kokoeva *et al.*, 2002), the overall role of this process in *Halobacteriales* osmodaptation has largely been unexplored.

Evolutionary and ecological dichotomy between trehalose-/2sulfotrehalose-producing versus non-producing Halobacteriales

Analysis of the phylogeny and distribution of *otsAB* genes revealed valuable insights into the evolution of osmoadaptive mechanisms, and the implications of such events on the ecological distribution of various *Halobacteriales* lineages. Phylogenetic analysis indicated that trehalose biosynthesis is an ancient trait that has subsequently been lost in some lineages during *Halobacteriales* evolution. We argue that the loss of *otsAB* genes was associated with the spread of *Halobacteriales* into hypersaline habitats, as this negative selection pressure event would alleviate the energetic costs associated with the synthesis of high level of trehalose to cope with the external hypersaline environments. The resulting energetic benefit of this mutation outweighs its anticipated drawback: the loss of osmoadaptive flexibility, as the benefit of such flexibility is highly diminished in permanently hypersaline habitats. Further, this loss of osmoadaptive flexibility could possibly be compensated for by the capability to uptake glycine betaine from the environment. Indeed, our analysis of *Halobacteriales* community in multiple habitats of various salinities demonstrated a clear preference and dominance of genera incapable of trehalose production in permanently hypersaline habitats.

In conclusion, this study highlights the role of compatible solute biosynthesis and uptake in the *Halobacteriales*, and provides ecological and evolutionary context to the observed variations and complexities of osmoadaptive strategies. The complexity and multiplicity of osmoadaptive mechanisms in the *Halobacteriales* reported here, as opposed to the prior view of sole dependence on a single solute (K^+ ions) for osmoadaptation, raise multiple yet-unanswered questions regarding the kinetics of compatible solutes production, as well as the relative contribution, interaction and regulation of various mechanisms in the overall process of osmoadaptation in the *Halobacteriales*.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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