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2-Sulfotrehalose, a Novel Osmolyte in Haloalkaliphilic Archaea

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A novel 1→1 α-linked glucose disaccharide with sulfate at C-2 of one of the glucose moieties, 1-(2-*O*-sulfo-α-D-glucopyranosyl)-α-D-glycopyranose, was found to be the major organic solute accumulated by a *Natronococcus* sp. and several *Natronobacterium* species. The concentration of this novel disaccharide, termed sulfotrehalose, increased with increasing concentrations of external NaCl, behavior consistent with its identity as an osmolyte. A variety of noncharged disaccharides (trehalose, sucrose, cellobiose, and maltose) were added to the growth medium to see if they could suppress synthesis and accumulation of sulfotrehalose. Sucrose was the most effective in suppressing biosynthesis and accumulation of sulfotrehalose, with levels as low as 0.1 mM being able to significantly replace the novel charged osmolyte. Other common osmolytes (glycine betaine, glutamate, and proline) were not accumulated or used for osmotic balance in place of the sulfotrehalose by the halophilic archaeons.

When cells encounter changed osmotic pressure due to alterations in external NaCl, they can respond in various ways. They can accumulate inorganic cations with the appropriate counterion (organic or inorganic), transport or synthesize a neutral organic compatible solute, or use some combination of the two strategies (2, 7, 11, 23, 47). Osmotic behavior has been studied with many different types of bacteria. In eubacteria, there are several different types of organic solutes that have been used as osmolytes, including sugars (glucose, sucrose, and trehalose), polyols (glycerol, mannitol, and glucosylglycerol), free amino acids (glutamate, glycine, and proline), and derivatives such as glycine betaine (16, 29, 44). Archaebacteria, a much less studied group, exhibit both types of osmotic strategies. Aerobic halophiles (e.g., *Halobacterium halobium*) have been found to accumulate high levels of potassium and sodium in the cell to balance external osmotic pressure (17, 18, 37, 40). Methanogens, an anaerobic subgroup of archaebacteria, not only accumulate inorganic cations (31) but also have been found to accumulate several organic solutes in response to changes in osmotic pressure (33). Notably, these organisms synthesize several β-amino acids (e.g., β-glutamate and β-glutamine) and derivatives (e.g., *N*ε-acetyl-β-lysine) as compatible solutes (21, 30, 34, 38). Betaine is another commonly found osmolyte that has been detected in several methanogens (32, 33) but has not been reported to play a role in other archaea.

Natronococcus and *Natronobacterium* spp. are a distinct group of haloalkaliphilic archaebacteria which grow under high-salt and high-pH conditions (17, 39). More specifically, optimum growth occurs at a pH of 9.0 to 10.0, an NaCl concentration of 3.0 to 4.0 M, and a very low Mg^{2+} concentration. These organisms are typically isolated from hypersaline soda lakes and are characterized by a distinct reddish color which is due to the presence of bacterioruberins (17). Currently, there is not much known about the physiology of these organisms, with most of the work done thus far concentrating on the lipid composition of these archaebacteria (9, 17, 39).

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Since *Natronococcus* and *Natronobacterium* spp., like all other bacteria, must have some sort of strategy to deal with osmotic stress, it was of interest to see if they chose to accumulate inorganic ions, like other aerobic halophilic archaea, or if they chose to synthesize and accumulate organic solutes (much like the halophilic *Methanohalophilus portucalensis* ([formerly called strain FDF1] [21, 30]). The present work involves the use of nuclear magnetic resonance (NMR) spectroscopy to identify and quantify the organic compatible solutes found in *Natronococcus* and *Natronobacterium* spp. Analysis of the data shows a novel 1→1 α-linked glucose disaccharide with sulfate at C-2 of one of the glucose moieties to be the major organic solute present in all these organisms grown in defined medium. The concentration of this novel disaccharide, termed sulfotrehalose, increased with increasing external NaCl, behavior consistent with its identity as an osmolyte in these archaea.

MATERIALS AND METHODS

Chemicals. D_2O (99.9%) was obtained from Sigma Chemical Company; D_2O (99.999%) was obtained from Cambridge Isotope Laboratories. Kojibiose, a 1→2-linked glucose disaccharide, was a gift from Irwin Goldstein, University of Michigan Medical School. Other disaccharides (including sucrose, trehalose, cellobiose, and maltose), amino acids (L-α-glutamate and glycine), and derivatives (glycine betaine) were obtained from Sigma Chemical Company.

Cell growth. *Natronococcus occultus* (ATCC 43101), *Natronobacterium gregoryi* (ATCC 43099), *Natronobacterium magadii* (ATCC 43098), and *Natronobacterium pharaonis* (ATCC 43100) were grown to mid-log phase in defined haloalkaliphilic (DH) medium at 40°C with shaking (200 rpm). The composition of DH medium was as follows (grams per liter): NaCl, 200; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.2; Casamino Acids, 5; Na_2CO_3, 18. Trace elements were added as 1 ml per liter of medium. The trace element solution contained (in grams per liter) $ZnSO_4 \cdot 7H_2O$, 0.10; $MnCl_2 \cdot H_2O$, 0.03; H_3BO_3, 0.3; $CoCl_2 \cdot 6H_2O$, 0.2; $CuCl_2 \cdot 2H_2O$, 0.01; $NiCl_2 \cdot 6H_2O$, 0.02; $Na_2MoO_4 \cdot H_2O$, 0.03. The cells were harvested by low-speed centrifugation (5,000 × *g*, 15 min, 4°C), and each pellet was washed three times with 3.4 M NaCl before lyophilization. This served to remove any organic medium adhering to the cells. Cells of *N. occultus* were also grown in DH medium with varying concentrations of NaCl.

Determination of cell volume. The intracellular volume of *N. occultus* grown in 3.4 M external NaCl was determined by measuring the differential retention of [^{14}C]inulin and ^3H_2O in cell pellets as described previously for methanogens (38).

Extraction of intracellular solutes. The lyophilized cell pellets were extracted with 70% ethanol as described previously (20, 32). The entire cell pellet was divided into four to six smaller tubes, and each tube was resuspended in 1 ml of 70% ethanol. This was critical for maximum disruption and extraction of small molecules from these cell pellets. Each tube was vortexed for approximately 5

min, bath sonicated for 5 to 10 min, and then centrifuged for 10 min at 10,000 rpm in an Eppendorf Microcentrifuge. The supernatants were collected, and the procedure was repeated four to five times. All of the ethanolic supernatants were collected, and the solvent was removed under vacuum. The remaining film was frozen and then lyophilized overnight to remove traces of ethanol. As an aid to identifying solutes by NMR spectroscopy, it is often useful to determine if observed components are acid labile. Spectral changes can indicate the presence of amide, ester, or glycosidic linkages. For NMR analyses of the sensitivity of organic solutes in *N. occultus* to acid, lyophilized ethanol extracts were dissolved in 1 M H_2SO_4 and incubated at 100°C for a period of 1 to 6 h. Samples were then neutralized and examined by ^{13}C NMR spectroscopy. The amounts of solutes found in different ethanol extracts were normalized to total cell protein determined from the cell pellet remaining after the ethanol extraction. This material was dissolved in 30 to 60 ml of water by bath sonication, and its protein content was measured by the Bradford assay (1). Values for solute concentrations were reported as micromoles per milligram of cell protein.

Purification of disaccharide. Pooled ethanol extracts (containing primarily the disaccharide and glutamate with smaller amounts of other sugars) were lyophilized and resuspended in water to a concentration of 10 to 20 mg/ml. Extracts were then applied to a column (2 by 1 cm) packed with ToyoPearl TSK quaternary aminoethyl resin and eluted with a gradient of 0.05 to 0.5 M ammonium bicarbonate. The column was then rinsed with 2 M NaCl to remove adsorbed bacterioruberins. ^1H NMR spectroscopy was used to ensure the purity of the disaccharide.

Paper chromatography and high-pressure liquid chromatography (HPLC) analysis of carbohydrates. Samples were spiked with a [^{14}C]glucose standard prior to hydrolysis. The purified disaccharide was hydrolyzed to its monosaccharide units by incubation in 70% formic acid overnight at 100°C. To analyze the constituent monosaccharides, the hydrolyzed sample was reduced with sodium [^3H]borohydride (6) to label all reducing sugars. The identity of the reduced sugars was determined by descending paper chromatography with Whatman no. 1 paper strips developed in an ethyl acetate-glacial acetic acid-formic acid-water (18:3:1:4) solvent system for 20 h (35). The R_{glc} value of the reduced glucose standard (distance migrated from the origin compared to the total distance between the origin and the solvent front) was compared to that from the disaccharide. The chemical composition of the disaccharide was also examined by HPLC analysis of the ^3H-labeled hydrolysis products on a Whatman Partisil PAC column equilibrated with 29% water in acetonitrile (14).

The total hexose content in a hydrolyzed disaccharide stock solution was estimated with a tetrazolium blue assay for reducing equivalents (25). A standard curve was constructed from glucose. The assay is sensitive over a range of 0 to 10 μg of glucose in 100 μl. Total sulfate in the hydrolyzed purified sample was measured by the $BaCl_2$-gelatin method (10) with K_2SO_4 to construct the standard curve. The sulfate (micromoles) in 10, 50, and 100 μl of disaccharide stock solution was compared to the amount of reducing equivalents (micromoles) in the same volumes of disaccharide stock and used to determine the ratio of sulfate to reducing equivalents in the disaccharide sample.

Preparation of samples for NMR. The lyophilized ethanol extract was dissolved in 1 to 2 ml of D_2O (99.9% for ^{13}C spectra, 99.999% for ^1H spectra). The pH of the dissolved sample was adjusted to be between 6.9 and 7.1. The sample was centrifuged for 10 min at 10,000 rpm in an Eppendorf Microcentrifuge. A thin top layer was discarded, and the rest of the aqueous layer was removed for NMR analysis (500-μl sample volume).

NMR spectroscopy. All NMR experiments were carried out on a Varian Unity 500 spectrometer. The ^1H total correlation spectroscopy (TOCSY), heteronuclear multiple quantum coherence (HMQC), and nuclear Overhauser effect spectroscopy (NOESY) and several ^{13}C experiments were performed with a 5-mm inverse probe. The remaining ^1H correlation spectroscopy (COSY) and natural abundance ^{13}C spectra were acquired with a broadband probe. ^1H WALTZ-decoupled ^{13}C NMR (125.7-MHz) spectra were acquired with the following parameters: 25-kHz sweep width, 65,024 datum points, 90° pulse width (18 μs at a transmitter power of 55 for the indirect probe and 11 μs at a transmitter power of 54 for the broadband probe), and a 1.0-s delay time. A total of 1,000 to 10,000 transients were acquired, and the resulting free induction decays were processed with 4-Hz line broadening. The ^1H NMR (500-MHz) spectra were acquired with an 8,000-Hz sweep width, 19,200 datum points, 90° pulse width (4.8 μs at transmitter power 63 for the indirect probe, 22.0 μs at transmitter power 63 for the broadband probe), a 1.0-s delay time, and 256 transients. The residual water was presaturated.

The amount of solute present in each extract sample (micromoles) was determined by integrating resonances for the organic solutes and comparing their intensities to that of 6 μmol of dioxane added as an internal standard. These values were then normalized (micromoles per milligram of protein) with the total protein content of the cells obtained from a Bradford assay on the resuspended pellet from the ethanol extraction. ^{13}C chemical shifts were referenced to dioxane at 67.4 ppm; ^1H chemical shifts were referenced to residual HDO at 4.75 ppm.

All of the two-dimensional experiments used standard Varian software, with suppression of residual water by presaturation. ^1H-^{13}C HMQC experiments used 56 scans per t1 increment, a 0.500-s delay time, a 0.171-s acquisition time, ^{13}C decoupling during acquisition, and a raw data matrix size of 512 by 256. A J_{CH} of 140 Hz was used to generate the delay period for evolution of C-H coupling. The matrix was zero filled and processed with 1,024 in f1 and 2,048 in f2. COSY experiments used a 1.600-s delay time, a 0.426-s acquisition time, decoupling on during predelay and pulse, 16 scans per t1 increment, and a raw data matrix size of 1,024 by 512. The matrix was zero filled with 1,024 in f1 and 1,024 in f2 prior to processing. TOCSY experiments used a 1.500-s delay time, a 0.197-s acquisition time, decoupler on during predelay only, 20 scans per t1 increment, a mixing time of 0.060 s, and a raw data matrix of 512 by 256. NOESY experiments used a 2.0-s delay time, a 0.189-s acquisition time, a 0.500-s mixing time, decoupler on during predelay, 16 scans per t1 increment, a raw data matrix size of 2,048 by 256, and zero filling with 2,048 in f1 and 1,024 in f2.

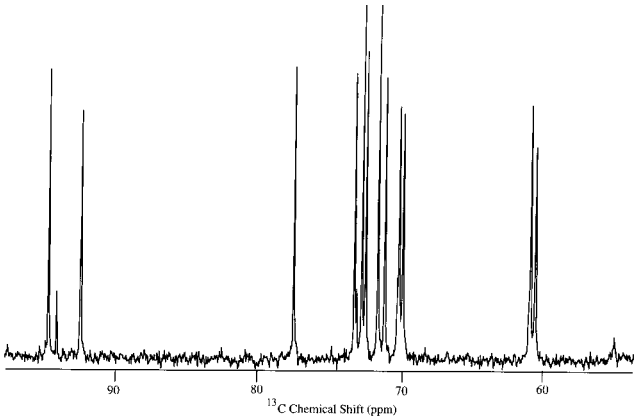

FIG. 1. ^1H-decoupled ^{13}C NMR spectrum of an ethanol extract of *N. occultus* grown in minimal medium in the presence of 3.4 M NaCl.

RESULTS

Intracellular organic solutes in *Natronococcus* and *Natronobacterium* spp. *N. occultus* cells grown in DH medium containing 3.4 M NaCl, the optimum salt concentration, were examined for the accumulation of organic solutes. The natural abundance ^{13}C NMR spectrum of an ethanol extract exhibited 12 major resonances between 62 and 100 ppm (Fig. 1). The occurrence of 12 distinct resonances of equivalent intensity in this chemical shift range could indicate that a disaccharide was the major solute present in these extracts. There were also minor ^{13}C resonances (1/10 to 1/5 of the major resonances) in the carbohydrate region, notably at 94.0, 73.3, and 70.4 ppm. These (and presumably others that overlapped with the major resonances) could represent a carbohydrate related to the major species. None of the major organic solutes used as osmolytes by other archaea (e.g., α- and β-glutamate, *N*ε-acetyl-β-lysine, glycine betaine, β-glutamine, di-*myo*-inositol-1,1′-phosphate and derivatives, and glucosylglycerate) appeared to be present at significant levels in this sample based on the absence of the corresponding resonances (4, 21, 30, 33, 38). ^{13}C NMR spectra of extracts of *N. pharaonis*, *N. magadii*, and *N. gregoryi* also exhibited the same 12 resonances, indicating that the same solute was present in all of the halophilic, alkaliphilic archaea under these growth conditions. These organisms also exhibited resonances for glutamate.

Characterization of the major organic solute by NMR spectroscopy. Since the chemical shifts of this solute did not match up with any of the common disaccharides (12), a number of two-dimensional NMR experiments were performed to characterize this compound. Analysis of the TOCSY spectrum indicated the presence of two major spin systems consistent with hexoses (Fig. 2A). In this experiment, spin magnetization is transferred through bonds along a spin system [for a typical carbohydrate, this would be C(1)-H to C(6)-H], and the crosspeaks indicate the identities of all the spins in a given hexose ring. The crosspeaks were fairly well resolved and easily pro-

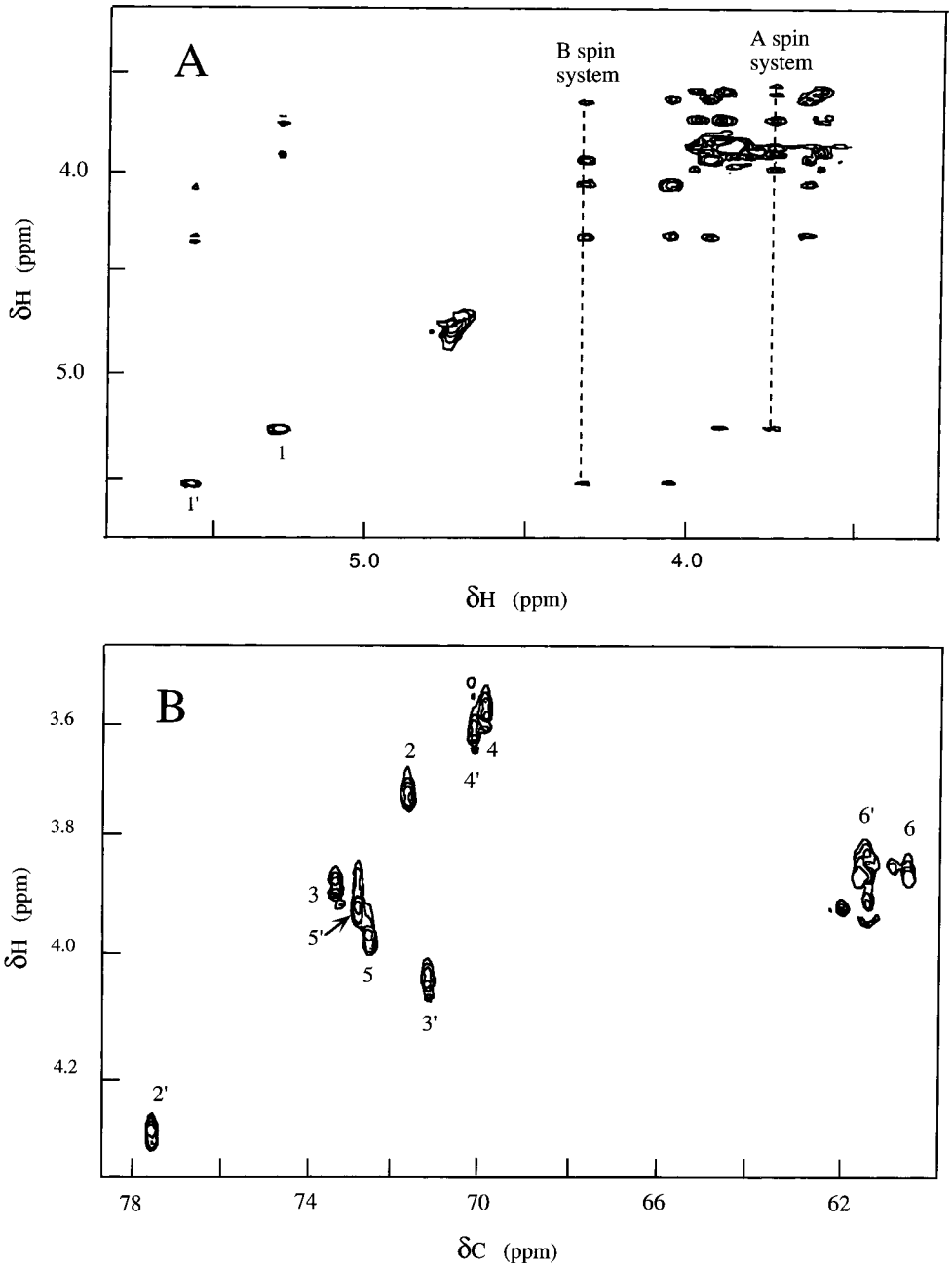

FIG. 2. Contour plots of TOCSY (A) and ^{1}H-^{13}C HMQC (B) experiments for an ethanol extract of *N. occultus* grown in the presence of 3.4 M NaCl. (A) Dotted lines illustrate how resonances belonging to a particular hexose spin system are identified. (B) Identities of each peak are labeled by the appropriate carbon number. The chemical shift region containing the two anomeric carbons is not shown; hence, these two carbons are not labeled on the plot.

vided 11 of the 12 proton chemical shifts (in Fig. 2A, the dotted lines connect resonances in each spin system). The results of the COSY spectrum provided connectivities between hydrogen atoms in each ring system, allowing one to assign each resonance in a spin system to a particular proton on the ring. This information together with the coupling constants derived from the ^{1}H spectrum indicated that the first four protons in each ring system were consistent with glucose stereochemistry with the C-1 oxygen in an α orientation in both rings. An extract from *N. occultus* was hydrolyzed in 1 M H_2SO_4; after 2 h, the ^{13}C spectrum showed no residual 12-line pattern but only resonances identified as the α- and β-anomers of glucose (data not shown). Since only one type of sugar monomer, glucose, was present, the 12 resonances in the unhydrolyzed sample must represent a glucose dimer.

An HMQC experiment was carried out in order to correlate ^{1}H with ^{13}C chemical shifts (Fig. 2B). This experiment uses ^{1}H-^{13}C coupling to identify protons attached to individual carbons (numbers on Fig. 2B label each C-H; the ^{1}H and ^{13}C chemical shifts are summarized in Table 1). A comparison of ^{1}H and ^{13}C chemical shifts of the unknown solute with those for glucose showed significant differences for both C-1 resonances (they occur at values consistent with only α-anomers present) and for one of the C-2 positions. The downfield-

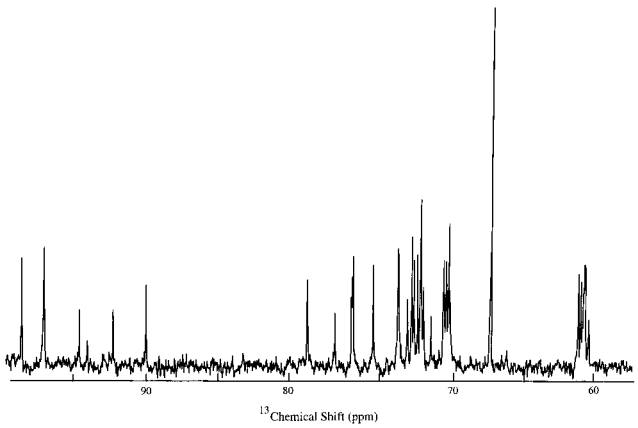

FIG. 3. ^{1}H-decoupled ^{13}C NMR spectrum of a sample containing an ethanol extract of *N. occultus* grown in 3.0 M NaCl with authentic kojibiose added. Dioxane (6 μmol) is present as an internal standard.

TABLE 1. ^{1}H and ^{13}C chemical shifts of the novel disaccharide accumulated by *N. occultus*

Spin system A			Spin system B		
Carbon	δ_C (ppm)[a]	δ_H (ppm)[b]	Carbon	δ_C (ppm)[a]	δ_H (ppm)[b]
C-1	94.56	5.28	C-1′	92.33	5.56
C-2	71.80	3.75	C-2′	77.63	4.35
C-3	73.45	3.92	C-3′	71.30	4.10
C-4	70.04	3.62	C-4′	70.30	3.68
C-5	72.64	4.00	C-5′	72.91	3.94
C-6	60.84	3.90	C-6′	61.21	3.88

[a] The ^{13}C chemical shifts were referenced with respect to added dioxane at 67.4 ppm.
[b] The ^{1}H chemical shifts were referenced with respect to residual HDO at 4.75 ppm.

shifted C-2 was either an indication of the position of a glycosidic linkage or a chemical modification of that hydroxyl group (42, 46).

If the abnormal C-2 chemical shift was due to an α(1→2) linkage, the compound could be α-kojibiose (15) or a derivative. In solution, kojibiose occurs with both α- and β-anomers at the reducing sugar of the disaccharide in equilibrium (15, 42). For comparison, the unknown glucose dimer exhibited only α orientations for both C-1 groups. When authentic kojibiose was added to the extract, its ^{13}C resonances clearly did not overlap with the resonances of the unknown disaccharide (Fig. 3). Hence, the unknown disaccharide in these archaea cannot be the neutral glucose dimer kojibiose.

Disaccharides show a strong positive NOE between protons attached to the carbons involved in the glycosidic bond. In the NOESY contour plot for the unknown disaccharide, there is a strong positive NOE between the C-1 protons of each spin system [Fig. 4; note the (1,1′) and (1′,1) crosspeaks]). This is diagnostic for a glycosidic bond involving C-1 of each spin system. The α(1→1)-linked glucose dimer is trehalose, a nonreducing sugar characterized by only six resonances since it is a symmetric molecule. In fact, the three distinct small resonances also observed in the carbohydrate region (Fig. 1) are consistent with the ^{13}C chemical shifts for authentic trehalose (C-1, 94.0; C-2, 73.0; C-3, 73.3; C-4, 70.5; C-5, 71.8; and C-6, 61.4 ppm). The abnormal ^{13}C shift of one of the C-2 carbons in the disaccharide could indicate chemical modification of the trehalose to generate a molecule in which the two glucose rings are differentiated by a chemical modification. No other carbons are detected in the ^{13}C spectrum, so that an acetylated or methylated group is also not an option. Phosphorylation would introduce ^{31}P-^{13}C (or ^{31}P-^{1}H) coupling, and none was ob-

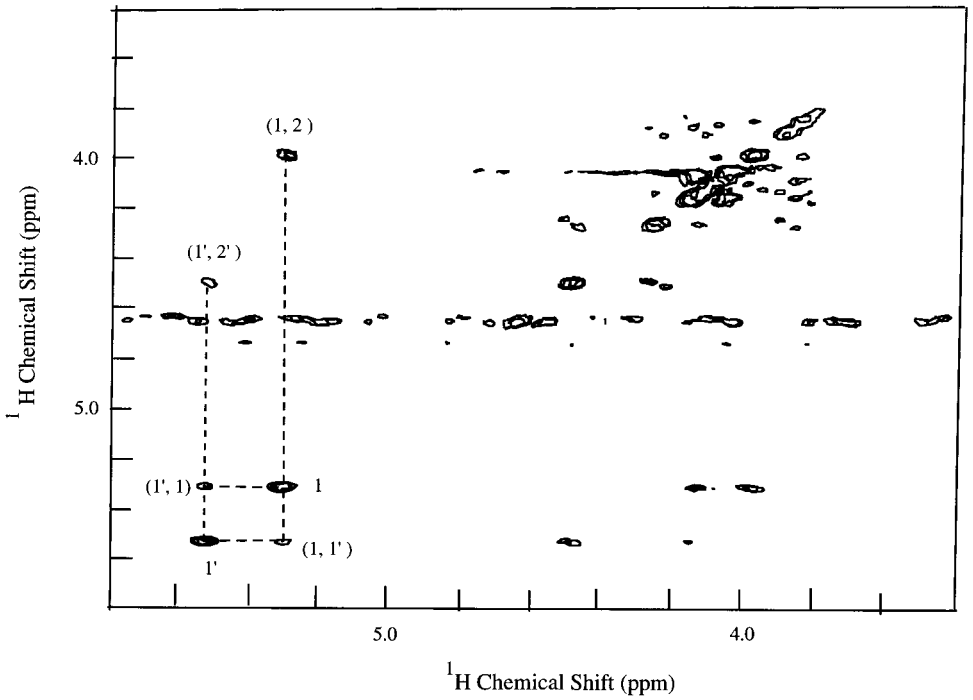

FIG. 4. Contour plot from a NOESY experiment for an extract of *N. occultus* grown in 4.0 M NaCl. The dashed lines identify crosspeaks indicative of NOEs between the C-1 and C-1′ protons [labeled as (1,1′) and (1′,1)]; crosspeaks representing NOEs between the C-1 and C-2 protons, labeled as (1,2) and (1′,2′), are also indicated.

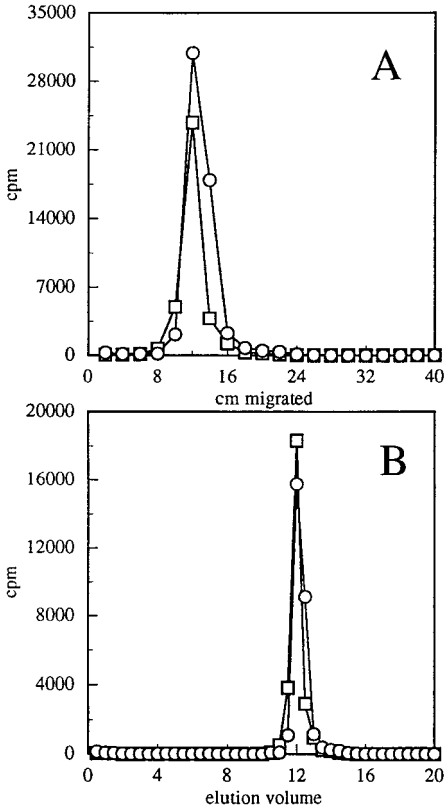

FIG. 5. Radioactivity profile of the paper chromatogram (A) and HPLC elution profile (B) of the hydrolyzed and reduced disaccharide labeled with ^{3}H (○) compared to the standard [^{14}C]glucitol (□).

served. A reasonable alternative is sulfation of the C-2 hydroxyl group. This would not introduce any coupling, and when it has occurred in carbohydrates, it shifted the carbon bearing the sulfate downfield by about 5 ppm compared to the parent sugar residue (27).

Chemical analysis of the disaccharide. Chemical methods were used (i) to confirm that the disaccharide was composed of only glucose moieties and (ii) to quantify the sulfate content. The disaccharide was purified from the extract (which could contain inorganic sulfate or other sulfated species) by quaternary aminoethyl anion-exchange chromatography. As expected for a compound with a negative charge, the disaccharide bound to the resin; it was eluted with 0.05 M ammonium bicarbonate. Fractions were monitored by ^{1}H NMR to ensure that there were no other organic solutes or that the disaccharide was not altered by this procedure. The sample was hydrolyzed in formic acid and then reduced with sodium [^{3}H]borohydride to yield labeled glycitols. Confirmation of the unknown compound as a glucose dimer was carried out with paper chromatography to separate all labeled monosaccharides. The labeled material formed from hydrolyzing and reducing the disaccharide exhibited a single peak that comigrated with [^{14}C]glucitol (Fig. 5A). Similarly, when the hydrolyzed sample was eluted from an HPLC PAC column, only a single ^{3}H-labeled peak comigrating with [^{14}C]glucitol was detected (Fig. 5B).

The total sulfate content of the hydrolyzed disaccharide stock solution was monitored by the $BaCl_2$-gelatin method. The stock solution contained 55 μg of SO_4^{2-} per 100 μl; this corresponded to 0.58 μmol of SO_4^{2-} per 100 μl (Fig. 6A). Reducing equivalents of glucose in the same hydrolyzed stock solution were also determined (Fig. 6B); the value for glucose was divided by two since the disaccharide is composed of two glucose units. There were 95 μg (0.53 μmol) of reducing equivalents per 100 μl of the disaccharide stock solution. The ratio of sulfate content to disaccharide was then estimated as 1.1:1.0. Thus, there is one sulfate group for the trehalose unit. The presence of a sulfate group typically causes a 5-ppm downfield shift of the carbon to which it is attached (27). The only carbon whose shift is markedly different from that of the carbons in trehalose is C-2. Hence, the combination of the NMR data with the sulfate assay identifies the novel osmolyte in haloalkaliphilic archaea as trehalose with a sulfate group on C-2, 2-sulfotrehalose or 1-(2-*O*-sulfo-α-D-glucopyranosyl)-α-D-glycopyranose.

Effect of external NaCl and exogenous solutes on sulfotrehalose. *N. occultus* was grown in media containing various NaCl concentrations (2.5, 3.4, and 4.0 M NaCl) in order to see if the amount of sulfotrehalose varied with external salt. The disaccharide was significantly lower in cells grown in 2.5 M NaCl and decreased slightly in cells grown with the higher level of NaCl (Fig. 7). Two or more extracts were examined at each external NaCl concentration to get a good estimate of the errors in determining the sulfotrehalose concentrations. Thus, this sulfated disaccharide exhibited an osmotic response (although not a simple linear response) in these cells. At the lower NaCl concentration (<3.0 M) at which there was less disaccharide, α-glutamate (also anionic) was also accumulated. The α-glutamate also contributed to osmotic balance, making up about 30% of the total organic solute present. While at the highest NaCl concentration examined, 4.0 M, the levels of sulfotrehalose decreased compared to what was detected in 3.4 M NaCl, no other organic solute was accumulated.

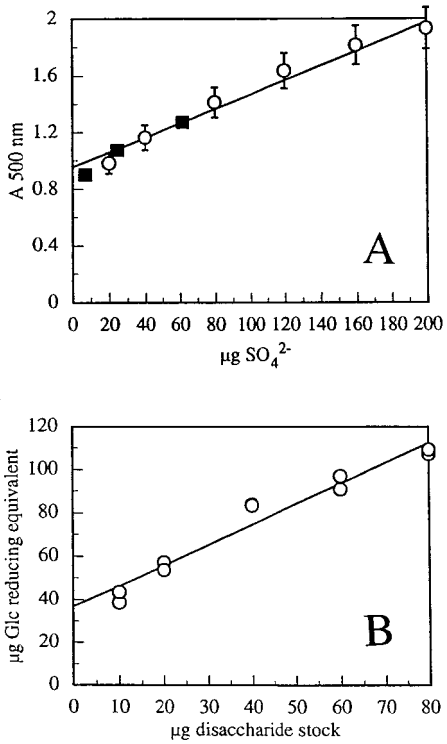

FIG. 6. (A) Determination of the sulfate content of the hydrolyzed disaccharide. ○, K_2SO_4 standard; ■, 10, 50, and 100 μl of the disaccharide stock solution. (B) Correlation of the microgram glucose-reducing equivalents in the disaccharide stock solution.

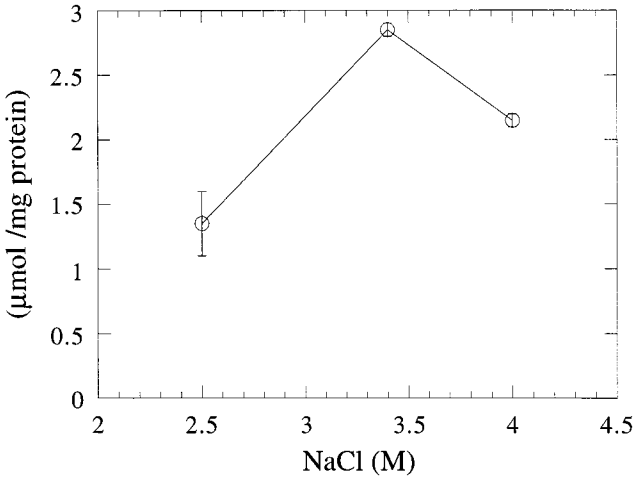

FIG. 7. Dependence of intracellular sulfotrehalose (micromoles per milligram of protein) on external NaCl (molars). Error bars represent the averages of two or more ethanol extracts of cells grown at a given NaCl concentration.

The cell volume of *N. occultus* grown in 3.4 M NaCl was estimated to be 3.12 μl/mg of protein by using ^3H_2O-[^{14}C]inulin to monitor permeable and impermeable volumes. Thus, the intracellular concentration of sulfotrehalose for these cells was estimated as 0.9 M. Initial estimates of internal K^+ concentration indicated that ~1 M K^+ was present in these cells (16a), making it the counterion of sulfotrehalose. This amount of K^+-sulfotrehalose is not enough to balance the osmotic pressure produced by 3.4 M external NaCl. Presumably, other species (e.g., large organic solutes that would be NMR invisible, Na^+, and inorganic anions) are present and also contribute to osmotic balance.

N. occultus was also grown in medium containing a fixed amount of NaCl (3.4 M) with 10 mM exogenous solutes added to see if other solutes could be internalized and suppress sulfotrehalose synthesis and accumulation. Cells grown in the presence of 10 mM glycine, glycine betaine, and L-α-glutamate all synthesized and accumulated the disaccharide rather than internalize these exogenous molecules. None of these compounds were accumulated to NMR-detectable levels. Given the typical size of these extracts and the signal-to-noise available, that would translate to a solute occurring in the cells at less than 50 mM.

Exogenous sugars, in contrast to amino acids, affected sulfotrehalose accumulation. Cells grown in the presence of trehalose, a likely precursor, exhibited a mix of the negatively charged sulfotrehalose and the neutral disaccharide. When the cells were grown in the presence of 10 mM sucrose, the sucrose was internalized, and disaccharide synthesis was completely suppressed. The natural abundance ^{13}C spectrum of the cell extract showed only sucrose resonances. Cells were grown in varying amounts of sucrose to determine in which concentration range sucrose could only partially suppress biosynthesis of sulfotrehalose. Cells grown in 1 mM sucrose only contained sucrose as a solute. However, when the cells were grown in 0.1 mM sucrose, both disaccharides were detected in roughly similar amounts. These results indicate that exogenous sucrose, a neutral disaccharide, can substitute for sulfotrehalose, a negatively charged disaccharide, as long as the external sucrose concentration is greater than 0.1 mM (Fig. 8). The total amount of organic solutes as a function of external sucrose concentration is shown in the inset in Fig. 8. Interestingly, when only a small amount of sucrose (0.1 mM) was present in the medium, the total amount of both solutes accumulated (~1.5 μmol/mg of protein) was about half of the sulfotrehalose accumulated when the cells were grown without added sucrose (2.8 μmol/mg of protein). However, with more and more sucrose available the total amount of organic solute increased well above the normal amount of sulfotrehalose at this NaCl concentration. The reason for this behavior is not clear, but it is compatible with changes in inorganic ions being a driving force in balancing different osmolytes. Cells were also grown in media supplemented with 10 mM cellobiose, *myo*-inositol, or maltose. Only the cells exposed to maltose showed significant internalization of exogenous solute, although maltose was not as effectively accumulated as sucrose and represented around 50% of the solute pool under these conditions.

The *N. occultus* cultures used for the previous experiments were all grown in a defined minimal medium. Cells were also grown in a rich medium (with yeast extract added) to see if organic solute composition varied. Natural abundance ^{13}C spectra showed no resonances for sulfotrehalose. In fact, there was little organic material present in these extracts. This suggests that under some growth conditions the cells can use inorganic ions exclusively as osmolytes. In rich medium, this strategy is preferred over de novo synthesis of the sulfotrehalose.

DISCUSSION

It was originally thought that halophiles used inorganic ions such as potassium ions as the solutes to balance external osmotic pressure (9, 17). However, a number of organic compounds serve as osmolytes in these organisms. Betaine and glutamate, both osmolytes in nonhalophilic eubacteria and eukaryotes, accumulate in a wide variety of halophilic eubacteria (16, 44). The halophile *Ectothiorhodospira halochloris* accumulated glycine betaine, trehalose, and ectoine (16), the last of these a solute more recently shown to be associated with a wide variety of halophilic organisms isolated from different environments (for an example, see reference 5). There are few examples of halophilic archaea that have been examined in any detail. Aerobic *Halobacteria* spp. appear to use inorganic ions, notably cations (17, 18, 37, 40), rather than any organic solutes

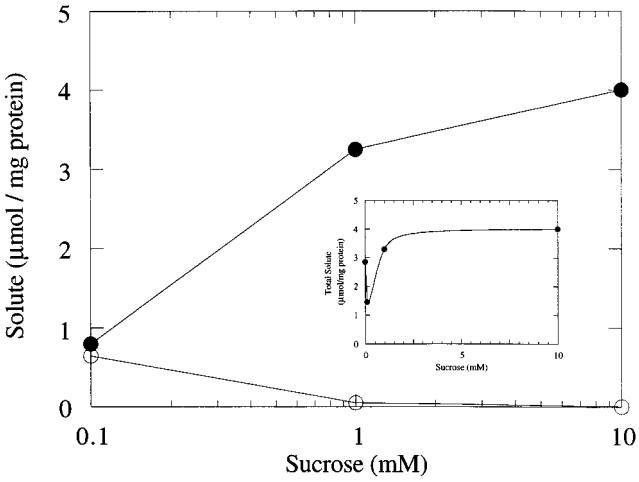

FIG. 8. Dependence of intracellular sulfotrehalose (○) and sucrose (●) levels (micromoles per milligram of protein) on the concentration of sucrose added to the medium. The inset shows the dependence of total organic solute (sulfotrehalose plus sucrose) on external sucrose concentration.

in response to osmotic stress. In contrast to those aerobic archaea, *Methanohalophilus* strain FDF1, a halophilic methanogen, synthesizes a number of organic compatible solutes. Its major organic osmolytes, *N*ε-acetyl-β-lysine, β-glutamine, and glycine betaine, are zwitterionic molecules, although anionic osmolytes (α-glutamate and glucosylglycerate) are also accumulated under some conditions (21, 30). This methanogen also accumulates high concentrations of K^+ (roughly 1 to 2 M) between 1.4 and 2.7 M external NaCl (21); that cation also plays a role in osmotic balance.

Sulfotrehalose is the first (and so far only) organic osmolyte detected in halophilic alkaliphilic archaea grown under optimum conditions in minimal medium. Small amounts (millimolars or micromolars) of glycine betaine had previously been detected in *Natronococcus* and *Natronobacterium* strains, but this material was associated with lipids in a complex that did not exhibit osmotic behavior (9). The choice of sulfotrehalose is also unique in that it is the first sulfated sugar to be used as an osmolyte by any organism. A variety of sugars have been found in other organisms as compatible solutes, but few have been detected in true halophiles. Sucrose and trehalose, both neutral molecules, have been accumulated in cyanobacteria such as *Anabaena* spp. as well as some plants and animals (29, 37), while the disaccharide β-fructofuranosyl-α-mannopyranoside was found to accumulate in some strains of the soil bacterium *Agrobacterium tumefaciens* in response to changes in osmotic strength (36). Anionic carbohydrates are rarer and include glucosylglycerate, mannosylglycerate, di-*myo*-inositol-1,1′-phosphate and related compounds (4, 30). In each case, the carbohydrates used for osmotic balance are ones in which the reducing end of the sugar is involved in a glycosidic bond or is otherwise modified. High intracellular concentrations of reducing sugars can result in nonenzymatic glycation of a wide spectrum of macromolecules. Glycation products form from reactive dicarbonyl intermediates that arise from oxidation of the so-called Amadori product (1-amino-1-deoxyketose), which is produced by the reaction of reducing sugars with protein amino groups (13). These glycation end products result in cross-linked proteins with severe physiological consequences (3, 24). To avoid these detrimental reactions, cells use a strategy that modifies the reducing end (e.g., formation of trehalose, glucosylglycerol, or glucosylglycerate) or reduces its reactivity. A 1→2 linkage as in sucrose is much less reactive than other linkages. However, sucrose is not a common osmolyte in extreme halophiles. The addition of a sulfate group to one of the glucose rings of trehalose is a novel strategy, and sulfotrehalose has not been detected in any other organisms thus far. The only sulfur-containing compound identified thus far that might have an osmotic role in cells is dimethylsulfoniopropionate. Dimethylsulfoniopropionate is synthesized and accumulated by algae and plants (8, 26, 28) and possibly by some cyanobacteria (43, 45). However, this zwitterionic methylsulfonium derivative is neither a carbohydrate nor a major osmolyte in bacteria.

Sulfotrehalose biosynthesis may be related to glycolipid biosynthesis since a number of carbohydrates, including sulfated moieties, have been detected in the glycolipids of halophiles. For example, a 1→6-linked diglucosyl sugar was identified in one of the glycolipids of a *Natronobacterium* strain from India (41). Extreme halophilic archaea isolated from Spanish salt ponds were found to contain a number of glycolipids, including a sulfated diglycosyl diether termed S-DGD (19). Sulfated lipids are not unique to halophilic archaea since a halophilic nonalkaliphilic archaebacterium called strain 172 was found to contain a phytanyl-sesterterpenyl-1-[2,6-$(HSO_3)_2$-α-Man*p*-1→2-Glc*p*]-*sn*-glycerol (22). Thus, the enzymology to prepare sulfated glycosyl derivatives as in sulfotrehalose may be linked to lipid biosynthesis in these organisms. Since intracellular sulfotrehalose levels respond to external NaCl, linking osmolyte enzymology with membrane components might generate a useful regulatory scheme.

The intracellular concentration of K^+-sulfotrehalose in *N. occultus* grown in 3.4 M external NaCl is ~1 M. This value is only one-third of that of the external NaCl, indicating that an osmotic gradient exists for the cells. This strongly suggests that there must be other inorganic ions (e.g., Na^+, Cl^-, and polyphosphate) or large organic solutes (possibly macromolecules) that are NMR invisible to help balance the 3.4 M external NaCl. What controls synthesis of the organic anion sulfotrehalose is not clear, although there are a few hints. If these halophilic alkaliphiles are grown in a rich medium, they no longer synthesize and accumulate sulfotrehalose. This may suggest an energy hierarchy in the cells. Accumulation and maintenance of an inorganic ion gradient are energetically costly. In rich medium, there is presumably a larger amount of substrate for growth (or more easily metabolized substrates), so that an ion gradient can be easily established and maintained. In the defined medium, the cost of the ion gradient may now be comparable to that of synthesizing sulfotrehalose. The enzymes of many halophiles have evolved to require high intracellular ion concentrations, and the *Natronococcus* and *Natronobacterium* spp. may have a similar dependency. Thus, while sulfotrehalose can replace some of the inorganic anions, it cannot totally replace them. Furthermore, the charge of the sulfotrehalose is less relevant because it can be effectively replaced by sucrose, which is a neutral sugar. Clearly, what regulates synthesis and accumulation of sulfotrehalose will be an interesting area to investigate in the future.

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REFERENCES

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
2. **Brown, A. D.** 1976. Microbial water stress. Bacteriol. Rev. **40:**803–846.
3. **Brownlee, M.** 1990. Advanced products of non-enzymatic glycosylation and the pathogenesis of diabetic complications, p. 279–293. *In* H. Rifkin and D. Porte, Jr. (ed.), Diabetes mellitus: theory and practice. Elsevier, New York, N.Y.
4. **Ciulla, R. A., S. Burggraf, K. O. Stetter, and M. F. Roberts.** 1994. Occurrence and Role of di-*myo*-inositol-1,1′-phosphate in *Methanococcus igneus*. Appl. Environ. Microbiol. **60:**3660–3664.
5. **Ciulla, R. A., M. R. Diaz, B. F. Taylor, and M. F. Roberts.** 1997. Organic osmolytes in aerobic bacteria from Mono Lake, an alkaline, moderately hypersaline environment. Appl. Environ. Microbiol. **63:**220–226.
6. **Conrad, H. E., E. Varcboncoeur, and M. E. James.** 1973. Qualitative and quantitative analysis of reducing carbohydrates by radiochromatography on ion-exchange papers. Anal. Biochem. **51:**486–500.
7. **Csonka, L. N.** 1982. A third L-proline permease in *Salmonella typhimurium* which functions in media of elevated osmotic strength. J. Bacteriol. **151:** 1433–1443.
8. **Dacey, J. W. H., G. M. King, and S. G. Wakeham.** 1987. Factors controlling emission of dimethyl sulfide from salt marshes. Nature **330:**3177–3187.
9. **DeRosa, M., A. Gambacorta, W. D. Grant, V. Lanzotti, and B. Nicolaus.** 1988. Polar lipids and glycine betaine from haloalkaliphilic archaebacteria. J. Gen. Microbiol. **134:**205–211.
10. **Dodgson, K. S.** 1961. Determination of inorganic sulfate in studies on the enzymic and nonenzymic hydrolysis of carbohydrate and other sulfate esters. Biochem. J. **78:**312–319.
11. **Erdmann, N., S. Fulda, and M. Hagemann.** 1992. Glucosylglycerol accumulation during salt acclimation of two unicellular cyanobacteria. J. Gen. Microbiol. **138:**363–368.
12. **Fan, T. N. M.** 1995. Metabolic profiling by one- and two-dimensional NMR

analysis of complex mixtures. Prog. NMR Spectr. **28:**161–219.
13. **Fu, M.-X., K. J. Wells-Knecht, J. A. Blackledge, T. J. Lyons, S. R. Thorpe, and J. W. Baynes.** 1994. Glycation, glycoxidation, and cross-linking of collagen by glucose: kinetics, mechanisms and inhibition of late stages of the Maillard reaction. Diabetes **43:**676–683.
14. **Gum, E. K., Jr., and R. D. Brown.** 1977. Two alternative HPLC separation methods for reduced and normal celloligosaccharides. Anal. Biochem. **82:** 372–375.
15. **Hoffman, R. E., J. C. Christofides, D. B. Davies, and C. J. Lawson.** 1986. Scope and limitations of the simple ^{13}C-N.M.R. method of structural analysis of carbohydrates: glucodisaccharides. Carbohydr. Res. **153:**1–16.
16. **Imhoff, J. F.** 1986. Osmoregulation and compatible solutes in eubacteria. FEMS Microbiol. Rev. **39:**57–66.
16a. **Jablonski, P.** Unpublished results.
17. **Javor, B.** 1989. Hypersaline environments, p. 101–123. Springer Verlag, New York, N.Y.
18. **Kushner, D. J.** 1985. Microbial life in extreme environments, p. 171–174. *In* C. R. Woese and R. S. Wolfe (ed.), The bacteria, vol. 8. Academic Press, Inc., New York, N.Y.
19. **Kushwaha, S. C., G. J. Perez, F. R. Valera, M. Kates, and D. J. Kushner.** 1982. Survey of lipids of a new group of extremely halophilic bacteria from salt ponds in Spain. Can. J. Microbiol. **28:**1365–1372.
20. **Lai, M.-C., R. Ciulla, M. F. Roberts, K. R. Sowers, and R. P. Gunsalus.** 1995. Extraction and detection of compatible intracellular solutes. p. 349–368. *In* K. R. Sowers and H. J. Schreier (ed.), Archaea: a laboratory manual. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
21. **Lai, M.-C., K. R. Sowers, D. E. Robertson, M. F. Roberts, and R. P. Gunsalus.** 1991. Distribution of compatible solutes in the halophilic methanogenic archaebacteria. J. Bacteriol. **173:**5352–5358.
22. **Matsubara, T., N. I. Tanaka, M. Kamekura, N. Moldoveanu, I. Ishizuka, H. Onishi, A. Hayashi, and M. Kates.** 1994. Polar lipids of a non-alkaliphilic extremely halophilic archaebacterium strain 172: a novel bis-sulfated glycolipid. Biochim. Biophys. Acta **1214:**97–108.
23. **Measures, J. C.** 1975. Role of amino acids in osmoregulation of non-halophilic bacteria. Nature **257:**398–400.
24. **Monnier, V. M.** 1989. Toward a Maillard reaction theory of aging, p. 1–22. *In* J. W. Baynes and V. M. Monnier (ed.), The Maillard reaction in aging, diabetes and nutrition: an NIH conference. Alan R. Liss, New York, N.Y.
25. **Mullings, R., and J. H. Parish.** 1984. New reducing sugar assay for the study of cellulases. Enzyme Microb. Technol. **6:**491–496.
26. **Paquet, L., B. Rathinasabathi, H. Saini, L. Zamir, D. A. Gage, Z.-H. Huang, and A. D. Hanson.** 1994. Accumulation of the compatible solute 3-dimethylsulfoniopropionate in sugarcane and its relatives, but not in other gramineous crops. Aust. J. Plant Physiol. **21:**37–48.
27. **Pavao, M. S. G., P. A. S. Mourao, B. Mulloy, and D. M. Tollefsen.** 1995. A unique dermatan sulfate-like glycosaminoglycan from ascidian, its structure and the effect of its unusual sulfation pattern on anticoagulant activity. J. Biol. Chem. **270:**27–34.
28. **Reed, R. H.** 1983. Measurement and osmotic significance of β-dimethylsulfoniopropionate in marine algae. Mar. Biol. Lett. **4:**173–181.
29. **Reed, R. H., L. J. Borowitzka, M. A. MacKay, J. A. Chudek, R. Foster, S. C. R. Warr, D. J. Moore, and W. D. P. Stewart.** 1986. Organic solute accumulations in osmotically stressed cyanobacteria. FEMS Microbiol. Rev. **39:**51–56.
30. **Robertson, D. E., M.-C. Lai, R. P. Gunsalus, and M. F. Roberts.** 1992. Composition, variation, and dynamics of major compatible solutes in *Methanohalophilus* strain FDF1. Appl. Environ. Microbiol. **58:**2438–2443.
31. **Robertson, D. E., D. Noll, and M. F. Roberts.** 1992. Free amino acid dynamics in marine methanogens. J. Biol. Chem. **267:**14893–14901.
32. **Robertson, D. E., D. Noll, M. F. Roberts, J. A. G. F. Menaia, and D. R. Boone.** 1990. Detection of the osmoregulator betaine in methanogens. Appl. Environ. Microbiol. **56:**563–565.
33. **Robertson, D. E., and M. F. Roberts.** 1991. Organic osmolytes in methanogenic archaebacteria. Biofactors **3:**1–9.
34. **Robertson, D. E., M. F. Roberts, N. Belay, K. O. Stetter, and D. R. Boone.** 1990. Occurrence of β-glutamate, a novel osmolyte in marine methanogenic bacteria. Appl. Environ. Microbiol. **56:**1504–1508.
35. **Roll, D. R., and H. E. Conrad.** 1977. Quantitative radiochromatographic analysis of the major groups of carbohydrates in cultured animal cells. Anal. Biochem. **77:**397–412.
36. **Smith, L. T., G. M. Smith, and M. Madkour.** 1990. Osmoregulation in *Agrobacterium tumefaciens*: accumulation of a novel disaccharide is controlled by osmotic strength and glycine betaine. J. Bacteriol. **172:**6849–6855.
37. **Somero, G. N., C. B. Osmond, and C. L. Bolis.** 1992. Water and life: comparative analysis of water relationships at the organismic, cellular, and molecular levels. Springer-Verlag, New York, N.Y.
38. **Sowers, K. R., D. E. Robertson, D. Noll, R. P. Gunsalus, and M. F. Roberts.** 1990. Nε-Acetyl-β-lysine: an osmolyte synthesized by methanogenic archaebacteria. Proc. Natl. Acad. Sci. USA **87:**9083–9087.
39. **Tindall, B. J., H. N. M. Ross, and W. D. Grant.** 1984. *Natronobacterium* gen. nov. and *Natronococcus* gen. nov., two new genera of haloalkaliphilic archaebacteria. Syst. Appl. Microbiol. **5:**41–57.
40. **Tindall, B. J., and H. G. Truper.** 1986. Ecophysiology of the aerobic halophilic archaebacteria. Syst. Appl. Microbiol. **7:**202–212.
41. **Upasani, V. N., S. Desai, N. Moldoveanu, and M. Kates.** 1993. Lipids of extremely halophilic archaeobacteria from saline environments in India: a novel glycolipid in *Natronobacterium* strains. Microbiology **140:**1959–1966.
42. **Usui, T., N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama, and S. Seto.** 1973. ^{13}C nuclear magnetic resonance spectra of glucobioses, glucotrioses, and glucans. J. Chem. Soc. Perkin Trans. **1:**2425–2432.
43. **Visscher, P. T., and H. van Gemerden.** 1991. Production and consumption of dimethylsulfoniopropionate in marine microbial mats. Appl. Environ. Microbiol. **57:**3237–3242.
44. **Vreeland, R. H.** 1987. Mechanisms of halotolerance in microorganisms. Crit. Rev. Microbiol. **14:**311–356.
45. **White, R. H.** 1982. Analysis of dimethylsulfonium compounds in marine algae. J. Mar. Res. **40:**529–536.
46. **Yamaoka, N., T. Usui, K. Matsuda, and K. Tuzimura.** 1971. ^{13}C nuclear magnetic resonance spectra of glucobioses. Tetrahedron Lett. **23:**2047–2048.
47. **Yancey, P. H., M. E. Clark, S. C. Hand, R. D. Bowlus, and G. N. Somero.** 1982. Living with water stress: evolution of osmolyte systems. Science **217:** 1214–1217.