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Osmotic adaptation of moderately halophilic methanogenic Archaeobacteria, and detection of cytosolic *N,N*-dimethylglycine

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Abstract. Methanohalophilus mahii SLP and *Methanohalophilus halophilus* Z-7982, two closely-related, moderately halophilic, methylotrophic methanogens, were tested for their adaptation to saline conditions. They grew in a wider range of salinities than previously reported, in a defined medium with as little as 0.1 M NaCl, and with a high as 4.0 M NaCl for *M. halophilus* and 4.5 M NaCl for *M. mahii*. Fastest growth occurred with 1.5 M NaCl for *M. mahii* and 1.0 M NaCl for *M. halophilus*. *M. mahii* also grew in media in which NaCl was replaced by sucrose or KCl as osmolytes up to the osmolal equivalent of 2 and 2.5 M NaCl (these media contained other sodium salts totaling about 0.1 M Na^+). In media with either sucrose of KCl replacing NaCl, *M. mahii* grew fastest at osmolalities approximately equiosmolal to 1 M NaCl. *M. mahii* not only grew well at a wide range of osmosities, it also tolerated rapid shifts in osmolality. Cells subjected to a rapid 10-fold hypertonic shift resumed growth without a prolonged lag. When cells were subjected to a rapid 10-fold hypotonic shift, 90% of cells lysed, but the remainder continued to swell with little further lysis during the next 45 min. Surviving cells resumed growth. *Methanohalophilus* strains grown in defined medium had low cytosolic Na^+ concentrations; K^+ concentrations were as high as 0.35 M. Organic osmotica in the cytosol include glycine betaine and larger amounts of N,N-dimethylglycine.

Key words. N,N-dimethylglycine; methanogens; compatible solutes; halophilic bacteria; osmotic adaptation.

Introduction

Methanogenic archaeobacteria inhabit hypersaline environments such as saline lakes[18,24,33], solar salterns[18,19], intertidal lagoons[34,35], estuaries[23], and saline ground water[15]. These methanogens growth by forming methane from methyl compounds such as trimethylamine (TMA)[15,24,34,35], methanol[15,18,24,34], methyl sulfide[12,20,22,23] and methane thiol[8,21]. Among these methanogens which grow well at neutral pH, one species is extremely halophilic (*Methanohalabium evestigatum*) and two species are moderately halophilic (*Methanohalophilus halophilus* and *Methanohalophilus mahii*). One other species of neutrophilic, halophilic methanogens (*Halomethanococcus doii*) was described[33] but the available type strain and its distinction from *Methanohalophilus halophilus* and *Methanohalophilus mahii* are in doubt[2].

When cells are subjected to hypotonic shock, elevated external A_w causes water to diffuse into the cell, thereby increasing the A_w of the cytosol, until internal A_w matches external A_w. The immediate effect of this water movement is an increase in cell volume and, depending on the elasticity and tensile strength of the cell wall, development of turgor pressure. Halophilic methanogens have weak cell walls[11,24,34], so they cannot develop significant turgor pressure. In the absence of turgor pressure, the cytosolic A_w can be rapidly raised only by dilution (and expansion of cell volume) or release of cytosolic osmolytes. Release of cytosolic osmolytes other than by lysis has not been described for methanogens, but possible mechanisms include pressure-sensitive channels and temporary breaches in the cell membrane which quickly re-seal[7].

The equilibration of water activity (A_w) between the cytosol and environment is almost instantaneous because water moves rapidly across microbial cell membranes. This equilibrium implies that cells growing in environments of low A_w must have high concentrations of cytosolic solutes. Otherwise, cytosolic A_w higher than that of the environment would cause efflux of water until the lowered cytosolic A_w balanced environmental A_w. Halophiles growing in the presence of high NaCl concentration exclude Na^+, probably because it would inhibit cytosolic enzymes[5]. In such organisms, other cytosolic solutes must lower the A_w to achieve osmotic balance. In methanogens, these solutes may be potassium salts[14] or compatible solutes[4], which are organic solutes such as glycine betaine (GB)[14,17] and β-amino acids and derivatives[26,32] (table 1). Glutamate is ubiquitous in methanogens and β-glutamate is common (table 1). Methanogens from saline environments or those which tolerate salinity (*Methanococcus voltaei*,

Table 1. Presence of organic compatible solutes in methanogens

Organism	α-glu	β-glu	β-gln	*N*-acetyl β-lysine	GB	Reference
Methanosarcina thermophila	+	−				32
Methanosarcina strain AN9	+	+				26, 27
Methanogenium cariaci	+	+		+	+	26, 27, 32
Methanococcus thermolithotrophicus	+	+		−		26, 27
Methanococcus igneus	+	+				26
Methanococcus jannaschii	+	+				26
Methanococcus voltaei	+	−				27
Methanococcus maripaludis	+	−				26
Methanolobus oregonensis GS-16	+	−	+	+	−	14
Methanohalophilus strain CAS-1	+	+	+	+	−	14
Methanohalophilus zhilinaeae	+	−			+	26, 27
Methanohalophilus mahii	+	−	+	+	±	14, 27
Methanohalophilus halophilus	+	+	+	+	−	14
Methanohalobium evestigatum	+	−	+	+	+	14

Methanogenium cariaci, *Methanogenium* strain AN9, *Methanohalophilus zhilinaeae*, and *Methanohalophilus mahii*) have significant cytosolic concentrations of GB when grown in saline medium[14,27]. However, GB was not detected in *Methanohalophilus* strain RET-1 and *Methanohalophilus halophilus*[14].

We report the presence of endogeneous *N*,*N*-dimethylglycine (DMG) as an important compatible solute in three methylotrophic and moderately halophilic methanogens: *Methanohalophilus mahii*, *Methanohalophilus halophilus*, and *Methanohalophilus* strain RET-1. The cytosol of these strains also contained GB and K^+, although at concentrations significantly lower than of DMG. We also report the osmotically adaptive features of the methanogenic archaeobacteria *M. mahii* and *M. halophilus*, and the ability of *M. mahii* to withstand both hypotonic and hypertonic osmotic shocks.

Materials and methods

Source of organisms

Methanohalophilus mahii SLP (OCM 68), *Methanohalophilus halophilus* Z-7982 (OCM 160), *Methanohalophilus* strain RET-1 (OCM 57), and *Methanohalobium evestigatum* strain Z-7303 (OCM 161) were from the Oregon Collection of Methanogens, Beaverton, Oregon.

Culture methods

Media preparation, culture procedures and anoxic manipulations were adapted from the Hungate technique[10]. Basal culture medium (Z medium) contained (per liter): 4 g of NaOH; 1.4 g of TMA·HCl; 5 g of $MgCl_2 \cdot 6H_2O$; 1.5 g of KCl; 1 g of NH_4Cl; 0.4 g of K_2HPO_4; 0.25 g of $Na_2S \cdot 9H_2O$; 0.05 g of $CaCl_2 \cdot 2H_2O$; 5 mg of Na_2-EDTA·$2H_2O$; 1.5 mg of $CoCl_2 \cdot H_2O$; 1.0 mg of $MnCl_2 \cdot 4H_2O$; 1.0 mg of $FeSO_4 \cdot 7H_2O$; 1.0 mg of $ZnCl_2$; 0.4 mg of $AlCl_3 \cdot 6H_2O$; 0.3 mg of $Na_2WO_4 \cdot 2H_2O$; 0.2 mg of $CuCl_2 \cdot 2H_2O$; 0.2 mg of $NiSO_4 \cdot 6H_2O$; 0.1 mg of H_2SeO_3; 0.1 mg of H_3BO_3; 0.1 mg of $NaMoO_4$; 0.5 g of mercaptoethanesulfonic acid (coenzyme M); and 2.5 ml of a vitamin solution[1]. Media were prepared by dissolving NaOH is distilled water and equilibrating with an O_2-free mixture of N_2 and CO_2(7:3). Residual O_2 was removed by bubbling the gas mixture for 10 min with stirring, while medium constituents other than sodium sulfide and vitamine solution were added, with coenzyme M added last. The medium was dispensed into individual vessels, sealed with butyl rubber stoppers, and autoclaved at 121 °C for 20 min, or 30 min for bottles with liquid volume of 500 ml or more. Vitamin solution and sodium sulfide were added prior to inoculation, from sterile, anoxic, stock solutions. After the medium cooled and the gas re-equilibrated, the pH was 7.25. For media of different osmolalities, the amount of NaOH added was adjusted empirically to obtain a final pH of 7.25 ± 0.05. Media of various osmolalities were prepared by varying the amount of NaCl added, or by replacing it with KCl or sucrose (table 2).

Osmolality was calculated according to the formula:

$$\text{osmodal} = \sum_j \phi_j m_j \qquad (1)$$

where values for ϕ, the osmotic coefficient, were from published values[29]. Cells were grown in 25-ml serum tubes containing 5 ml of medium, and 70- and 2000-ml bottles containing 20 and 1000 ml of medium, respectively. Inoculation volumes were 5% (v/v) and incubation was static and at 37 °C for *Methanohalophilus* and 50 °C for *Methanohalobium evestigatum*. Inocula were transferred at least four times in medium with the same composition as used in each experiment.

Methanohalophilus strains were routinely grown in ZV medium with 1 M NaCl and *Methanohalobium evestigatum* was grown in ZV medium with 4 M NaCl.

Determination of specific growth rates

For experiments in which specific growth rates are reported, inocula were grown in medium with the same

Table 2. Molar concentration of osmolytes and Na^+, osmolality and osmosity of culture media

Medium	(NaCl)	(KCl)	(sucrose)	(Na^+)	osmolality	osmosity
ZV-0	0	0	0	0.1	0.18	0.09
ZV-0.1	0.1	0	0	0.2	0.36	0.19
ZV-0.5	0.5	0	0	0.61	1.12	0.6
ZV-1	1	0	0	1.15	2.17	2.16
ZV-1.5	1.5	0	0	1.68	3.27	1.67
ZV-2	2	0	0	2.17	4.4	2.16
ZV-2.5	2.5	0	0	2.69	5.66	2.68
ZV-3	3	0	0	3.19	7.03	3.18
ZV-3.5	3.5	0	0	3.68	8.49	3.67
ZV-4	4	0	0	4.12	9.99	4.11
ZV-4.5	4.5	0	0	4.65	11.8	4.64
ZV-5	5	0	0	5.17	13.75	5.16
S-1	0	0	1.4	0.13	2.58	1.33
S-1.5	0	0	1.89	0.09	4.32	2.09
S-2	0	0	2.31	0.07	6.61	2.97
Sa-0.5	0	0	0.73	0.12	1.14	0.61
Sa-1	0.5	0	0.73	0.64	2.11	1.1
Sa-1.5	1	0	0.73	1.16	3.11	1.57
Sa-2	1.5	0	0.73	1.65	4.14	2.02
Sb-1.5	0.5	0	1.27	0.65	3.24	1.63
Sb-2	0.5	0	1.64	0.65	4.35	2.11
ZK-0.5	0	0.51	0	0.13	1.12	0.6
ZK-1	0	1.03	0	0.16	2.17	1.14
ZK-1.5	0	1.57	0	0.14	3.27	1.67
ZK-2	0	2.09	0	0.14	4.38	2.16
ZK-2.5	0	2.65	0	0.14	5.63	2.68

composition as in the experiments. Growth rates were calculated from the rate of CH_4 accumulation in headspace during the exponential phase[3].

Cell harvesting

Cells were harvested from 2500-ml cultures in late exponential phase. Cultures were centrifuged at 4 °C for 45 min at 7,000 × *g* to form a soft pellet, which was washed 3 times in 5 ml of isotonic phosphate buffer (pH 7.2, with 50 mM phosphate, 10 mM cellobiose, Mg^{2+} and Ca^{2+} concentrations the same as those of the culture medium, and with the NaCl concentration sufficient to provide the same A_w as the culture medium). When cytosolic Na^+ was measured, cells were washed in buffer with K^+ salts replacing Na^+ salts. After the last centrifugation the supernatant was removed with a Pasteur pipet, and a strip of filter paper was inserted into the pellet for 5 min to withdraw residual buffer. *Methanohalobium evestigatum* cells did not form a pellet under these conditions, perhaps because the specific gravity of the cells was similar to that of the culture medium. Therefore, cells were obtained by diluting the culture with 250 ml of deionized water per liter before centrifuging.

Lysates and aqueous extracts of cells

Lysates were prepared by sonicating (30 W, 30 s) cell pellets in an ice bath. Aqueous extracts were prepared by diluting cell pellets 10 fold (w/v) with deionized water (18 $M\Omega\,cm^{-1}$), and stirring at 100 °C for 10 min. The lysate was centrifuged at 7,000 × *g* and 4 °C for 30 min^{30}, and the supernatant (aqueous extract) was stored at −20 °C.

Cell-volume measurements

Our calculations of cytosolic volumes assumed that the volume of the particulate cell fraction was negligible, so the cytosolic volume in cell pellets was the total volume of each cell pellet minus the volume of buffer retained in the intercellular space. The total volume of a cell pellet was calculated from the mass of the pellet and its density, determined at 25 °C by using a 10-ml volumetric flask as a pycnometer. The intercellular volume was determined by measuring the amount of cellobiose (a component of the cell-washing buffer) retained in the pellet.

Microscopy and detection of lysis

Morphology, size and integrity of cells were examined in fresh wet mounts, with a Zeiss Axioskop equipped with phase-contrast, epifluorescence and microphotography. Phase-contrast microphotographs were taken from fresh wet mounts of exponentially growing cultures. Cells were counted in a Petroff-Hauser chamber. Lysis of cells by detergents were determined by visual changes in turbidity[3] and by microscopic examination of wet-mounts. Cell integrity was indicated by retention of epifluorescence.

Analytical methods

A_w was measured with a psychrometer (model SC-10, Decagon, Pullman, Washington). In order to avoid effects of temperature variations on A_w measurements, the psychrometer was loaded with samples from the

culture medium, cell lysate, and standards. After 1-h equilibration, 3 sets of readings were made at 30-min intervals. Absorbance (A_{450}) of cell suspensions was measured with a Spectronic 21 colorimeter (Milton Roy, Rochester, New York). Methane was analyzed by gas chromatography with flame-ionization detection[16]. Cellobiose concentration was measured enzymatically with cellobiose oxidase[25]. pH was measured with a combination electrode Orion model 91-04 (Boston, Massachusetts), which we found to be relatively insensitive to interference by Na^+. DMG and GB were analyzed by cation-exchange high-pressure liquid chromatography with a column of Partisil 10-SCX (Whatman) and detected by UV absorbance at 195 nm[9]. The mobile phase was phosphate buffer (50 mM KH_2PO_4, pH 4.6) with 5% methanol (v/v). The presence of DMG and GB was confirmed by thin-layer chromatography on 0.2-mm silica gel with various solvent systems; best separation of GB, DMG, and sarcosine was obtained with ethanol, n-butanol, and 5% (v/v) aqueous ammonia (10:5:1) or with ethanol and water (5:1). Chromatograms were developed in iodine vapor.

Results

Halophilia and halotolerance of M. mahii *and* M. halophilus

M. mahii grew rapidly in medium with osmolality of 2.2 to 4.4 osmol (equiosmolal to 1.1 and 2.2 M NaCl) when NaCl was the major solute in the medium, with fastest growth (0.043 h^{-1}) at 3.3 osmol (equiosmolal to 1.7 M NaCl) (fig. 1). *M. halophilus* was slightly less halophilic than *M. mahii*, growing rapidly at 2.2 to 3.3 osmol (equiosmolal to 1.14 M to 1.67 M NaCl), with fastest growth (0.045 h^{-1}) also as a slightly lower osmolality (2.2 osmol, equiosmolal to 1.14 M NaCl) than was found for *M. mahii* (fig. 1). Both of these methanogens grew (though slowly) in medium with an osmolality as low as 0.36 osmol (equiosmolal to 0.19 M NaCl). Not only was *M. mahii* slightly more halophilic than *M. halophilus*, but it was also more halotolerant, growing at osmolalities up to 11.8 osmol (equiosmolal to 4.6 M NaCl). *M. halophilus* did not grow above 10 osmol (equiosmolal to 4.1 M NaCl).

When we determined the lowest salinity which could support growth, we found that *M. mahii* was usually able to withstand 2-fold hypertonic shocks during inoculation. However, cells inoculated into media with salinities near the lower limit for growth for *M. mahii* (0.25 M or less) were more sensitive. When such media were inoculated with cultures grown at 2-fold higher osmolality, growth was not reproducible. For instance, cultures grown in 0.5 M NaCl inoculated into medium with 0.25 M NaCl did not grow consistently even though cultures grew well in 0.25 M NaCl from inocula grown in 0.25 M NaCl. Thus, to determine the lowest salinity which allows growth, it was necessary to change medium salinity in small steps. This may have allowed us to document growth at lower salinity than was found previously.

Growth of M. mahii *in media with sucrose as the dominant osmolyte*

When NaCl was omitted from culture medium and replaced by sucrose as the main osmolyte, rapid growth occurred at lower osmolalities (fig. 2). Growth was faster in sucrose medium than NaCl medium at 1.1 osmol and slower at 4.3 osmol. The growth of *M. mahii* in sucrose media was as rapid at osmolalities of 1.1 osmol to 2.6 osmol (250.5 g to 518.8 g of sucrose per liter) as the most rapid growth in NaCl media. Media without added NaCl contained 70 mM to 120 mM Na^+ from other medium salts. The specific growth rate of

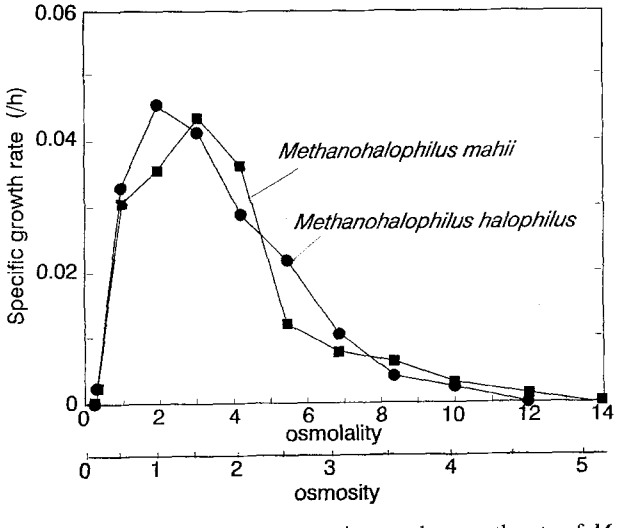

Figure 1. Effect of NaCl concentration on the growth rate of *M. mahii* and *M. halophilus*.

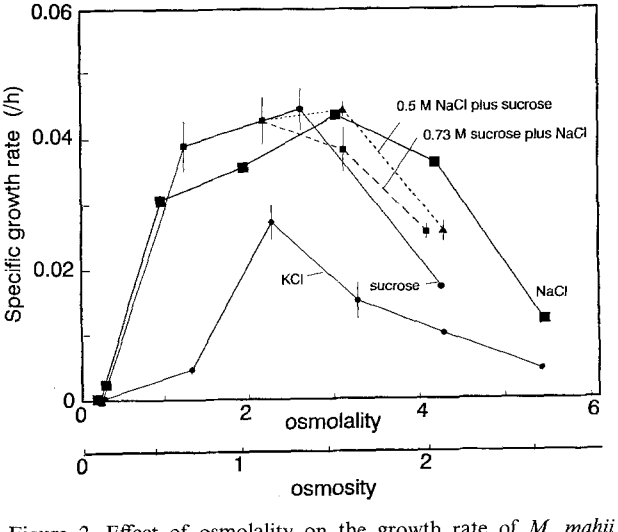

Figure 2. Effect of osmolality on the growth rate of *M. mahii* when the concentration of various solutes is varied.

M. mahii was not greatly affected when NaCl partially replaced sucrose as medium osmolyte (fig. 2).

Growth of M. mahii *in media with KCl*

M. mahii also grew in media having KCl as the major osmolyte (fig. 2). These media had 120 to 138 mM Na^+ from other medium salts. Growth was slower than in equiosmolal media with NaCl at all concentrations tested. Growth occurred in media with 0.51 M to 2.65 M KCl. Fastest growth occurred at lower osmolality (2.2 osmol [1.031 M KCl]) with KCl as the major medium osmolyte than when NaCl was the major osmolyte (fig. 2).

Cell size and morphology of M. mahii *and* M. halophilus *growing at low and high osmolalities*

The morphology of *M. mahii* and *M. halophilus* varied according to salinity. When these two species grew at osmolalities of 2.2 osmol or higher, they appeared microscopically as very irregular cocci, but when they grew at 0.36 osmol they were large and spherical, appearing to be turgid (fig. 3). Changes in morphology have been reported for other halophilic bacteria as well. These cells tend to be larger and rounder at lower osmolalities[4]. At osmolalities below 1.14 (equiosmolal to 0.5 M NaCl), *M. mahii* was sensitive to agitation. Growth of cultures incubated on a shaker was not reproducible, whereas growth with static incubation was reproducible.

Turgor pressure in M. mahii

We measured turgor pressure in *M. mahii* by measuring the A_w of the culture medium and comparing to the A_w of the cytosol of lysed cells. During growth, the A_w equilibrates across the cell membrane, so the cytoplasmic A_w is equal to that of its environment. A change in cytosolic A_w upon lysis would indicate that a significant component of this A_w was derived from turgor pressure. We grew *M. mahii* in medium with 2 M NaCl and collected cell pellets. The volume of extracellular water in these pellets was about 3% (v/w). We lysed the cells by sonication, measured A_w, and compared it with the A_w of the culture medium. Two independent measurements of the turgor pressure of *M. mahii* indicated 0 ± 0.5 atm and 0.6 ± 0.4 atm.

Susceptibility of M. mahii *to detergents*

M. mahii was tested for susceptibility to sodium dodecyl sulfate and Triton X-100, at 0.5% and 0.05% (w/v), under iso- and hypotonic conditions. We grew a 20-ml culture to late exponential phase in medium with 2 M NaCl, collected the cells by centrifugation, and resuspended in 2 ml of the same medium. We added 0.1 ml of this suspension to 0.9 ml of: a) isotonic medium; b) water c) isotonic medium plus detergent or d) water plus detergent. In the absence of detergent, cell suspensions diluted into water or into isotonic medium remained turbid. Some lysis may have occurred, but a substantial fraction of cells remained intact and retained their epifluorescence. The effect of detergents was the same at either concentration (0.5% or 0.05%). Triton X-100 lysed cells in both water and in isotonic medium, as determined by loss of turbidity and microscopic examination. However, sodium dodecyl sulfate lysed cells diluted into water, but not those in isotonic medium.

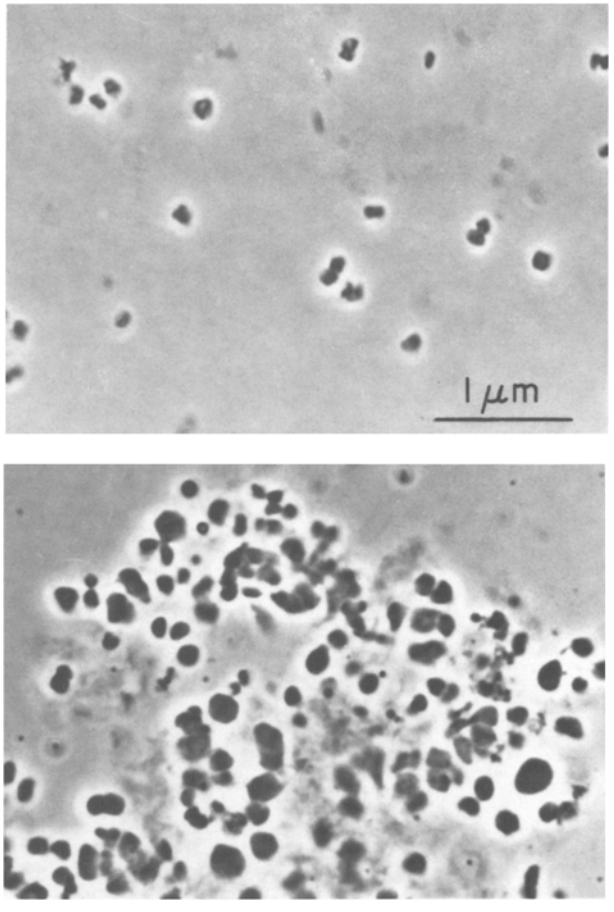

Figure 3. Photomicrograph of *M. mahii* growing in media with 4 M (top) and 0.1 M (bottom) NaCl.

Resistance of M. mahii *to hypertonic shock*

M. mahii cultures were grown to late exponential phase in media with 0.5 M NaCl, and inoculated (10% v/v) into media with various NaCl concentrations ranging from 0.5 to 4 M NaCl. Within 3 weeks all of these cultures grew to completion, as indicated by accumulated methane approximating the amount stoichiometrically expected from the catabolic substrate. These results indicated that at least some *M. mahii* cells could survive large, rapid shifts to higher osmolality.

Resistance of M. mahii *to hypotonic shock*

M. mahii was grown in media with various concentrations of NaCl (1 to 3 M) and exposed to osmotic shock by a 1:10 dilution in media without NaCl. For each initial concentration of NaCl tested, two diluted cultures were mixed by gently inverting the dilution tube,

and two others were immediately stirred with a vortex-mixer for 15 s. After 3 weeks of incubation, growth was complete in all samples. *M. mahii* growing in medium with 4 M NaCl gave erratic results when diluted in this way, with some cultures not growing and becoming oxidized (indicated by resazurin turning pink) before the conclusion of the experiment. We modified the experiment to give a higher number of cells in the inoculum: A culture was grown in 4 M NaCl, concentrated 10-fold by centrifugation and resuspension in the same medium, and 10 10-fold dilutions of this suspension were made in medium without NaCl. Two of these were vortexed immediately, the others were mixed by gentle inversion, and all were incubated 30 min at room temperature, and the NaCl concentration was adjusted to 1.5 M. Near complete growth (>50% of expected methane) occurred within 1 week of incubation in four samples, within 6 weeks in four other samples (including the two vortexed samples), but no methane was formed in the last two samples.

We used optical density to investigate the time-course of cell swelling after hypotonic shock. Light scattering of a cell suspension is a function of cell size, with larger cell having lower turbidity[13]. A 20-ml culture grown in medium with 4 M NaCl was centrifuged to form a dense cell suspension of about 1 ml. This suspension was diluted 1:10 under non-anoxic conditions, either with isotonic medium or with water. Microscopic observation indicated that cells swelled and may lysed within 30 s when the culture was diluted with water. During the next hour, the unlysed cells appeared to swell and a few cells were seen to lyse. No swelling or lysis occurred in isotonic medium during the same time period. The experiment was repeated, but this time cells were counted after 30 s and after 1 h (about 10 min was required to count the cells) and A_{450} was determined (fig. 4). These results indicate that minimal lysis occurred after the first 30 s, and that the cells continued to swell for about 45 min. In no case were the counts of a culture significantly different (95% confidence) when measurement began 30 s or 1 h after hypotonic shift. This slowness of the swelling was unexpected, so we vortexed the hypotonically shocked cells for 7 s and for 30 s (fig. 4). This treatment caused slightly greater lysis of cells during the first 30 s than in gently mixed suspensions. Those cells vortexed for 7 s continued to swell for 45 min, but cells vortexed for 30 s did not swell further (fig. 4). To determine whether enzymatic activity was necessary for cells to withstand 10-fold hypotonic shock, we chilled the cell suspensions on ice, and diluted them with cold water. Microscopic observations indicated a similar pattern of rapid lysis of some cells during the first 30 s to 1 min, and continued slow swelling afterward.

K^+ *and* Na^+ *in* Methanohalophilus

We determined cytosolic concentrations of K^+ and Na^+ in *Methanohalophilus* strain RET-1, *Methanohalophilus*

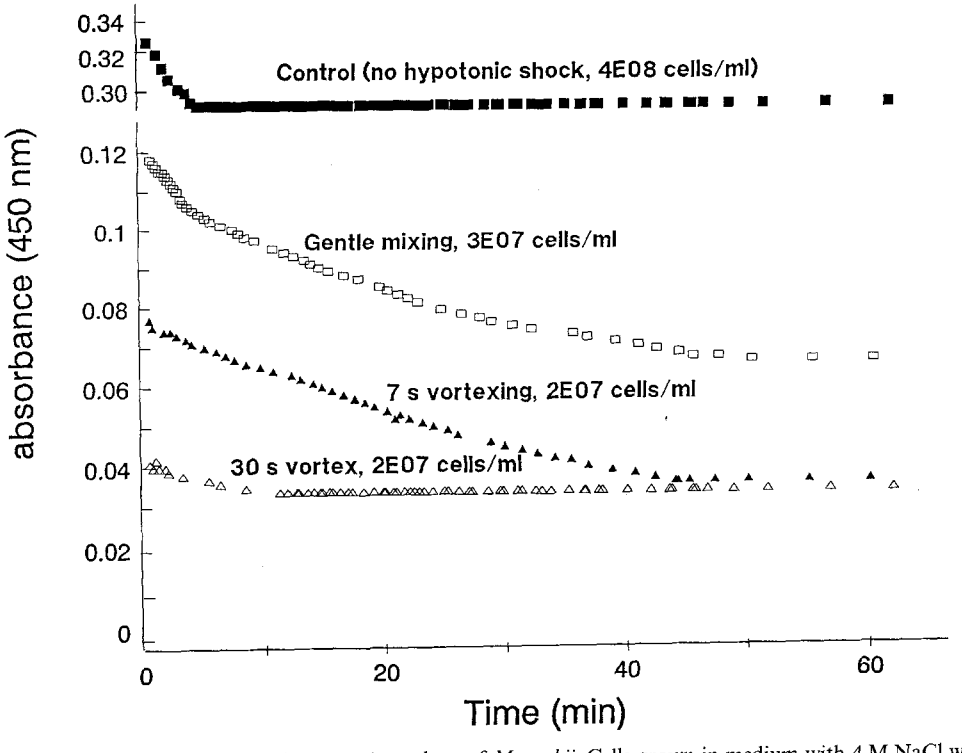

Figure 4. Effect of hypotonic shock on absorbance and numbers of *M. mahii*. Cells grown in medium with 4 M NaCl were diluted 10× (final NaCl concentration of 0.4 M), and mixed gently or vortexed for 7 s or 30 s.

mahii, and *Methanohalophilus halophilus*. Cytosolic K^+ concentration never exceeded 0.35 M when *Methanohalophilus mahii* and *Methanohalophilus halophilus* were grown in media with total osmotic strengths of 2.2, 4.4, and 7.0 osmol (ZV medium with 1, 2, and 3 M NaCl added). Intracellular Na^+ concentrations were 0.025 ± 0.008 M in both of these two strains grown in each of these three media. When grown in medium with 1.5 NaCl, *Methanohalophilus* strain RET-1 had intracellular concentrations of 0.12 M K^+ and 0.01 M Na^+.

GB in Methanohalophilus *and* Methanohalobium

When *Methanohalophilus mahii* was grown in ZV medium with 1 M NaCl (2.2 osmol), 2 M NaCl (4.4 osmol) and 3 M NaCl (7.0 osmol), cytosolic GB concentration was 0.11 M, 0.30 M, and 0.36 M, respectively.

GB was detected in aqueous extracts from all four halophilic methanogens. The intracellular concentration of GB in *Methanohalophilus* strains grown in ZV medium with 2 M NaCl (4.4 osmol) did not significantly vary (0.24 ± 0.07 M) either among strains or when the catabolic substrate was varied (TMA or methanol). The intracellular concentration of GB in *Methanohalobium evestigatum* grown in ZV medium with 4 M NaCl (9.99 osmol) was similar (0.29 M).

DMG in halophilic methanogens

We detected DMG in aqueous extracts from each of the 3 *Methanohalophilus* strains. Similar cytosolic levels of DMG (1.3 ± 0.3 M) were found in all *Methanohalophilus* strains grown in medium with 2 M NaCl (4.4 osmol), whether they were grown on TMA or methanol. Intracellular DMG concentration of *Methanohalophilus mahii* increased with medium osmolality. When medium osmolality was 2.2, 4.4, and 7.0 the DMG concentration was 0.58 M, 1.24 M, and 2.00 M, respectively. GB but not DMG was detected in *Methanohalobium evestigatum*.

Thin-layer chromatography of n-methyl glycines

We examined cell extracts of *Methanohalophilus mahii*, *Methanohalophilus halophilus*, and *Methanohalobium evestigatum* by thin-layer chromatography. GB was found in all three extracts, but DMG was found only in the *Methanohalophilus* strains. Sarcosine in prepared solutions could be separated from other methylated glycines and detected, but it was not detectable in any of these cells extracts.

Effects of compatible solutes on the growth of Methanohalophilus mahii

We grew *Methanohalophilus mahii* in the presence of various potential compatible solutes to determine their effect on the growth rate of *Methanohalophilus mahii*. Growth media were amended with 5 mM of DMG, GB, sarcosine, glycolate, oxalate, acrylate, choline, glycine, urea, proline, glutamate, β-glutamate, glycerol, trehalose, dimethylsulfoniopropionate, acetate, γ-aminobutyrate, putrescine, spermine, or spermidine. We also tested the effect of yeast extract, Trypticase peptone (BBL Microbiology Systems, Cockeysville, Maryland), and casein hydrolysate (Difco, Detroit, Michigan) at 2 g per liter of medium. Only DMG, GB, sarcosine, and yeast extract stimulated the growth of *Methanohalophilus mahii*.

Discussion

M. mahii and *M. halophilus* grew over a wider range of osmolalities than previously published ranges[24,34]. Possible reasons why we found growth over a wider range of osmolalities were that we decreased osmolality in small steps and that glycine betaine was absent from our culture medium. Glycine betaine is present in yeast extract[6], a component of the culture medium used in the initial characterization of *M. mahii*[24]. Glycine betaine may inhibit growth at low salinity because it is accumulated as a compatible solute of *Methanohalophilus*[27].

The survival of intact cells of *M. mahii* after a 10-fold hypotonic shift was unexpected, because osmotic equilibrium of water across cell membranes takes place within a few milliseconds[13,17]. This flux of water results in cell bursting unless the influx is managed in one of three ways: swelling, increased tugor pressure, or loss of osmolytes from the cytosol[5]. Cell swelling is certain one of the mechanisms by which *M. mahii* dealt with hypotonic shock, as we observe microscopically. The highly irregular morphology of halophilic methanogens may allow significant increases in cell volume as the cells swell and become more spherical. However, cells would have had to increase 10-fold in volume (approximately 2-fold in radius) to achieve equilibrium if swelling were the only adaptive response.

The development of a significant turgor pressure in *M. mahii* is unlikely to be an important factor in tolerance of hypotonic shock. *M. mahii's* cell wall appears to be a single glycoprotein layer (S layer), like that of *M. halophilus*[34] and many other methanogens[11], and S layers lack the tensile strength to withstand significant turgon pressure[11,31]. Also, *M. mahii* was fragile when grown in media of low osmolarity, and the shear stress of agitation was probably much less than the turgor pressure which would result from a 10-fold hypotonic shift (approximately 200 atm of turgor pressure) unless other responses were also at work.

The loss of osmolytes from the cytoplasm might be an adaptive response of these methanogens. Examples of this response in other cells include the presence of pressure-sensitive membrane channels in *Escherichia coli* or even a breach of the cell membrane which rapidly recloses[7]. Other responses such as polymerization of cytoplasmic constitutents are unlikely to be swft enough to prevent lysis caused by the rapid influx of water.

Our findings corroborated published results showing that GB is a compatible solute in moderately and extremely halophilic methanogens[14,27], including three strains (*Methanohalophilus mahii*, *Methanohalophilus halophilus*, and *Methanohalophilus* strain RET-1) in which previous studies were unable to detect cytosolic GB. Another important contrast between our results and those of Lai et al.[14] was that they found substantially higher cytosolic concentrations of GB and K^+ in cells grown in medium with 2.7 M NaCl than in cells grown in media of lower osmolalities, but we found that cytosolic GB was only slightly higher in medium with 3 M NaCl than in medium with 2 M NaCl.

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