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Halotolerance in *Methanosarcina* spp.: Role of N^{ε}-Acetyl-β-Lysine, α-Glutamate, Glycine Betaine, and K^+ as Compatible Solutes for Osmotic Adaptation

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The methanogenic *Archaea*, like the *Bacteria* and *Eucarya*, possess several osmoregulatory strategies that enable them to adapt to osmotic changes in their environment. The physiological responses of *Methanosarcina* species to different osmotic pressures were studied in extracellular osmolalities ranging from 0.3 to 2.0 osmol/kg. Regardless of the isolation source, the maximum rate of growth for species from freshwater, sewage, and marine sources occurred in extracellular osmolalities between 0.62 and 1.0 osmol/kg and decreased to minimal detectable growth as the solute concentration approached 2.0 osmol/kg. The steady-state water-accessible volume of *Methanosarcina thermophila* showed a disproportionate decrease of 30% between 0.3 and 0.6 osmol/kg and then a linear decrease of 22% as the solute concentration in the media increased from 0.6 to 2.0 osmol/kg. The total intracellular K^+ ion concentration in *M. thermophila* increased from 0.12 to 0.5 mol/kg as the medium osmolality was raised from 0.3 to 1.0 osmol/kg and then remained above 0.4 mol/kg as extracellular osmolality was increased to 2.0 osmol/kg. Concurrent with K^+ accumulation, *M. thermophila* synthesized and accumulated α-glutamate as the predominant intracellular osmoprotectant in media containing up to 1.0 osmol of solute per kg. At medium osmolalities greater than 1.0 osmol/kg, the α-glutamate concentration leveled off and the zwitterionic β-amino acid N^{ε}-acetyl-β-lysine was synthesized, accumulating to an intracellular concentration exceeding 1.1 osmol/kg at an osmolality of 2.0 osmol/kg. When glycine betaine was added to culture medium, it caused partial repression of de novo α-glutamate and N^{ε}-acetyl-β-lysine synthesis and was accumulated by the cell as the predominant compatible solute. The distribution and concentration of compatible solutes in eight strains representing five *Methanosarcina* spp. were similar to those found in *M. thermophila* grown in extracellular osmolalities of 0.3 and 2.0 osmol/kg. Results of this study demonstrate that the mechanism of halotolerance in *Methanosarcina* spp. involves the regulation of K^+, α-glutamate, N^{ε}-acetyl-β-lysine, and glycine betaine accumulation in response to the osmotic effects of extracellular solute.

In response to changes in extracellular solute concentration, osmoregulation in species of the *Bacteria* and *Eucarya* is mediated by the accumulation of low-molecular-weight compounds, termed compatible solutes (44). Compatible solutes function by maintaining the intracellular osmotic potential at a value that is greater than that in the extracellular milieu, thus maintaining a constant turgor pressure (8, 16, 44). The fundamental characteristic of compatible solutes is their ability to be accumulated and to reduce intracellular water activity without perturbing protein function. Extremely halophilic, nonmethanogenic *Archaea* regulate intracellular K^+ concentration in response to changes in extracellular solute concentration, while the halotolerant and halophilic methanogenic *Archaea*, in addition to accumulating K^+, synthesize and regulate intracellular concentrations of organic solutes that include α-glutamate,*N*,*N*-dimethylglycine and glycine betaine (18, 27, 30–34, 39). The methanogenic *Archaea* also synthesize and accumulate a unique class of compatible solutes, β-amino acids, that include N^{ε}-acetyl-β-lysine, β-glutamate, and β-glutamine.

Species of *Methanosarcina* exhibit one of the widest ranges of halotolerance among the methanogenic *Archaea*, occurring in environments as osmotically diverse as freshwater lakes and rivers, sewage digestors, and marine sediments (see Table 1). *Methanosarcina barkeri*, *Methanosarcina thermophila*, *Methanosarcina mazei*, and *Methanosarcina vacuolata* strains used in this study were from nonmarine sources and were isolated with media that contained a relatively low concentration of extracellular solutes (≤0.3 osmol/kg) (21, 46). *Methanosarcina acetivorans* was from marine sediment and was isolated with media that contained marine solutes (typically <1.0 osmol/kg) (35). It has been reported that all *Methanosarcina* spp. can be adapted to grow in saline concentrations as high as 1.0 M (2.0 osmol/kg) (21, 22, 36, 37). Concomitant with osmotic adaptation to highly saline medium, *M. thermophila* has been shown to have high intracellular concentrations of α-glutamate and N^{ε}-acetyl-β-lysine (39). However, there has not been a comprehensive report on the coaccumulation of all compatible solutes, and the effects of exogenous compatible solutes on their de novo synthesis, over the entire range of extracellular solute concentrations that support growth of *Methanosarcina* spp. In this study, we characterized the distribution and quantified the accumulation of compatible solutes in *M. thermophila* and four additional species of *Methanosarcina* in response to extracellular osmolalities ranging from 0.3 to 2.0 osmol/kg. Results of this study demonstrate the physiological strategy used by the halotolerant *Methanosarcina* spp. for uptake and

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TABLE 1. Sources of *Methanosarcina* strains used in this study

Species	Strain	DSM no.[a]	OCM no.[b]	Source	Reference
M. barkeri	MS^T[c]	800	38	Sewage digestor	3
	227[d]	1538	35	Sewage digestor	24
	Fusaro[d]	804	83	Freshwater sediment	14
	UBS[e]	1311	27	Freshwater sediment	42
M. mazei	S-6^T[d]	2053	90	Sewage digestor	23
	LYC[e]		34	Alkaline drilling sediment	19
M. thermophila	TM-1^T[c]	1825	12	Thermophilic sewage digestor	46
M. acetivorans	C2A^T[c]	2834	95	Marine sediment	35
	C2E[c]			Marine sediment	35
M. vacuolata	Z-761^T[d]		85	Sewage digestor	45

[a] DSM, German Collection of Microorganisms.
[b] OCM, Oregon Collection of Methanogens.
[c] Strain source described previously (38).
[d] Provided by R. A. Mah.
[e] Provided by G. Gottschalk.

synthesis of compatible solutes in response to changes in osmotic pressures.

MATERIALS AND METHODS

Bacterial strains. Strain sources are described in Table 1.

Media and growth conditions for single cells. Media were prepared anaerobically in an N_2-CO_2 (4:1) atmosphere by a modification of the Hungate technique (1). Gases were passed through a column of reduced copper turnings at 350°C to remove traces of oxygen. Cultures were grown in disaggregating medium that contained 0.05 M methanol as a growth substrate (36). The osmolality of the disaggregating medium was varied from 0.3 to 2.3 osmol/kg by the addition of NaCl and measured with a vapor pressure osmometer (Wescor, Logan, Utah) prior to methanol addition. The pH of the medium was adjusted to 6.6 ± 0.1 after addition of NaCl. Media were dispensed into culture tubes (16 by 160 mm) or serum bottles (54 by 107 mm), and the containers were sealed with butyl rubber septa secured by aluminum crimp collars (Bellco Glass, Inc., Vineland, N.J.). All glassware was acid cleaned prior to use.

Throughout this study, *Methanosarcina* spp. were grown and maintained as single cells, rather than as multicellular aggregates, as described previously (36). Briefly, cultures of aggregated cells were adapted to higher extracellular osmolalities by sequentially transferring late-exponential-phase cultures (10%, vol/vol) into disaggregating medium containing progressively higher NaCl concentrations. All strains of *Methanosarcina* underwent a transition from aggregates to single cells after adaptation and growth in medium containing osmolalities between 1.0 and 2.0 osmol/kg. After undergoing transition, single cells were readapted to osmolalities as low as 0.3 osmol/kg by sequentially transferring cultures into disaggregating medium containing progressively lower NaCl concentrations. Microscopic examination indicated that single-cell morphology was retained at the osmolalities indicated, which allowed the use of optical density (550 nm; path length = 18 mm) for growth assays and direct cell counts for calculating cell volume.

Water-accessible cytoplasmic volume. Cultures were grown at 37°C, and cells were harvested during mid-exponential growth by centrifugation at 6,000 × *g* for 15 min. Water-accessible cytoplasmic volumes were determined by measuring the differential retention of [^{14}C]glucose, which is able to permeate archaeal S-layers but not cell membranes, and ^3H_2O, which is able to permeate the cells (17). Cytoplasmic water volumes were calculated from the cell number and differential radioactive counts retained by the two supernatants (7). Cell numbers were determined by using a Petroff-Hausser chamber (C.A. Hausser and Son, Philadelphia, Pa.) by standard methods (15). Dilutions were made in media that had an osmolal concentration equivalent to that of the growth medium of the culture being counted. Since water-accessible volume does not exclude osmotically inactive bound water, which has been shown to be constant in *Escherichia coli*, the concentration of cytoplasmic solutes would be progressively underestimated with increasing hypertonicity of the medium if only water-accessible volume is used (4). In this study the osmotically effective concentration of cytoplasmic solutes was calculated by subtracting the estimated concentration of bound water (0.4 μl/mg [dry wt]) for *E. coli* from the water-accessible volume in *M. thermophila* (4). Bound-water concentrations are assumed to be similar in *M. thermophila* and *E. coli* since both the *Archaea* and the *Bacteria* have procaryotic cell structure. In addition, a report on the methanogen *Methanospirillum hungatei* indicates that the cellular composition of this methanogen is similar to the composition of bacterial species (2).

Analysis of the intracellular elemental ion concentration. Cultures (1,000 ml) were grown in medium containing the indicated osmolality and always harvested at the same phase of growth (i.e., mid-exponential) by centrifugation for 15 min at 7,000 × *g* at 4°C. Pellets containing approximately 2×10^{10} cells were washed once with 10 ml of anaerobic sucrose solution poised at an osmolal concentration equivalent to that of the growth medium. Cells were lysed by suspending the pellet in 1 ml of perchloric acid (10%, vol/vol) and stored overnight at 4°C. Cell debris was removed by centrifugation, and then the pellets were washed once with deionized water. Pooled supernatants were filtered through a 0.2-μm-pore-size PTFE membrane filter (Gelman Sciences), and nitric acid was added to the filtrate at a final concentration of 1% (vol/vol). Multielement analysis was done simultaneously for each sample with a multichannel inductively coupled 1.5-m optical emission spectrometry system equipped with an ultrasonic nebulizer (Applied Research Laboratories, Valencia, Calif.) at the UCLA Laboratory of Biomedical Sciences. Spectral parameters were as follows: 1,500 W, 21.1-MHz plasma field, 35-s flushing time, 10-s integration time, three integrations. To account for the interaction of K^+ with macromolecules as bound counterions, the osmotically effective unbound fraction of K^+ was determined by subtracting the fraction of bound K^+, which is estimated in *E. coli* to be 43 to 52% in 0.17 to 0.9 osM medium, respectively (25, 29). Intracellular K^+ is reported as the unbound ion fraction.

Cell extraction. Ethanol extracts were prepared from cells as previously described (30, 39). Cell pellets that contained approximately 10^{10} cells were extracted twice by heating for 5 min at 65°C in 0.5 ml of 70% (vol/vol) ethanol-water. The ethanol extracts were combined, centrifuged at 5,000 × *g* for 5 min, and dried in a rotary evaporator. Dried extracts were dissolved in 0.5 ml of deionized water, eluted from a Sep-Pak C_{18} cartridge (Millipore Corp.) with 0.1% trifluoroacetic acid in water-methanol (7:3) as described by the manufacturer, and filtered through a 0.2-μm-pore-size PTFE membrane filter (Gelman Sciences).

Quantification of compatible solutes. Primary amines were separated with a high-pressure liquid chromatography (HPLC) dual pump system (Waters Associates) that was equipped with a gradient programmer (model 720). Extract (10 μl) was eluted from a cation-exchange column (25 by 0.46 cm; 9 (±0.5)-μm particle size; Waters Associates) with a linear pH gradient ranging from 3.17 to 9.94. Eluent was monitored with a fluorescence spectrophotometer (excitation wavelength = 340 nm, absorption wavelength = 455 nm) after *o*-phthalaldehyde postderivatization. Proline was detected and quantified by absorbance after treatment with acidic ninhydrin (41). Glycine betaine was detected and quantified by absorbance after periodide derivatization (18).

Chemicals. Ion-exchange elution buffers, *o*-phthalaldehyde, and amino acid standards were HPLC grade and obtained from Pierce Chemicals (Rockford, Ill.). [U-^{14}C]D-glucose and ^3H_2O were obtained from ICN Radiochemicals (Irvine, Calif.). Casamino Acids and yeast extract were obtained from Difco Laboratories, Detroit, Mich. All other chemicals were of reagent grade.

RESULTS

Effect of extracellular osmolality on cell growth and water-accessible volume. The species of *Methanosarcina* used in this study were previously isolated from environments that represent a wide range of solute concentrations (Table 1). To document how these microorganisms respond to different osmotic pressures, we examined growth rates in media poised at osmolalities ranging from 0.3 to 2.3 osmol/kg (disaggregating medium with 0 to 1.2 M NaCl, respectively). After three serial transfers at each of the indicated osmolalities, all *Methanosarcina* spp. exhibited reproducible growth rates. Although maximum growth rates occurred in osmolalities between 0.6 and 1.0 osmol/kg, they ranged from an average of 0.15 h^{-1} for *M. barkeri* UBS to 0.10 h^{-1} for *M. barkeri* Fusaro (Fig. 1). Strains showed varying degrees of tolerance to the lowest extracellular osmolality tested (0.3 osmol/kg), ranging from specific growth rates of 0.045 ± 0.0012 h^{-1} for *M. barkeri* MS, 227, and W to 0.085 ± 0.0078 h^{-1} for *M. barkeri* UBS, *M. mazei* S-6, and *M. thermophila* TM-1. Significantly reduced growth of all strains occurred at osmolalities greater than 2.0 osmol/kg.

As determined by phase-contrast microscopy, all species examined grew as spherical cocci (diameter, 1.5 ± 0.2 μm) in medium poised at 0.3 osmol/kg; they grew as smaller irregularly shaped cocci (diameter, 1.2 ± 0.4 μm) in osmolalities of 0.6 to 2.0 osmol/kg. Similar changes in morphology in response to medium osmolality have been reported for moderately halo-

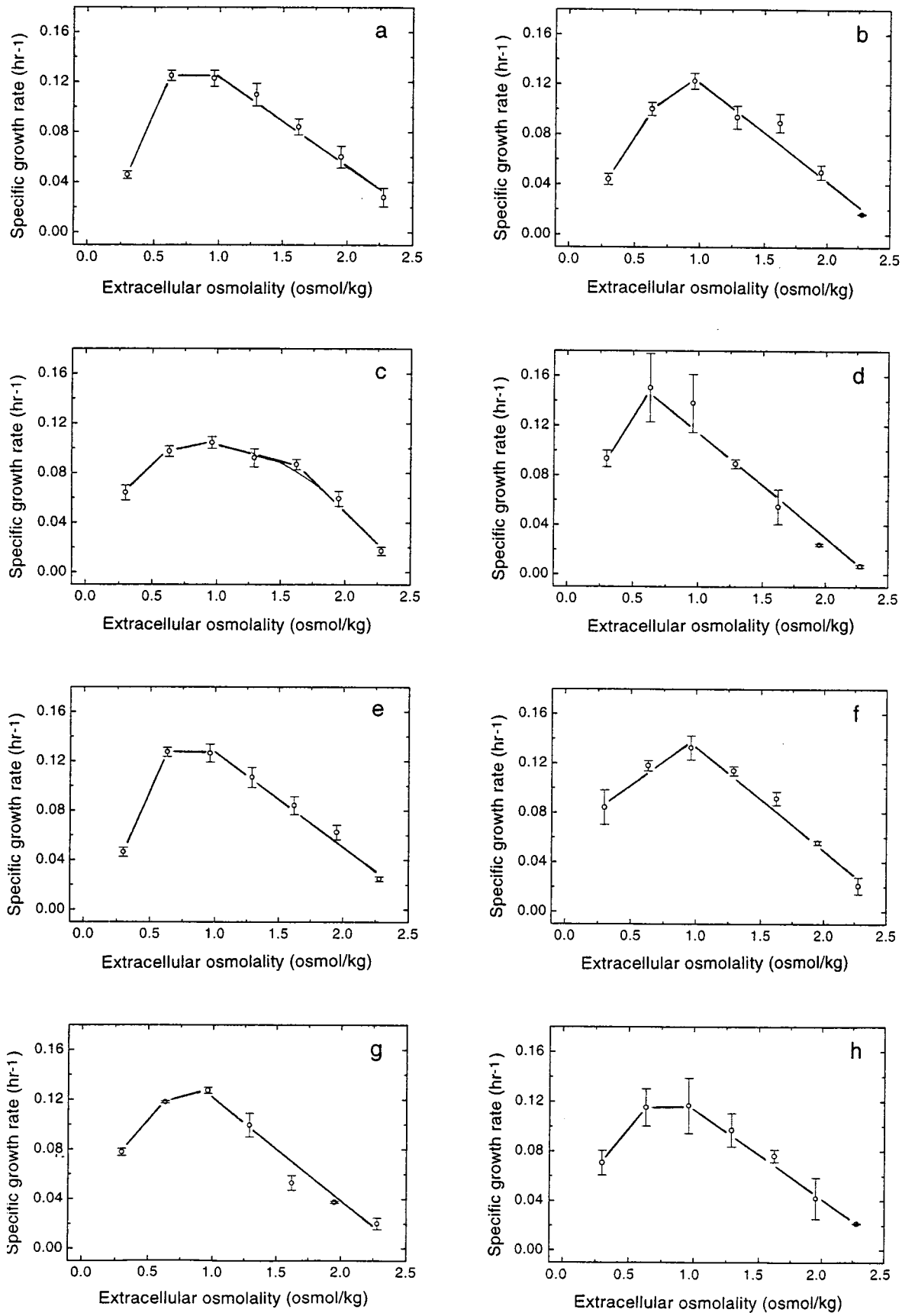

FIG. 1. Effect of extracellular solute concentration on the growth rate of *M. barkeri* MS (a), 227 (b), Fusaro (c), UBS (d), and W (e), *M. mazei* S-6 (f), *M. thermophila* TM-1 (g), and *M. acetivorans* C2A (h). Cells were cultured in media containing the indicated solute concentrations, and the growth rates were determined by measuring A_{550}. Values are means and standard deviations for three to six replicate cultures.

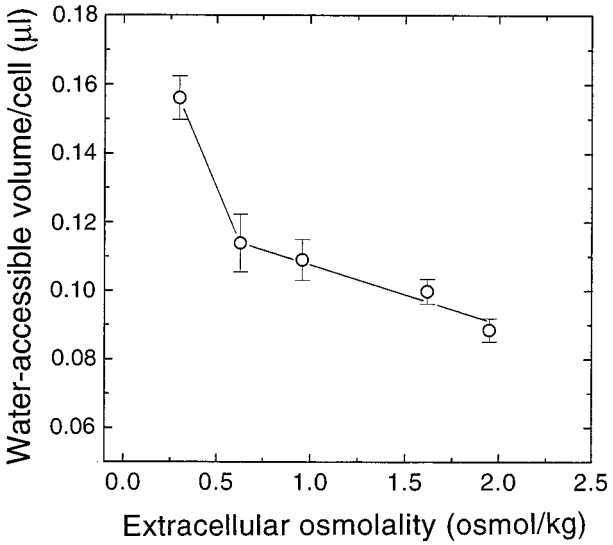

FIG. 2. Effect of extracellular solute concentration on the cell volume of *M. thermophila*. Cells were cultured in media containing the indicated solute concentrations, and the cell volumes were determined as described in Materials and Methods. Values are means and standard deviations for three replicate cultures.

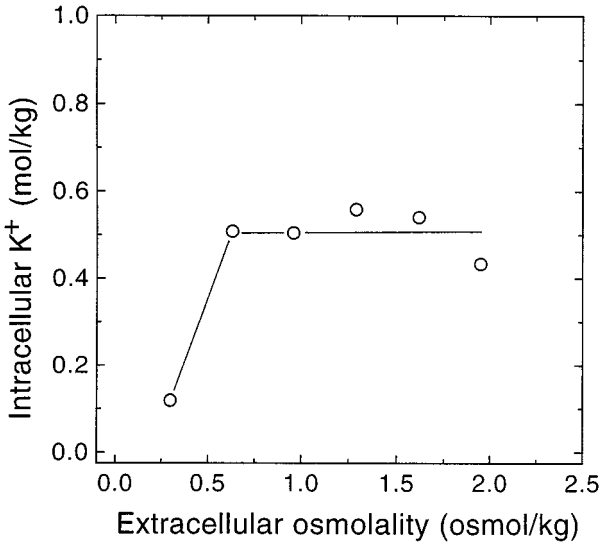

FIG. 3. Effect of extracellular solute concentrations on intracellular levels of potassium ion in *M. thermophila*. Cells were cultured in media containing the indicated solute concentrations. After three serial transfers, the growth rates were determined by measuring A_{550}. Values are means for three replicate integrations from a single culture.

philic *Methanohalophilus* spp. (27). Water-accessible volumes of *M. thermophila* determined at different osmolalities showed an inverse relationship with extracellular solute concentration (Fig. 2). Cells grown in medium poised at 0.6 osmol/kg had 30% less volume than cells grown in medium at 0.3 osmol/kg, and the volume decreased an additional 22% between osmolalities of 0.6 and 2.0 osmol/kg.

Effect of osmolality on intracellular potassium concentration. The total intracellular concentration of potassium was measured in *M. thermophila* following adaptation and growth in different extracellular osmolalities. In medium poised at 0.3 osmol/kg, K^+ was the predominant intracellular cation, at a concentration of 0.12 ± 0.02 mol/kg (Fig. 3). When the osmotic strength of the medium was increased to 0.6 osmol/kg, the intracellular K^+ concentration increased approximately fourfold (0.51 ± 0.03 M) and then remained above 0.4 mol/kg at extracellular osmolalities greater than 1.0 osmol/kg. Total intracellular concentrations of other bound and unbound cations remained relatively constant in media ranging from 0.3 to 2.0 osmol/kg and included the following, in millimoles per kilogram (mean ± standard deviation for six cultures): Mg, 163 ± 62; Na, 17 ± 11; B, 12 ± 5; Zn, 11 ± 5; Ca, 3.8 ± 1.5; Fe, 3.5 ± 1.9; Ni, 1.1 ± 0.5; Co, 1.0 ± 0.6; and Mn, Mo, Al, Cu, Si, Cr, As, Ag, and Pb, <1.

Effect of extracellular osmolality on the intracellular pool of organic compatible solutes. The intracellular concentrations of α-amino acids, β-amino acids, and the imino acid proline were compared in *M. thermophila* grown in extracellular osmolalities of 0.3 and 2.0 osmol/kg. α-Glutamate (0.09 mol/kg) was the predominant amino acid in the soluble fraction of cells grown in 0.3 osmol/kg, whereas α-glutamate (0.62 mol/kg) and N^{ε}-acetyl-β-lysine (1.01 mol/kg), the only β-amino acid detected, were the predominant intracellular solutes, at 2.0 osmol/kg. Other amino acids detected at concentrations higher than 0.01 mol/kg include the following, in millimoles per kilogram (mean ± standard deviation for six cultures grown in extracellular osmolalities of 0.3 and 2.0 osmol/kg, respectively): Ala, 11 ± 5 and 33 ± 13; Asp, 6.3 ± 2 and 22 ± 3; Gly, 7.8 ± 4 and 17 ± 6; and Val, 11 ± 5 and 18 ± 6. The intracellular concentrations of all other primary amines and proline were less than 0.01 mol/kg. Total intracellular solute concentrations (in moles per kilogram) were greater than the extracellular solute concentrations (in osmoles per kilogram) in media ranging from 0.6 to 2.0 mol/kg, but the values were similar at 0.3 mol/kg (Table 2). Glycine betaine, which is a compatible solute in several species of the *Bacteria* and *Archaea* (18, 32, 33, 44), was not detected in *M. thermophila* grown in disaggregating medium poised at any of the osmolalities tested. No other low-molecular-weight organic compounds are detected at significant concentrations by ^{13}C nuclear magnetic resonance spectroscopy (39).

Effect of exogenous osmolytes on the intracellular concentration of compatible solutes. The effects of yeast extract, Casamino Acids, and glycine betaine on de novo synthesis of compatible solutes by *M. thermophila* are shown in Table 3. When grown in disaggregating medium that contained 2.0 osmol of solute per kg, α-glutamate and N^{ε}-acetyl-β-lysine were the predominant intracellular solutes. Upon addition of yeast extract (0.1%, wt/vol) to the culture medium, glycine betaine accumulated in the cells and there was a concomitant reduc-

TABLE 2. Effect of extracellular solute concentration on total intracellular solute concentration in *M. thermophila*

Extracellular NaCl concn (M)[a]	Extracellular solute concn (osmol/kg)[b]	Total intracellular solute concn (mol/kg)[c]
0.0	0.30	0.27
0.2	0.63	0.94
0.4	0.96	1.26
0.6	1.29	1.70
0.8	1.62	1.84
1.0	1.95	2.30

[a] NaCl added to basal medium.
[b] Total osmolality of medium based on vapor pressure relative to NaCl standard.
[c] Solutes measured include K^+, α-amino acids, N^{ε}-acetyl-β-lysine, and glycine betaine.

TABLE 3. Effect of extracellular growth factors on the intracellular levels of compatible solutes in *M. thermophila*

Addition (concn [wt/vol])[a]	Intracellular solute concentration (mol/kg)[b]			
	α-Glutamate	N^{ε}-Acetyl-β-lysine	α-Amino acids[c]	Glycine betaine
None	0.60 ± 0.04	1.11 ± 0.05	0.069 ± 0.004	<0.007[d]
Casamino Acids (0.1%)	0.59 ± 0.06	1.06 ± 0.07	0.063 ± 0.010	<0.007[d]
Yeast extract (0.1%)	0.39 ± 0.03	0.31 ± 0.03	0.057 ± 0.004	0.30 ± 0.04
Glycine betaine (0.006%)	0.37 ± 0.02	0.33 ± 0.03	0.034 ± 0.005	0.29 ± 0.03

[a] Added to disaggregating medium (total solutes = 1.95 osmol/kg).
[b] Total of all α-amino acids except glutamate.
[c] Values are means ± standard deviations.
[d] Not detected (limit of detection, <0.007 mol/kg).

tion in the levels of α-glutamate and N^{ε}-acetyl-β-lysine. When glycine betaine (0.05%, wt/vol) was added to the medium, the intracellular concentrations of glycine betaine, α-glutamate, and N^{ε}-acetyl-β-lysine were similar to those observed in cells grown with yeast extract. The similarity of these responses is likely due to the accumulation of the quaternary amine from the yeast extract preparations which has been reported to occur in other methanogenic *Archaea* (18, 27, 31–33, 39). The intracellular concentrations of other amino acids were not significantly affected by the presence of glycine betaine (data not shown). The addition of choline to disaggregating medium, which is oxidized to glycine betaine by some species of bacteria, did not affect the intracellular levels of α-glutamate or N^{ε}-acetyl-β-lysine, nor did glycine betaine accumulate.

The effect of glycine betaine on the intracellular accumulation of other compatible solutes was determined in medium osmolalities ranging from 0.3 to 2.0 osmol/kg (Fig. 4). The α-glutamate concentration increased as the extracellular osmolality was raised from 0.3 to 1.0 osmol/kg and then increased only slightly at extracellular osmolalities between 1.0 and 2.0 osmol/kg (Fig. 4a). N^{ε}-Acetyl-β-lysine was first detected at between 0.6 and 1.0 osmol/kg, and its concentration continued to increase as the extracellular osmolality approached 2.0 osmol/kg. When *M. thermophila* was grown in medium that contained 0.5 mM glycine betaine, this quaternary amine began to accumulate in cells grown in extracellular osmolalities of between 0.6 and 1.0 osmol/kg, and its concentration continued to increase as the osmolality approached 2.0 osmol/kg (Fig. 4b). Although significant accumulation of α-glutamate and N^{ε}-acetyl-β-lysine was observed at extracellular osmolalities ranging from 0.03 to 1.0 osmol/kg and 1.0 to 2.0 osmol/kg, respectively, in the presence of glycine betaine, the intracellular concentrations of both of these primary amines were lower than in cells grown without the addition of glycine betaine.

When each of the strains shown in Table 1 was grown in disaggregating medium containing osmolalities of 0.3 and 1.0 osmol/kg, either with or without the addition of glycine betaine, the distribution and concentrations of amino acids and glycine betaine were similar to those observed for *M. thermophila* (data not shown).

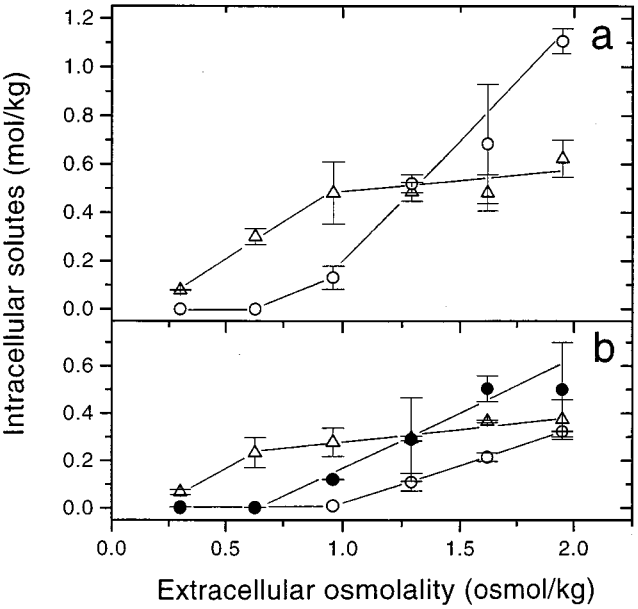

FIG. 4. Effect of extracellular solute concentrations on the synthesis of α-glutamate and N^{ε}-acetyl-β-lysine and the uptake of glycine betaine. The intracellular accumulation of α-glutamate (△), N^{ε}-acetyl-β-lysine (○), and glycine betaine (●) without (a) and with (b) the addition of 0.5 mol of betaine per kg to the medium was measured. Values are means and standard deviations for three replicate cultures.

DISCUSSION

The methanogenic *Archaea* grow in osmotically diverse environments ranging from the relatively low solute concentrations in freshwater sediments to saturation level of solutes found in brine ponds (10, 12). Although the reported "optimum" solute concentration for growth of microorganisms is often similar to that of their source of isolation, this study and others have shown that individual species of methanogens can adapt and proliferate in a range of extracellular osmolalities (18, 21, 22, 27, 31, 33, 36, 37, 39). Some earlier reports indicated that individual species of *Methanosarcina* tolerate a more limited range of NaCl concentrations for maximum growth (10, 12, 19, 35). In this study, five *Methanosarcina* spp., including strains from both freshwater and marine sources, were preadapted and grown in extracellular osmolalities ranging from 0.3 to 2.3 osmol/kg and shown to grow exponentially over the entire range of solute concentrations tested (Fig. 1). We observed that there were a lag in growth and lower growth rates following the initial inoculation into media with an osmolality different from that of the inoculum. However, after the second serial transfer at the same respective osmolality, there was no lag in growth and the growth rates reached their maximum values. The limited ranges previously reported likely resulted from hyperosmotic shock to cells that were not preadapted to each new NaCl concentration before growth was measured. Interestingly, the growth responses to extracellular solute of *M. acetivorans* C2A and C2E, the only marine isolates, were nearly identical to those observed for *M. mazei* S-6 and *M. thermophila* TM-1. This observation is consistent with an earlier report showing that *M. acetivorans* C2A and several *M. mazeii* strains have similar ranges of halotolerance (22). Likewise, Maestrojuán and Boone (22) showed that preadapted cultures of *M. barkeri* MS and 227, *M. mazei* S-6, and *M. vacuolata* Z had maximum growth rates over a range of medium solute concentrations similar to those reported here, although the overall maximum growth rates of *Methanosarcina* spp. in this report were greater than those observed previously. This difference may reflect a difference in medium compositions or a higher rate of nutrient uptake into the single cells used in this study than for methanochondroitin-bound multicellular aggre-

gates used in the prior study (1a). The similarity of their growth response to a range of extracellular solute concentrations indicates that all *Methanosarcina* spp. are halotolerant and is consistent with the close phylogenetic relationship reported to exist between strains isolated from nonmarine and marine sources (38).

In order to adapt to different extracellular osmolalities, cells must maintain turgor pressure for growth by regulating the intracellular levels of K^+ and organic osmolytes in response to the osmotic potential created by the extracellular milieu (44). Results presented here and in a previous report suggest that the *Methanosarcina* spp. regulate cell turgor pressure by synthesis or uptake of compatible solutes in response to changes in extracellular osmolalities (39). The decrease in steady-state water-accessible volume of *M. thermophila* as medium osmolality was increased from 0.6 to 2.0 osmol/kg (Fig. 2) is similar to the response reported for *E. coli* grown in medium ranging from 0.1 to 1.11 osmol/liter (4). However, unlike in *E. coli*, the decrease in water-accessible volume of *M. thermophila* (30%) between 0.3 and 0.6 osmol of extracellular solute per kg is disproportionately greater than the decrease in volume of cells grown between 0.6 and 2.0 osmol of solute per kg. The apparent distension of the cells coincides with a decrease in the growth rates of *Methanosarcina* spp. (Fig. 1) and suggests that the nonaggregated, single-cell forms of these species are hypotonically stressed at 0.3 osmol/kg. In contrast, Maestrojuán and Boone (21) showed that *Methanosarcina* spp. did not exhibit a decline from their maximum growth rates in medium with low osmolality (≈0.3 osmol/kg). However, *Methanosarcina* spp. in the previous study were grown as aggregates in medium of low osmolality (1a), and higher growth rates of those cells may have resulted from mechanical protection by the rigid methanochondroitin outer layer. This observation supports the hypothesis that nonrigid single cells are hypotonically stressed below this solute threshold. Consistent with this observation, *Methanosarcina* spp. from hypotonic environments such as freshwater lakes and rivers have generally been isolated as multicellular aggregates with a rigid methanochondroitin extracellular matrix, while species from hypertonic environments of 0.6 osmol/kg and more are usually isolated as single cells without a rigid cell wall (14, 21, 35, 36, 38). Furthermore, when single-cell *Methanosarcina* spp. are adapted to media with a low osmolality (<0.6 osmol/kg) and a magnesium concentration below 0.01 M, cells begin synthesizing a rigid methanochondroitin outer layer that would presumably enable them to maintain a higher turgor pressure created by hypoosmotic media (36, 37, 43). The role of aggregation as a potential survival mechanism for *Methanosarcina* spp. has been discussed previously (21, 38).

Methanosarcina spp. maintain turgor pressure in extracellular osmolalities greater than 0.3 osmol/kg by accumulating intracellular solutes to regulate their osmotic potential in response to the immediate environment. Potassium and α-glutamate were the predominant intracellular solutes in *M. thermophila* grown at 0.3 osmol/kg (Fig. 4). The total K^+ concentration (161 ± 10 mol/kg) in *M. thermophila* grown in 0.3 osmol/kg, when not corrected for bound water and K^+, is similar to values reported by other investigators for *Methanosarcina* spp. grown in media with low osmolality (13, 20). The simultaneous accumulation of K^+ and glutamate as the medium osmolality approached 1.0 osmol/kg would partially neutralize the net charge contributed by these ionic species as well as contribute to the osmotic potential of the cell cytoplasm (33, 39). The ratio of glutamate to K^+ estimated in this study (0.7 to 0.9 at 0.96 to 1.29 osmol/kg, respectively) and the pattern of K^+ and glutamate accumulation to a threshold level are similar to responses reported for *E. coli* and *Klebsiella aerogenes* and suggest that these charged ionic species may have an adverse affect on the stability of cell protein at higher concentrations (9, 25, 26). In addition, the pattern of intracellular glutamate accumulation is similar to that reported for marine *Methanococcus* spp. (31). As K^+ and glutamate accumulation levels off, *Methanosarcina* spp. begin to synthesize N^{ε}-acetyl-β-lysine, which continues to accumulate to intracellular concentrations as high as 1.1 ± 0.05 mol/kg as the extracellular osmolality approaches 2.0 osmol/kg. In medium with glycine betaine, de novo synthesis and accumulation of glutamate and N^{ε}-acetyl-β-lysine are reduced as glycine betaine begins to accumulate to an intracellular concentration as high as 0.5 ± 0.05 mol/kg at an extracellular osmolality of 2.0 osmol/kg. A similar reduction in glutamate accumulation in the presence of glycine betaine occurs in *E. coli* (5). These zwitterionic amines are presumed to be less inhibitory to enzyme function than charged ionic species at equivalent osmolal concentrations and can therefore accumulate to relatively high intracellular concentrations without perturbing cell function (11, 44). In support of this hypothesis, this class of compatible solutes has previously been reported to protect proteins from denaturation at low water activities (28). The partial repression of α-glutamate and N^{ε}-acetyl-β-lysine synthesis when glycine betaine is available for uptake may be an energy-conserving mechanism that enables cells to transport compatible solute in lieu of biosynthesis.

The total intracellular concentration was slightly greater than the extracellular solute concentration, with the exception of cells grown in 0.3 osmol/kg, indicating that *Methanosarcina* spp. had a positive turgor pressure (Table 3). Even at 0.3 osmol/kg, the effective intracellular osmolality would be greater than that of the medium because of the osmotic contribution of other cytoplasmic ions, small molecules, and macromolecules, as indicated by the increased water-accessible volume. In contrast to *Methanobacterium* spp., which have a rigid cell wall and accumulate intracellular solutes that make them highly hypertonic, *Methanosarcina* spp., like other osmotically sensitive methanogenic species with nonrigid S-layer cell walls, maintain a lower turgor pressure by accumulating solutes to concentrations that are closer to the osmolality of the medium (6, 13, 27, 40). Although the intracellular solute concentrations in Table 3 suggest that cells grown in media with solute concentrations higher than 0.3 osmol/kg have a high turgor pressure, the effective osmotic potential of intracellular solutes is likely less than indicated since the osmotic potential of organic solutes can be reduced at low water activities by molecular association of solute molecules with other solutes and macromolecules (4, 5, 25, 26, 44).

Synthesis of both α-glutamate and N^{ε}-acetyl-β-lysine and uptake of glycine betaine are regulated by *Methanosarcina* spp. in response to changes in osmotic pressure. Although other methanogenic *Archaea* synthesize and/or accumulate compatible solutes, each genus described has exhibited a unique combination of solutes. Nonmarine *Methanobacterium* spp. show no evidence of solute accumulation in response to increasing osmotic pressure (6, 33). However, the marine species *Methanogenium cariaci* and *Methanococcus thermolithotrophicus* accumulate K^+, α- and β-glutamate, and N^{ε}-acetyl-β-lysine, and hyperhalophilic *Methanohalophilus* spp. accumulate K^+, α-glutamate, N^{ε}-acetyl-β-lysine, β-glutamine, glycine betaine, and *N*,*N*-dimethylglycine in response to changing osmotic pressures (18, 27, 33). The pattern of compatible solute accumulation by *Methanosarcina* spp. is different from responses reported thus far for any other methanogenic archaeon and is universal for all described species of this genus. The ability of these species to sense and respond to extracellular solute con-

centrations by synthesizing or transporting compatible solutes enables *Methanosarcina* spp. to tolerate osmotic pressures encountered in a wide range of environments.

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