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Distribution of Compatible Solutes in the Halophilic Methanogenic Archaebacteria

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Accumulation of compatible solutes, by uptake or de novo synthesis, enables bacteria to reduce the difference between osmotic potentials of the cell cytoplasm and the extracellular environment. To examine this process in the halophilic and halotolerant methanogenic archaebacteria, 14 strains were tested for the accumulation of compatible solutes in response to growth in various extracellular concentrations of NaCl. In external NaCl concentrations of 0.7 to 3.4 M, the halophilic methanogens accumulated K^+ ion and low-molecular-weight organic compounds. β-Glutamate was detected in two halotolerant strains that grew below 1.5 M NaCl. Two unusual β-amino acids, N_ε-acetyl-β-lysine and β-glutamine (3-aminoglutaramic acid), as well as L-α-glutamate were compatible solutes among all of these strains. De novo synthesis of glycine betaine was also detected in several strains of moderately and extremely halophilic methanogens. The zwitterionic compounds (β-glutamine, N_ε-acetyl-β-lysine, and glycine betaine) and potassium were the predominant compatible solutes among the moderately and extremely halophilic methanogens. This is the first report of β-glutamine as a compatible solute and de novo biosynthesis of glycine betaine in the methanogenic archaebacteria.

Prokaryotic and eukaryotic microorganisms have evolved mechanisms to adapt to osmotic stress ranging from low solute concentrations in spring water to saturated solutes in salt brines (38). In environments where the extracellular solute concentration exceeds that of the cell cytoplasm, microorganisms accumulate low-molecular-weight organic compounds, known as compatible solutes, that enable them to minimize water loss and maintain cell turgor pressure (42). These compounds function by reducing the difference between the osmotic potentials of the cell cytoplasm and the extracellular environment, thus maintaining a constant turgor pressure, and by protecting enzymes from the low water activity caused by solute accumulation (4, 9, 16, 37). Compatible solutes in eukaryotic organisms include proline and betaine in plants (15, 35), glycerol in lower fungi (22), and polyols, proline, and amino acids in marine algae (39). Eubacteria also contain a broad spectrum of osmotically active solutes, including potassium, proline, glutamic acid, glutamine, γ-aminobutyric acid, alanine, and glycine betaine (9, 40).

Several types of compatible solute have been identified in archaebacteria. The predominant compatible solute in extremely halophilic archaebacteria such as *Halobacterium* and *Halobium* species is potassium (11); organic compatible solutes do not accumulate to a large extent in these organisms. Methanogenic archaebacteria, however, accumulate β-amino acids as compatible solutes in response to external NaCl concentrations. β-Glutamate accumulates in thermophilic strains of methanococci as well as the mesophile *Methanogenium cariaci* (26, 28), and the β-amino acid derivative N_ε-acetyl-β-lysine accumulates in *Methanosarcina thermophila* and *M. cariaci* (30). Glycine betaine also accumulates as a compatible solute in several marine methanogens (27). In *M. cariaci*, the betaine was not synthesized de novo but was transported into the cells from the medium (27).

In previous studies, methanogens were cultured between 0.05 and 1.5 M NaCl, which is an optimal NaCl range for growth of slightly halotolerant organisms (28, 30). The present work is a survey of compatible solutes in halotolerant (<1.5 M NaCl), moderately halophilic (1.5 to 3 M), and extremely halophilic (>3 M) methanogenic archaebacteria grown in various salt concentrations. The occurrence and intracellular concentrations of several unusual β-amino acids, including β-glutamine (3-aminoglutaramic acid) and N_ε-acetyl-β-lysine, and de novo biosynthesis of glycine betaine were documented in response to changes in the extracellular concentration of NaCl.

MATERIALS AND METHODS

Bacterial strains. Strains used in this study (Table 1) were provided by I. M. Mathrani, D. R. Boone, and R. A. Mah. *Methanohalophilus* strains Z7301, Z7302 (=OCM130), Z7401 (=OCM131), and Z7404 (=OCM 132) and *Methanococcus halophilus* Z7982 (=OCM160, =DSM3094) were described by Zhilina (43, 44). Strains FDF1 (=OCM59), FDF2 (=OCM66), SD1 (=OCM134), SF2 (=OCM133), Ret-1 (=OCM57), and Cas-1 (=OCM135) were described by Mathrani et al. (19, 20). Strain GS-16 (=OCM58) was described by Kiene et al. (12), strain SF-1 (=OCM13, =DSM3243) (18) was isolated by R. A. Mah, and *Methanohalophilus mahii* SLP (=OCM68, =DSM5219) was described by Paterek and Smith (23).

Media. Sterile media were prepared under an N_2-CO_2 atmosphere (4:1) by a modification of the Hungate technique (1). The basal medium consisted of the following additions in deionized water (grams per liter): $MgCl_2 \cdot 6H_2O$, 3; KCl, 2; $CaCl_2 \cdot 2H_2O$, 0.1; K_2HPO_4, 0.4; NH_4Cl, 1; cysteine-HCl $\cdot H_2O$, 0.5; $NaHCO_3$, 4; and resazurin, 0.001. Vitamin (41) and trace element (5) solutions were each added to a

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TABLE 1. Occurrence of compatible solutes in halophilic and halotolerant methanogens

Strain	NaCl growth range (M)[a]	Medium salinity[b]	Presence of compatible solute[c]				
			α-Glu	β-Glu	NABL	β-Gln	Gly-B
Halotolerant							
Methanohalophilus strain Cas-1	0.5–1.5	0.7	+	+	+	+	−
Methanococcus halophilus Z7982	0.5–1.5	0.7	+	+	+	+	−
Methanohalophilus strain GS-16	0.2–1.2	0.7	+	−	+	+	−
Moderately halophilic							
Methanohalophilus strain FDF1	1.2–2.9	2.0	+	−	+	+	+
Methanohalophilus strain FDF2	1.2–2.9	2.0	+	−	+	+	−
Methanohalophilus strain SF1	1.7–2.6	2.0	+	−	+	+	+
Methanohalophilus strain SF2	1.7–2.6	2.0	+	−	+	+	+
Methanohalophilus strain SD1	1.0–3.4	2.0	+	−	+	+	+
Methanohalophilus strain Ret-1	1.4–2.9	2.0	+	−	+	+	−
Methanohalophilus strain Z7301	1.4–3.0	2.0	+	−	+	+	+
Methanohalophilus strain Z7401	0.7–3.1	2.0	+	−	+	+	+
Methanohalophilus mahii SLP	1.0–2.6	2.0	+	−	+	+	−
Extremely halophilic							
Methanohalophilus strain Z7302	1.7–4.3	3.4	+	−	+	+	+
Methanohalophilus strain Z7404	1.7–4.3	2.0	+	−	+	+	+

[a] Data from references 19, 20, 43, and 44.
[b] Molar NaCl concentration used for screening.
[c] α-Glu, L-α-glutamic acid; β-Glu, β-glutamate; NABL, N_ϵ-acetyl-β-lysine; β-Gln, β-glutamine; Gly-B, glycine betaine.

final concentration of 1% (vol/vol). The medium pH was adjusted to 7.2 before sterilization. The substrates trimethylamine (TMA), methanol, and $Na_2S \cdot 9H_2O$ were sterilized individually by autoclaving and added to the medium at final concentrations of 20, 50 mM, and 1 mM, respectively, immediately prior to inoculation. NaCl, glycine, choline, and glycine betaine were added to the basal medium at the indicated concentrations. All glassware was acid cleaned prior to use.

Cell growth. Cells were grown at 37°C and harvested during mid-exponential growth by centrifugation at 6,000 × *g* for 15 min. Cell growth rates were monitored by measuring the rate of methane production with a gas chromatograph equipped with a thermal conductivity detector. Prior to high-pressure liquid chromatography (HPLC) analysis for compatible solutes, six sequential culture transfers were made with each strain in a defined basal medium.

Cell volume determination. Cell volumes were determined by measuring the differential retention of [^{14}C]glucose and ^3H_2O in cell pellets as described previously (3, 30). Cell counts were determined with a Petroff-Hausser counting chamber under a phase-contrast microscope.

Determination of intracellular potassium ion concentration. Cultures (100 ml) were harvested under anaerobic conditions and centrifuged for 15 min at 6,000 × *g* at 4°C. Pellets (approximately 10^{11} cells) were washed once with 10 ml of an anaerobic LiCl solution equiosmolar to the growth medium and then extracted with *n*-butanol at 95°C (33, 34). The supernatant was separated from cell debris by centrifugation. The cell debris pellets were washed once with deionized water, and the supernatants were pooled. Nitric acid (15.4 N) was added to the pooled supernatant to a final concentration of 1% (vol/vol). Potassium analysis was performed with a multichannel inductively coupled argon emission spectrometry system equipped with an ultrasonic nebulizer. The intracellular potassium ion concentration (millimolar) was calculated by the following equation: [(micromoles of ion in pellet)/(molecular weight of ion)]/[total pellet volume (cell volume × total cell number)].

Extraction of compatible solutes. Cell pellets that contained approximately 10^{11} cells (prepared as described above) were extracted twice by heating for 5 min at 65°C in 1 ml of 70% (vol/vol) ethanol-water. Significant amounts of compatible solutes were not detected in the supernatant, indicating that cell leakage was minimized during the cell harvesting process. Pooled extracts were centrifuged at 5,000 × *g* for 5 min, filtered through 0.2-μm-pore-size PTFE membrane filters (Gelman Sciences), and lyophilized (30).

Ion-exchange HPLC analysis of primary amines. Dried ethanol cell extracts were dissolved in deionized H_2O, applied to a Sep-Pak C18 column (Waters Associates), and eluted with 0.1% trifluoroacetic acid in water-methanol (7:3). A dual-pump HPLC system (Waters Associates) equipped with a gradient programmer (model 720) was used to deliver a linear pH gradient ranging from pH 3.17 to 9.94. The eluant was monitored after *o*-phthalaldehyde postderivatization with a fluorescence spectrophotometer (excitation at 340 nm and emission at 455 nm) (30).

NMR spectroscopy. Dried ethanol extracts were suspended in 0.5 ml of nuclear magnetic resonance (NMR) buffer (10 mM potassium phosphate [pH 7.2], 0.1 mM EDTA, 50% D_2O) for NMR analysis. ^{13}C NMR spectra were obtained at 75.4 MHz on a Varian XL-300 spectrometer with acquisition parameters as described previously (26). Under these conditions, all carbons with directly bonded protons have comparable nuclear Overhauser effects (nOe's) and are not saturated. ^{13}C chemical shifts were standardized with respect to external dioxane at 67.4 ppm. ^{1}H NMR spectra (300 MHz) of the extracts (with presaturation of the bulk water resonance) were determined by adjusting the sample pH to 5.0 and changing the solvent to 15% D_2O so that amide protons could be detected. ^{15}N NMR spectra (30.4 MHz) were obtained from extracts in NMR buffer that contained 85% H_2O and 15% D_2O. Acquisition parameters were 12,005-Hz sweep width, 6,000 points, 90° pulse angle, 15-s recycle delay, WALTZ decoupling of the protons, and 500 transients.

Glycine betaine assay. Glycine betaine concentrations in the ethanol extracts (5 to 10 μl) were detected and quantified by absorbance of their periodide derivatives (6, 31). Briefly,

samples were diluted 1:1 with 2 N H_2SO_4, to which cold KI-I_2 (40 μl) was added per 50 μl of acidified sample in a cold tube, and the reactants were vortexed. The samples were stored for 16 h at 4°C and then centrifuged (13,000 rpm for 15 min at 4°C). The supernatant was removed, and the periodide crystals were dissolved in 1.0 ml of 1,2-dichloroethane (reagent grade) for 2 h at 20°C. The A_{365} of the supernatant was measured with a UVIKON model 810 spectrometer. Glycine betaine standards and unknowns were analyzed in triplicate. Although this method is nonspecific for glycine betaine and other quaternary amines, ^{13}C NMR spectroscopy confirmed that these samples contained no quaternary ammonium compounds other than glycine betaine. Concentrations of glycine betaine obtained by this method were consistent with those determined by NMR.

Chemicals. Ion-exchange elution buffers, *o*-phthalaldehyde, and amino acid standards were HPLC grade (Pierce Chemical Co., Rockford, Ill.). All other chemicals were reagent grade. [^{14}C]glucose and ^3H_2O were obtained from ICN Radiochemicals (Irvine, Calif.). $^{15}NH_4Cl$ (99% ^{15}N) was obtained from Cambridge Isotope Laboratories (Cambridge, Mass.) and used without further purification.

RESULTS

Identification of β-glutamine as a compatible solute. The soluble pool of primary amines in 14 strains of halotolerant and halophilic methanogens was examined by HPLC analysis. Strains were grown in a basal medium with either methanol or TMA as a substrate. Yeast extract was excluded from the medium because it contains glycine betaine, which has an inhibitory effect on the de novo biosynthesis of compatible solutes in some methanogenic organisms (27, 30). The HPLC elution profile of the extract from one representative moderate halophile, *Methanohalophilus* strain FDF1, grown in 2.4 M NaCl is shown in Fig. 1. Peaks corresponding to aspartic acid, α-glutamate, and N_ε-acetyl-β-lysine, as well as one peak that did not correspond to any known amino acid, were obtained (Fig. 1A). N_ε-acetyl-β-lysine and the unknown compound were the predominant primary amines in these cells. The elution of the unidentified compound at 31.5 min, immediately before leucine (31.8 min), suggested that it was zwitterionic. Acid hydrolysis of the extract prior to HPLC analysis caused a shift in the retention time of the unidentified peak to that of β-glutamate; the N_ε-acetyl-β-lysine peak was converted to β-lysine (Fig. 1B).

The ^{13}C NMR spectrum of *Methanohalophilus* strain FDF1 cell extract (Fig. 2A) revealed three organic molecules, which were identified from their chemical shifts (parts per million from tetramethylsilane). These compounds included the primary amines N_ε-acetyl-β-lysine (178.8, C-1; 39.2, C-2; 50.0, C-3; 30.3, C-4; 25.1, C-5; 39.5/39.7, C-6; 174.9, C-7; 22.7, C-8) and L-α-glutamate (175.6, C-1; 55.4, C-2; 27.7, C-3; 34.1, C-4; 182.3, C-5) and the quaternary amine glycine betaine (169.7, C-1; 66.9, C-2; 54.1, C-3). Five other carbons with chemical shifts which did not correlate with known compounds were also detected: 178.3, 175.5 (see insert of Fig. 2A), 47.3, 39.0, and 37.2. Since HPLC analysis detected only primary amines, the unknown peak was not glycine betaine. The five unassigned ^{13}C chemical shifts were characteristic of the shifts yielded by β-glutamine. To confirm this, an ^1H NMR spectrum of the extract in 85% H_2O and 15% D_2O at pH 5.0 was obtained. Under these conditions, amide protons (e.g., NH of N_ε-acetyl-β-lysine) do not exchange rapidly with water and give rise to resonances of ~7 to 8 ppm. Besides the amide proton of the N_ε-acetyl-β-lysine (30) (Fig. 2B), two other amide resonances were detected. These resonances were consistent with the N-H bonds of β-glutamine (3-aminoglutaramic acid); two resonances indicated that there was a barrier to rotation around the C-N bond and that the two protons were nonequivalent. This is a common occurrence for amides, and the ^1H chemical shift difference for β-glutamine is comparable for what is observed with L-α-glutamine.

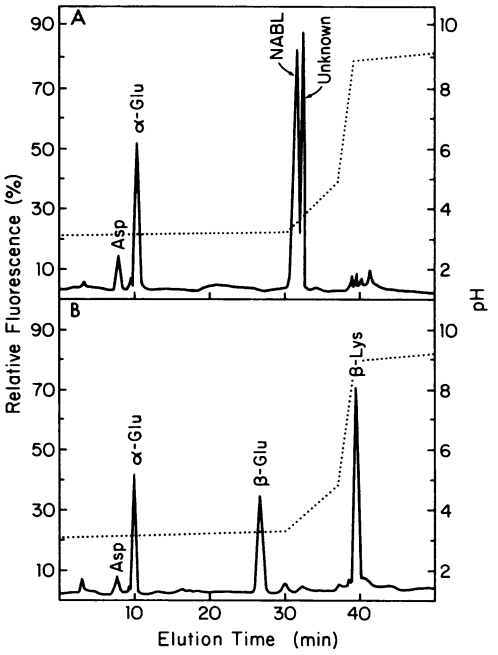

FIG. 1. HPLC ion-exchange chromatography of ethanol extracts of *Methanohalophilus* strain FDF1 grown in basal medium containing 2.4 M NaCl before (A) and after (B) acid hydrolysis with 6 N HCl at 95°C for 12 h. The dashed line represents the pH of the elution buffer. Abbreviations: Asp, α-aspartic acid; α-Glu, α-glutamate; β-Glu, β-glutamate; β-Lys, β-lysine; NABL, N_ε-acetyl-β-lysine.

Biosynthesis of betaine as a compatible solute. Glycine betaine was detected in several species of moderately and extremely halophilic methanogens that were grown in defined basal medium with TMA or methanol as the sole source of carbon and energy (Table 1). To confirm glycine betaine's synthesis de novo, *Methanohalophilus* strain FDF1 was grown in basal medium that contained methanol and $^{15}NH_4Cl$ instead of $^{14}NH_4Cl$. The ^1H-decoupled ^{15}N NMR spectrum (Fig. 3) of an extract from these cells indicated incorporation of the label into glycine betaine. Identification of the glycine betaine ^{15}N was verified by comparing extract shifts with those of a concentrated solution of authentic glycine betaine. Since the betaine nitrogen has no directly bonded protons, its ^{15}N resonance will not display a large nOe like the NH resonances of β-glutamine, L-α-glutamate, and N_ε-acetyl-β-lysine. Therefore, its intensity compared with that of the β-amino acids does not reflect the relative concentration of that species.

When *Methanohalophilus* strain FDF1 was grown with TMA at a salt concentration of 2.4 M, the intracellular ratio of glycine betaine to N_ε-acetyl-β-lysine was about 1:1. This ratio changed to 2.5:1 when methanol rather than TMA was used as a substrate. The basis for the different ratios of those zwitterionic compounds is not known.

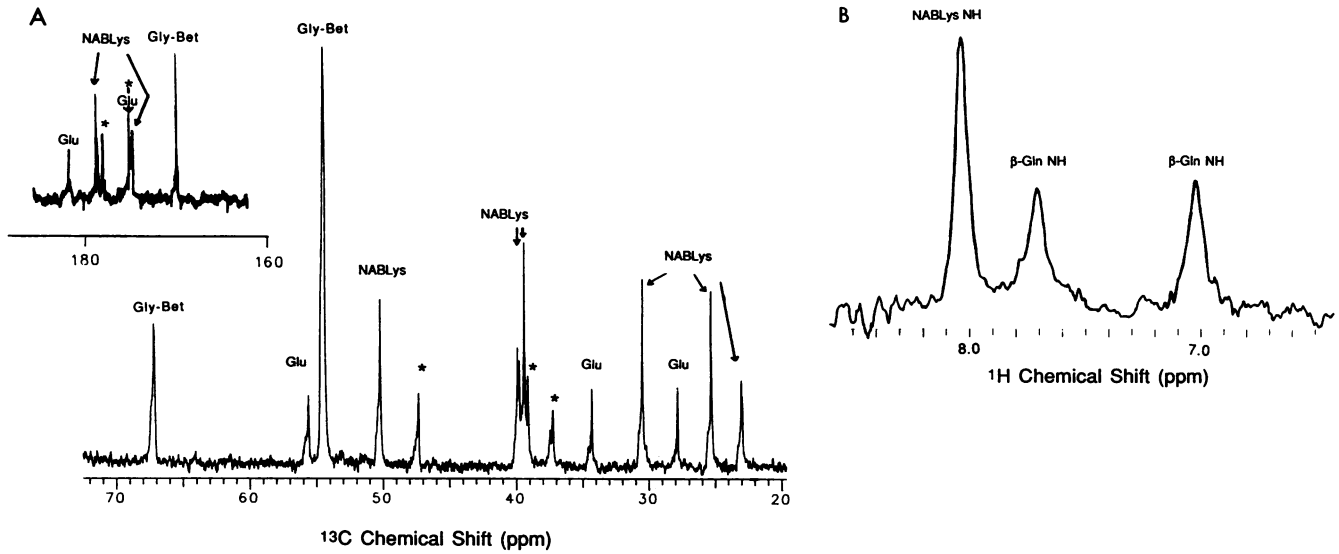

FIG. 2. (A) ^{13}C NMR spectrum (75.4 MHz) of an extract from *Methanohalophilus* strain FDF1 grown in 2.4 M NaCl. Carbons belonging to N_ϵ-acetyl-β-lysine (NABLys), glycine betaine (Gly-Bet), and glutamate (Glu) are labelled. Carbons marked with an asterisk are assigned to β-glutamine. (B) ^1H NMR spectrum (300 MHz) of an acidic (pH 4.5) extract of *Methanohalophilus* strain FDF1, showing two nonequivalent amide protons assigned to the β-glutamine (β-Gln) as well as the single N_ϵ-acetyl-β-lysine (NABLys) amide resonance in the 7- to 8-ppm region of the spectrum. At this pH, only the amide protons are observed. The amino protons exchange too rapidly with water and are thus not detected.

Compatible solutes of halotolerant and halophilic methanogens. In two halotolerant methanogens, *Methanohalophilus* strain Cas-1 and *Methanococcus halophilus* Z7982, L-α-glutamate and N_ϵ-acetyl-β-lysine were the predominant compatible solutes (Table 1 and data not shown). β-Glutamate and β-glutamine were also present but at lower levels relative to N_ϵ-acetyl-β-lysine. The intracellular concentrations of N_ϵ-acetyl-β-lysine and β-glutamine increased when the NaCl concentration in the medium increased from 0.7 to 1.7 M, whereas the amounts of α- and β-glutamate remained

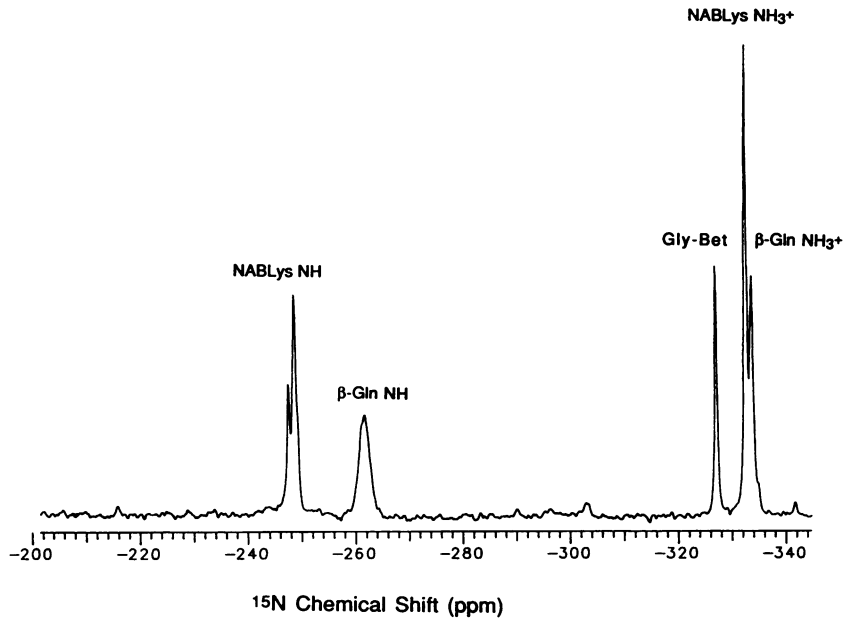

FIG. 3. ^{15}N NMR spectrum (30.4 MHz) of an ethanol extract of *Methanohalophilus* strain FDF1 grown on $^{15}NH_4Cl$ in 2.4 M NaCl. The major resonances are identified as belonging to the free amino ($-NH_3^+$) and amide ($-NH$) nitrogens of β-glutamine (β-Gln) and N_ϵ-acetyl-β-lysine (NABLys) and the $-N(CH_3)_3^+$ group of glycine betaine (Gly-Bet). The free amino groups belonging to aspartate and L-α-glutamate (which are detected considerably lower concentrations by HPLC), overlap the major resonances in the −322- to −335 ppm region, and so are not easily distinguished. Chemical shifts are referenced to external HNO_3.

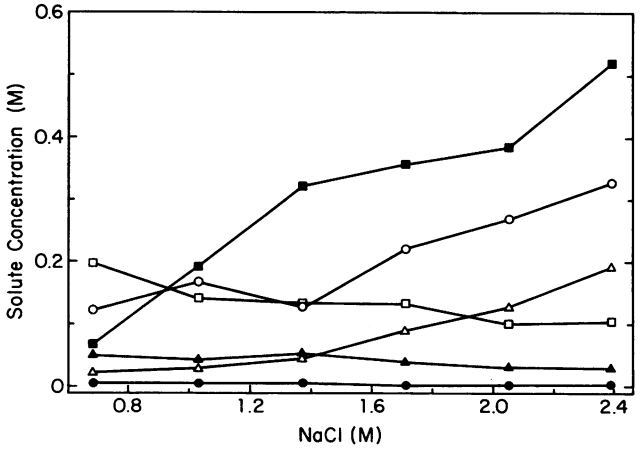

FIG. 4. Intracellular concentrations of compatible solutes of *Methanohalophilus* strain Z7401 grown in basal medium with the indicated concentrations of NaCl. Symbols: ▲, aspartate; ●, arginine; □, α-glutamate; ○, N_ϵ-acetyl-β-lysine; △, β-glutamine; ■, glycine betaine.

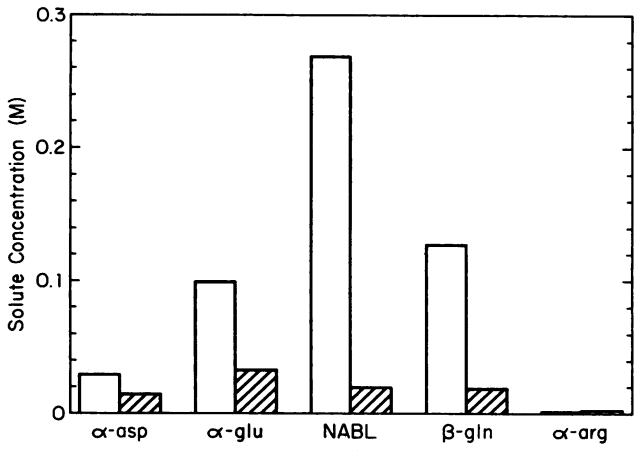

FIG. 5. Intracellular concentrations of compatible solutes in *Methanohalophilus* strain Z7401 grown in 2.1 M NaCl basal medium with (▨) and without (□) the addition of 0.5 mM glycine betaine. Abbreviations: α-asp, aspartic acid; α-glu, α-glutamic acid; NABL, N_ϵ-acetyl-β-lysine; β-gln, β-glutamine; α-arg, arginine.

constant. A third halotolerant strain, *Methanohalophilus* strain GS-16, exhibited similar behavior except that β-glutamate was not detected.

The HPLC profiles of all nine strains of moderately halophilic methanogens, including *Methanohalophilus* strain FDF1 (Fig. 1A), were similar; α-glutamate, β-glutamine, N_ϵ-acetyl-β-lysine, and glycine betaine were the predominant compatible solutes (Table 1). Glycine betaine was detected in six of the eight strains of moderate halophiles examined. Within the salt range for optimal growth of these organisms (1.4 to 2.7 M), the concentration of each zwitterionic species increased in response to an increase in the extracellular NaCl concentration in the medium. The ratio of N_ϵ-acetyl-β-lysine to β-glutamine was typically 5:1 at NaCl conditions less than 1.4 M and then gradually decreased to 2:1 within the NaCl range of 1.7 to 2.75 M.

The profile of compatible solutes in the extremely halophilic methanogen *Methanohalophilus* strain Z7302 was similar to that in the moderately halophilic methanogens (Table 1) except for the difference in the ratios of N_ϵ-acetyl-β-lysine to β-glutamine, which were 1:1 and 1:2 at 1.7 and 4.3 M NaCl, respectively.

Intracellular concentrations of compatible solutes. The intracellular concentration of each compatible solute was determined in strains of moderate halophiles grown in an NaCl range of 0.7 to 2.4 M. In *Methanohalophilus* strain Z7401, the level of β-glutamine increased gradually from 0.02 to 0.2 M as the external NaCl concentration increased from 0.7 to 2.4 M (Fig. 4). The intracellular concentration of N_ϵ-acetyl-β-lysine, which ranged from 0.1 to 0.34 M in *Methanohalophilus* strain Z7401, was higher than that of β-glutamine at each NaCl concentration tested (Fig. 4). Also, the intracellular levels of glycine betaine increased from 0.07 to 0.6 M. In contrast, the intracellular concentration of α-glutamate decreased slightly between 0.7 and 1.0 M NaCl and then remained essentially constant at 0.1 to 0.13 M. The three zwitterionic species, β-glutamine, N_ϵ-acetyl-β-lysine, and glycine betaine, were the predominant compatible solutes in this strain. The low levels of aspartate and arginine remained constant over 0.7 to 2.4 M NaCl (Fig. 4). Similar results were observed with the other moderate halophilic strains, FDF1 and FDF2, except that FDF2 lacked the ability to synthesize glycine betaine (data not shown).

Effects of exogenous compatible solutes on the intracellular concentrations of compatible solutes. When *Methanohalophilus* strain Z7401 was grown in medium containing 0.5 mM glycine betaine, the intracellular concentrations of β-glutamine, N_ϵ-acetyl-β-lysine, and α-glutamate were less than in the absence of added glycine betaine (Fig. 5). This observation indicated that this strain preferentially accumulated glycine betaine from the medium rather than synthesize other primary amines as compatible solutes. The presence of neither choline, which can be converted to glycine betaine by various aerobic eubacteria, nor glycine caused lower intracellular concentrations of β-glutamine, N_ϵ-acetyl-β-lysine, or α-glutamate.

Intracellular concentration of potassium ions. The intracellular concentration of potassium ions in *Methanohalophilus* strain FDF1 was examined in cells grown in various concentrations of NaCl (Table 2). When the extracellular NaCl concentration was in the range of 1.7 to 2.7 M, intracellular potassium increased from 0.6 to 1.1 M, which was 20- to 40-fold higher, respectively, than the extracellular K^+ concentration (0.031 M).

Balance of extracellular and intracellular osmolarity. The

TABLE 2. Extracellular and intracellular osmolarity of *Methanohalophilus* strain FDF1

NaCl concn of medium (M)	Osmolarity	Intracellular compatible solute concn (M)[a]						
		α-Glu	NABL	β-Gln	Betaine	K+	Zwitterions[b]	Total[c]
1.4	2.7	0.17	0.24	0.07	0.16	ND		
1.7	3.4	0.20	0.47	0.20	0.30	0.61	0.97	1.77
2.0	4.1	0.17	0.46	0.17	0.31	0.73	0.94	1.84
2.4	4.8	0.13	0.54	0.25	0.40	0.76	1.19	2.08
2.7	5.5	0.17	0.81	0.39	0.65	1.13	1.85	3.15

[a] For abbreviations, see Table 1, footnote *c*.

[b] Concentration of all zwitterions such as β-glutamine, N_ϵ-acetyl-β-lysine, and glycine betaine.

[c] The intracellular osmolarity, which includes K^+, L-α-glutamate, and all detected zwitterionic species.

moderate halophile *Methanohalophilus* strain FDF1 synthesized α-glutamate, glycine betaine, β-glutamine, and N_ε-acetyl-β-lysine as compatible solutes (Table 1). When the saline concentration was increased from 1.4 to 2.7 M, the intracellular levels of β-glutamine, N_ε-acetyl-β-lysine, and glycine betaine increased about six-, three-, and fourfold to 0.39, 0.81, and 0.65 M, respectively (Table 2). In contrast, the concentration of α-glutamate remained at approximately 0.2 M over this NaCl range. The intracellular osmolarity as determined by the sum of α-glutamate, total zwitterions, and K^+ was within 40 to 60% of the extracellular osmolarity (Table 2).

DISCUSSION

The methanogenic archaebacteria, like many eubacteria and eukaryotes, accumulate compatible solutes, thus reducing the osmotic potential between the cell interior and its exterior in environments with high solute concentrations (26, 28, 30). In this study, nine moderately halophilic, two extremely halophilic, and three halotolerant methanogens were screened for compatible solutes. These halophilic methanogenic archaebacteria were originally isolated from various hypersaline environments all over the world (e.g., Arabat Peninsula, Austriala, Portugal, Senegal, and United States). All strains tested synthesized α-glutamate, N_ε-acetyl-β-lysine, and β-glutamine, a primary amine that has not been previously identified as a compatible solute (Table 1). Several strains also synthesized β-glutamate or glycine betaine. However, glycine betaine was not observed in the three halotolerant strains, and of all strains tested, β-glutamate was detected in only two halotolerant strains. The intracellular concentration of every compatible solute increased with the osmolarity of the growth medium except for α-glutamate and β-glutamate, which remained at relatively constant concentrations in all strains examined. At high extracellular NaCl concentrations, the levels of β-glutamine and N_ε-acetyl-β-lysine, as well as those of L-α-glutamate and glycine betaine, attained high intracellular concentrations. One of the moderate halophiles, *Methanohalophilus* strain FDF1, at the upper range of its salt tolerance (2.7 M), contained 0.4 M β-glutamine, 0.8 M N_ε-acetyl-β-lysine, and 0.7 M glycine betaine (Table 2). The total intracellular osmolarity of this strain, which comprised all organic and inorganic compatible solutes, was approximately 40 to 60% of the osmolarity of extracellular solutes over the entire range (1.7 to 2.7 M) of NaCl levels tested. One of the few extremely halophilic methanogens thus far isolated, *Methanohalophilus* strain Z7302, can grow in a medium containing 4.3 M NaCl (25%, wt/vol), and it also synthesizes α-glutamate, β-glutamine, N_ε-acetyl-β-lysine, and glycine betaine as compatible solutes.

A stepwise synthesis of compatible solutes in response to the extracellular osmolarity was observed in the halophilic methanogens. L-α-glutamate, the predominant intracellular solute at low salt levels, increased to a threshold concentration and remained relatively constant. This threshold concentration may have been maintained to prevent perturbation to cell physiology due to the accumulation of charged species (30). N_ε-acetyl-β-lysine synthesis occurred at an intermediate salt concentration (>0.3 M [30]), whereas β-glutamine and glycine betaine accumulated only at higher NaCl concentrations (>0.5 M). N_ε-acetyl-β-lysine, β-glutamine, and glycine betaine are zwitterionic species at physiological pH, which suggests that they can accumulate as intracellular solutes without perturbing cell metabolism (21).

β-Amino acids, while rare in nature, have been reported in biological systems (7). β-Glutamate and β-lysine have been detected in marine eubacteria and eukaryotes, but their concentrations and physiological roles have not been determined (8, 36). Besides establishing the presence of N_ε-acetyl-β-lysine and β-glutamate in the halophilic methanogenic archaebacteria, we also identified a third β-amino acid, β-glutamine. This is the first report of β-glutamine as a compatible solute. Since β-amino acids are not considered to be good substrates for L-α-amino acid biosynthetic enzymes, their accumulation should not interfere with cell metabolism (25). The accumulation of the β-amino acids and N_ε-acetyl-β-lysine in the methanogenic archaebacteria demonstrates that these species have evolved alternative biosynthetic pathways for generating compatible solutes to adapt to osmotic stress (30).

Nonmarine eubacteria (24), marine (13, 14), and slightly halotolerant methanogens (27, 30) preferentially accumulate glycine betaine as a major compatible solute. Exogenous addition of glycine betaine to *Methanohalophilus* strains Z7401 and FDF2 partially suppressed intracellular accumulation of β-glutamine, N_ε-acetyl-β-lysine, and α-glutamate, indicating that these halophilic methanogens preferentially accumulate glycine betaine rather than synthesize it or the three β-amino acids (Fig. 5). These halophilic methanogens and possibly others possess both a biosynthetic pathway for de novo synthesis of glycine betaine and a transport system for glycine betaine accumulation. Although high levels of glycine betaine can be achieved by both means, glycine betaine transport rather than synthesis would be bioenergetically more efficient.

The moderate halophilic methanogenic archaebacterium *Methanohalophilus* strain FDF1 accumulates potassium in the range of 0.6 to 1.1 M (Table 2). The high internal potassium ion concentration also occurs in the extremely halophilic archaebacteria *Halobacterium* sp. and *Halobium* sp. (2, 17) and in the nonhalophilic methanogenic archaebacteria (10, 29, 32). Like the extremely halophilic archaebacteria, the halophilic methanogens exhibit a high intracellular salt requirement. Unlike the extreme halophiles that accumulate only potassium in response to osmotic changes, the halophilic methanogens use both organic compatible solutes, N_ε-acetyl-β-lysine, β-glutamine, glycine betaine, and inorganic ions in response to the extracellular solute concentration. This is the first report of de novo synthesis of glycine betaine by the methanogenic bacteria. The synthesis of β-amino acids as compatible solutes, in this case β-glutamine and N_ε-acetyl-β-lysine, appears to be a unique characteristic of methanogenic archaebacteria.

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