*Proc. Natl. Acad. Sci. USA*
Vol. 87, pp. 9083–9087, December 1990
Biochemistry

# $N^{\varepsilon}$-Acetyl-β-lysine: An osmolyte synthesized by methanogenic archaebacteria

(methanogens/osmoregulation/β-amino acids)

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*Communicated by Ralph S. Wolfe, August 27, 1990*

ABSTRACT **_Methanosarcina thermophila_, a nonmarine methanogenic archaebacterium, can grow in a range of saline concentrations. At less than 0.4 M NaCl, _Ms. thermophila_ accumulated glutamate in response to increasing osmotic stress. At greater than 0.4 M NaCl, this organism synthesized a modified β-amino acid that was identified as $N^{\varepsilon}$-acetyl-β-lysine by NMR spectroscopy and ion-exchange HPLC. This β-amino acid derivative accumulated to high intracellular concentrations (up to 0.6 M) in _Ms. thermophila_ and in another methanogen examined—_Methanogenium cariaci_, a marine species. The compound has features that are characteristic of a compatible solute: it is neutrally charged at physiological pH and it is highly soluble. When the cells were grown in the presence of exogenous glycine betaine, a physiological compatible solute, $N^{\varepsilon}$-acetyl-β-lysine synthesis was repressed and glycine betaine was accumulated. $N^{\varepsilon}$-Acetyl-β-lysine was synthesized by species from three phylogenetic families when grown in high solute concentrations, suggesting that it may be ubiquitous among the methanogens. The ability to control the biosynthesis of $N^{\varepsilon}$-acetyl-β-lysine in response to extracellular solute concentration indicates that the methanogenic archaebacteria have a unique β-amino acid biosynthetic pathway that is osmotically regulated.**

Microorganisms can proliferate in a diverse range of saline concentrations from low saline environments such as freshwater lakes to saturated brines found in solar salterns (1, 2). Cell size and the intracellular water activity must remain relatively constant to maintain physiological processes. Eukaryotic and eubacterial microorganisms have evolved mechanisms that enable them to minimize water loss when the extracellular solute concentration exceeds that of the cell cytoplasm (1–3). The mechanism of this adaptation involves the uptake or synthesis of low molecular weight organic compounds known as physiological compatible solutes (4, 5). These compounds reduce the osmotic potential between the extracellular milieu and the cytoplasm and protect enzymes from the low water activity that results from solute accumulation.

Methanogenic archaebacteria have been isolated from environments with NaCl concentrations ranging from <0.05 M for many nonmarine species to >4 M for halophilic species (6). Individual species can also adapt to a range of saline concentrations. *Methanosarcina thermophila*, which was isolated from a thermophilic sludge digestor, grows in medium containing 0.05–1.2 M NaCl (7–9), and the marine species *Methanogenium cariaci* (10) grows in medium containing 0.17–1.4 M NaCl (D.N. and M.F.R., unpublished results). Compatible solutes have been detected in methanogenic bacteria (11–13). Several species of marine methanogens synthesize β-glutamate in response to external NaCl (11, 13). Methanogens have also been reported to accumulate glycine betaine if it is provided in the medium (12). However, the mechanism for adaptation to high osmotic stress has not been investigated. Here we report the structure of a modified β-amino acid that is synthesized as the predominant compatible solute by both marine and nonmarine species of methanogenic archaebacteria in response to high extracellular solute concentrations

## MATERIALS AND METHODS

**Reagents.** Ion-exchange elution buffers, *o*-phthalaldehyde, and amino acid standards were obtained from Pierce. All other chemicals were reagent grade.

**Bacterial Strains.** *Ms. thermophila* TM-1 (DSM 1825) was a laboratory stock originally obtained from R. A. Mah (University of California, Los Angeles). *Mg. cariaci* JR1 (DSM 1497) was obtained from D. R. Boone (Oregon Graduate Center).

**Media Growth and Conditions.** Sterile media were prepared under an $N_2/CO_2$ (4:1) atmosphere by a modification of the Hungate technique (14). *Ms. thermophila* was grown in basal medium as described (7) without trimethylamine and supplemented with 100 mM methanol, 50 mM $MgCl_2$, 10.2 mM KCl, and 0.95 mM $CaCl_2$. *Mg. cariaci* was grown as described in medium prepared without yeast extract (12). NaCl was added to the basal medium at the concentrations indicated. Each strain was adapted for growth at the selected NaCl concentrations by sequentially transferring cultures into medium that contained increasing or decreasing NaCl concentrations. Cultures were grown at 40°C and harvested during midexponential growth by centrifugation at 6000 × *g* for 15 min. Cell growth was measured by optical density at 550 nm. Cell counts were determined with a Petroff–Hauser counting chamber (15).

**Osmolyte Extraction.** Ethanol extracts of cells were prepared for analyses as described (11). Cell pellets that contained $2 \times 10^{10}$ cells were extracted twice by heating for 5 min at 65°C in 1 ml of 70% ethanol/water. Pooled extracts were centrifuged at 5000 × *g* for 5 min, filtered through a 0.2-μm (pore size) polytetrafluoroethylene (PTFE) membrane filter (Gelman), and freeze-dried.

**Ion-Exchange HPLC Analyses for Primary Amines.** Cell extracts were dissolved in doubly distilled $H_2O$ and eluted from a Sep-Pak $C_{18}$ column (Waters) with 0.1% trifluoroacetic acid in water/methanol, 7:3 (vol/vol). The HPLC dual pump system (Waters) was equipped with a gradient programmer (model 720). Extract (10 μl) was eluted from an anion-exchange column with a linear pH gradient ranging from pH 3.17 to 9.94. Eluent was monitored with a fluorescence spectrophotometer (model 420E; excitation wavelength = 340 nm and emission wavelength = 455 nm) after *o*-phthalaldehyde postderivatization.

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Abbreviation: COSY, chemical shift correlation spectroscopy.

**NMR Spectroscopy.** $^{13}C$ NMR spectra were obtained at 75.4 MHz on a Varian model XL-300 spectrometer. The dry residue from each ethanol extract was suspended in NMR buffer consisting of 10 mM potassium phosphate, 0.1 mM EDTA, and 50% (vol/vol) $D_2O$ (pH 7.2). Spectral parameters included: $^1H$ WALTZ decoupling, 16,500-Hz sweep width, 54,080 data points, 40° flip angle (6.1 μs), 1.6-s recycle delay, and 5000 to 10,000 transients. $^1H$ NMR spectra of the same extracts were obtained at 300 MHz on the same spectrometer. For chemical shift correlated spectroscopy (COSY) experiments, spectral parameters included 1283-Hz sweep width and 1024 data points in the $F_1$ dimension and 1283 Hz and 512 data points in the $F_2$ dimension, a 90° flip angle of 22 μs, 3-s recycle delay, and 16 transients.

**Cell Volume Determination.** Cell volumes were determined by measuring the differential retention of [$^{14}C$]glucose and $^3H_2O$ in cell pellets using a modification of a published method (16). Cell suspension buffer consisted of growth medium that did not contain substrate. Approximately $1 \times 10^{10}$ *Ms. thermophila* cells were equilibrated in buffer that contained 0.001 M spermine for 30 min to stabilize the protein cell wall (K.R.S., unpublished results). Cells were centrifuged at 5000 × *g* for 5 min in a tabletop microcentrifuge and resuspended in buffer (0.5 ml) that contained 0.15 μCi of [$^{14}C$]glucose and 0.4 μCi of $^3H_2O$ (1 Ci = 37 GBq). Cells were allowed to equilibrate for 10 min at room temperature prior to centrifugation and separation of the supernatant. Cells were then resuspended in buffer, equilibrated, and centrifuged as described above, and the supernatant was retained. The radioactivities of the two supernatants were determined by liquid scintillation using channels set for $^3H$ and $^{14}C$. Volumes were determined from the differential cpm retained by the two supernatants as described (16).

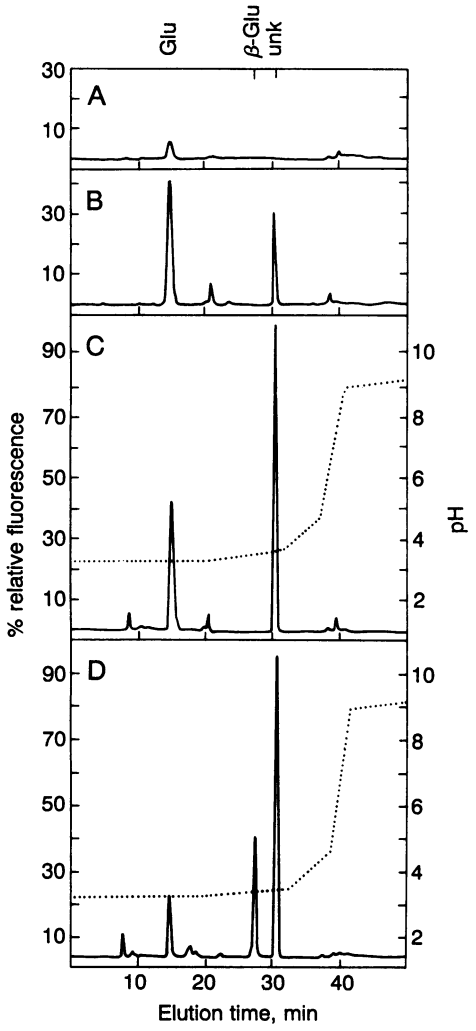



FIG. 1. HPLC ion-exchange chromatography of ethanol extracts from *Ms. thermophila* TM-1 grown in medium that contained 0.05 M (*A*), 0.4 M (*B*), and 1.0 M (*C*) NaCl and from *Mg. cariaci* grown in medium that contained 0.5 M NaCl (*D*). The ratio of peak height to area increases with elution time. The dashed line indicates the pH of the elution buffer. unk, Unknown.

## RESULTS

An HPLC ion-exchange elution profile of cell extracts from *Ms. thermophila* grown in medium of low osmotic strength (<0.035 M NaCl) showed that intracellular free amino acids occurred at relatively low concentrations with glutamate as the major species (Fig. 1*A*). When the osmotic strength of the medium was raised by increasing the NaCl concentration to 0.4 M, glutamate and another primary amine that did not elute with known amino acids were the predominant species in the intracellular amino acid pool (Fig. 1*B*). At 1.0 M NaCl, the unidentified compound was the major species (Fig. 1*C*). The same unknown compound, in addition to aspartate, glutamate, and β-glutamate, was also detected by ion-exchange HPLC in extracts of *Mg. cariaci* grown in 0.5 M NaCl (Fig. 1*D*). When *Ms. thermophila* or *Mg. cariaci* were grown in medium that contained 0.05% yeast extract or 0.5–10 mM betaine, the unidentified compound was significantly reduced or undetectable. The unidentified compound possessed a primary amine group, indicated by derivatization with *o*-phthalaldehyde, and was eluted with a relative retention time of 30.5 min, between isoleucine (29.9 min) and leucine (31.2 min), indicating that it was likely zwitterionic.

An $^{15}N$ NMR spectrum of a cell extract from *Ms. thermophila* grown in 0.8 M NaCl with $^{15}NH_4Cl$ exhibited resonances for glutamate (−335.2 ppm) and two unidentified species: a resonance at −334.6 ppm, consistent with an α- or β-amino acid free amino group, and a resonance at −250 ppm, consistent with an amide linkage (data not shown). Since HPLC detected only two major species, glutamate and a neutrally charged unknown compound, the $^{15}N$ NMR results suggested that in addition to the free amino group, a second nitrogen was present in the unidentified compound, presumably an acid-labile amide. An HPLC analysis of an acid-hydrolyzed cell extract from *Ms. thermophila* indicated

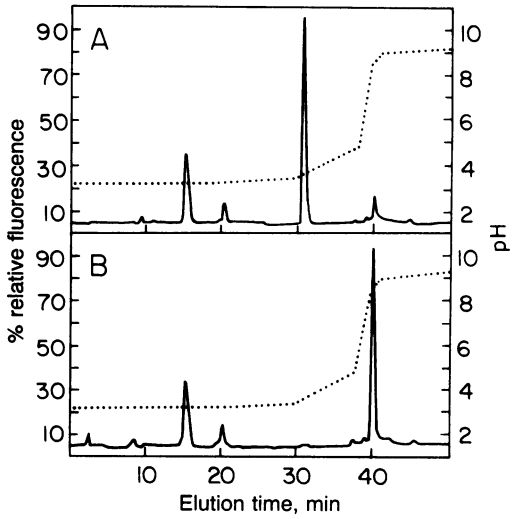



FIG. 2. HPLC ion-exchange chromatogram of ethanol extract from osmotically stressed *Ms. thermophila* before (*A*) and after (*B*) acid hydrolysis with 6 M HCl at 95°C for 12 hr. The dashed line represents the pH of the elution buffer.

that the zwitterionic species at 30.5 min was quantitatively converted to a basic species that eluted at 40.7 min, between lysine (39.7 min) and arginine (45.5 min) (Fig. 2). This result was consistent with the presence of an acid-labile amide moiety.

Natural abundance $^{13}C$ spectra extracts from *Ms. thermophila* grown to late exponential phase in 1 M NaCl showed resonances (ppm from dioxane at 67.4 ppm) consistent with glutamate (27.7, C-3; 34.2, C-4; 55.5, C-2; 175.1, C-1; and 182.8, C-5) (Fig. 3*A*). The $^{13}C$ spectrum also showed eight carbons, two of which were clearly doublets, with chemical shifts that did not correspond to known amino acids. This suggested that the unidentified carbons (indicated in Fig. 3*A* by triangles) corresponded to the unknown species. Minor amounts of aspartate (53.0, C-2, and 37.3, C-3) and alanine (58.3, C-2, and 17.5, C-3) were also detected in the extract spectrum of *Ms. thermophila* (consistent with the low levels of these amino acids detected by HPLC). $^{13}C$ spectra of extracts from *Ms. thermophila* grown on $^{15}NH_4Cl$ (99%) showed that for the eight unidentified carbon resonances, two of the carbons with directly bonded protons (50.0 and 39.5/39.7 ppm) exhibited $^{13}C$–$^{15}N$ splitting of ($J$ = 4.6 Hz). This identified these two carbons as -$CH_n$N- groups; the difference in the $^{13}C$ chemical shifts was consistent with -$CH_2$N- (≈39.6 ppm) and -CHN- (50 ppm) groups. The same eight-carbon resonances were also detected at significantly higher amounts in $^{13}C$ NMR spectra of extracts from *Mg. cariaci* (Fig. 3*B*). Under the conditions used for growth of this organism, the eight-carbon unknown occurred at much higher levels relative to glutamate, β-glutamate (39.2, C-2/4; 47.8, C-3; and 178.5, C-1/5) (11), and aspartate. A $^{13}C$ NMR spectrum of the unknown compound isolated from ethanol-extracted cells by ion-exchange HPLC yielded the same $^{13}C$ chemical shifts marked in Fig. 3, indicating that all eight carbons belonged to the same molecule. The proton multiplicities of these carbons were determined on a *Mg. cariaci* extract with a distortionless enhancement by polarization transfer (DEPT) pulse sequence (18). Coupled with the $^{15}N$ splitting of carbon, this led to the following assignments for the unknown: 22.7 ($CH_3$), 25.1 ($CH_2$), 30.3 ($CH_2$), 39.2 ($CH_2$),

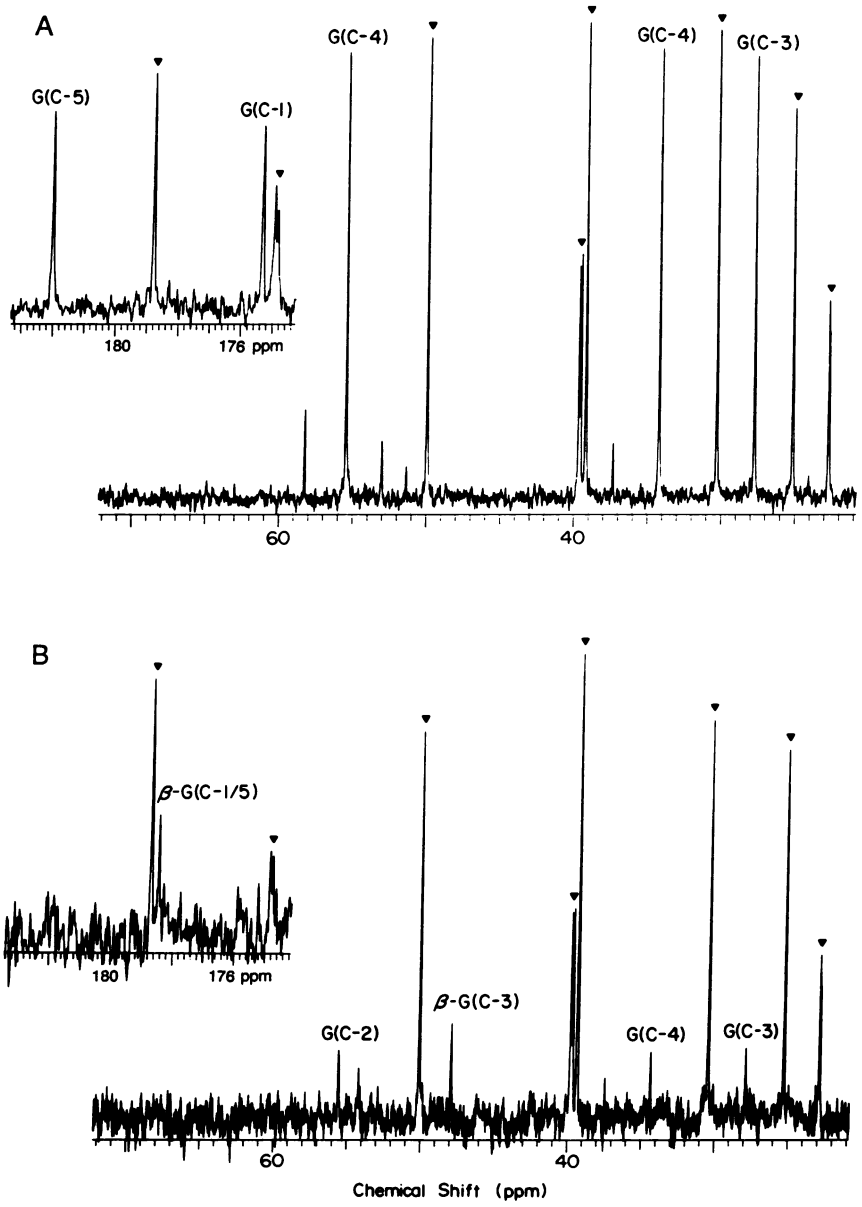
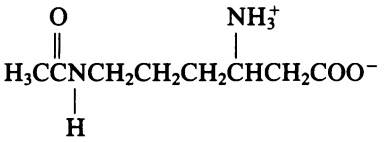



FIG. 3. $^1H$-decoupled $^{13}C$ NMR (75.4 MHz) spectra of 70% ethanol extracts of *Ms. thermophila* grown in 1.0 M NaCl to late exponential phase (*A*) and *Mg. cariaci* grown in 0.5 M NaCl (8, 17) without yeast extract (*B*). Spectral parameters include $^1H$ WALTZ decoupling, 16,500-Hz sweep width, 54,080 data points, 40° flip angle (6.1 μs), 1.6-s recycle delay, 5000 transients for *A* and 10,000 transients for *B*. Resonances in each extract whose chemical shifts do not correspond with known amino acids are indicated (▼).

39.5/39.7 ($CH_2N$), 50.0 (CHN), 174.8/174.9 (COX), and 178.8 (COX) ppm (where X is likely an amide nitrogen). The methyl carbon was slightly broad, possibly indicating a doublet (two nearly overlapping species) and its integrated intensity was lower than that of the other carbons because of a smaller nuclear Overhauser effect.

A $^1H$ NMR (300 MHz) spectrum of ethanol-extracted *Mg. cariaci* is shown in Fig. 4*A*. When the $^1H$ spectrum was compared with the $^{13}C$ spectrum (Fig. 3*B*), it was apparent that the methyl group belonged to an acetyl linkage. Other connectivities derived from a $^1H$ COSY experiment in $D_2O$ (Fig. 4*A*) indicated both $-CH_2-CH(N)-CH_2-$ and $-NCH_2-CH_2-$ moieties as well as the $CH_3CO-$ group. These, plus the $^{13}C$ shifts and the behavior of the hydrolyzed compound on HPLC, suggested that the unknown metabolite was $N^{\epsilon}$-acetyl-β-lysine. To determine the connectivity of the acetyl group a $^1H$ COSY was performed in 85% $H_2O$ at pH 5.5. Under these conditions a cross peak was detected between the amide N—H (8.0 ppm) and the $CH_2$ resonance at 3.2 ppm (Fig. 4*B*). The eight-carbon osmolyte in methanogens, identified as $N^{\epsilon}$-acetyl-β-lysine, has the following structure:



$^{13}C$ chemical shifts of the hydrolyzed material were also consistent with the formation of β-lysine from the original material (chemical shifts of authentic β-lysine were supplied by Perry Frey, University of Wisconsin). As observed in Fig. 3, the $CH_2N$ and side-chain acetyl carboxyl resonances in $N^{\epsilon}$-acetyl-β-lysine were resolved as doublets. The acetyl methyl was broader than other carbons, which was also consistent with two overlapping resonances. Since the splitting of these carbons varied with magnet field strength, it represents two conformations of the compound with respect to the amide linkage. This implies that at room temperature there is hindered rotation around the amide C—N bond.

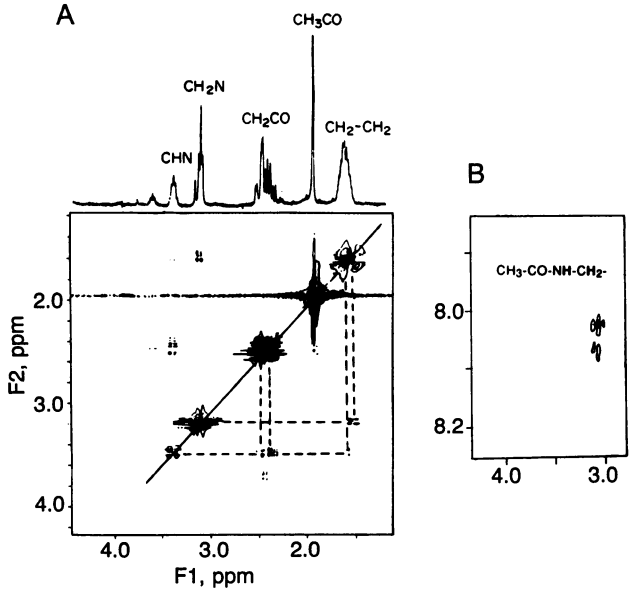



FIG. 4. $^1H$ NMR (300 MHz) spectra of 70% ethanol extract of *Mg. cariaci* grown without yeast extract (8). (*A*) One-dimensional spectrum in 99.9% $D_2O$ and contour plot from a COSY experiment on the same sample showing connectivities of the different protons. (*B*) COSY cross peak for the same extract adjusted to pH 5 and solubilized in 85% $H_2O$, showing the connectivity of the amide N—H with the $CH_2N$ protons.

Table 1. Effect of NaCl levels on the intracellular concentrations of solutes in *Ms. thermophila*

| NaCl, M | Intracellular amino acid concentration, mM | | |
|---|---|---|---|
| | Aspartate | L-α-Glutamate | $N^{\epsilon}$-Acetyl-β-lysine |
| 0.05 | 4 | 69 | ND |
| 0.40 | 30 | 290 | 107 |
| 1.0 | 20 | 357 | 597 |

ND, not detected.

When the extract was heated to 65°C, the carbon doublets collapsed to a singlet, which supports the interpretation of two conformations.

Having established the identity of $N^{\epsilon}$-acetyl-β-lysine, we determined the intracellular concentrations of aspartate, glutamate, and $N^{\epsilon}$-acetyl-β-lysine in *Ms. thermophila* as a function of NaCl in the medium. As shown in Table 1, glutamate was the predominant small molecular weight organic molecule in the soluble cell fraction when *Ms. thermophila* was grown in mineral medium with a low concentration of NaCl (<0.05 M). Furthermore, the intracellular concentrations of glutamate (0.07 M), $K^+$ (19, 20), and other cytoplasmic components indicated that the cells maintained a high osmotic potential relative to low saline medium (<0.150 osmolar). Intracellular glutamate increased with increasing levels of NaCl but leveled off in medium with NaCl ≥0.4 M. $N^{\epsilon}$-Acetyl-β-lysine was detected at NaCl ≥0.4 M and its intracellular concentration continued to increase with up to 1.0 M NaCl in the medium. For comparison, aspartate, which is not thought to act as a compatible solute, showed only a small change in intracellular concentration over the same NaCl range. Although intracellular volumes have not been determined for *Mg. cariaci*, the amounts of $N^{\epsilon}$-acetyl-β-lysine synthesized by this organism and *Ms. thermophila*, both grown in 0.5 M NaCl, were 860 ± 100 and 94 ± 10 nmol/mg of protein, respectively.

## DISCUSSION

Methanogenic archaebacteria have been isolated from diverse environments ranging in solute concentration from freshwater to saline, hypersaline, or concentrated brines. Although their "optimal" solute concentration for growth is usually similar to that of their isolation source, many species of methanogens tolerate a wide range of solute concentrations (6). Like many eubacteria and eukaryotes, the apparent mechanism for osmotic adaptation in the methanogens involves the accumulation of osmolytes known as compatible solutes that reduce the osmotic potential between the cell and medium without perturbing the cell metabolism. The structures of the physiological osmolytes synthesized by methanogens are β-amino acids, unusual compounds that have rarely been described in biological systems. L-β-Glutamate has been detected and characterized (11, 13) as an osmolyte in thermophilic methanococci and mesophilic methanogenium sp. In this report we have shown that a β-amino acid derivative, $N^{\epsilon}$-acetyl-β-lysine, is synthesized by marine and nonmarine methanogens when grown in high extracellular solute concentrations. The presence of these two compounds in methanogenic archaebacteria suggests that these species have taken a unique approach in synthesizing β-amino acids and derivatives as compatible solutes. Most β-amino acids are not substrates for enzymes that utilize L-α-amino acids and presumably would not interfere with cell metabolism.

Although β-amino acids have been detected in some organisms, they are relatively rare in nature (21). To our knowledge, $N^{\epsilon}$-acetyl-β-lysine has not been described in any other biological system. β-Lysine has been identified as an intermediate in lysine fermentation by *Clostridium* SB4 (22)

and is an important moiety in the formation of several antibiotics (23). The few other β-amino acids that have been detected occur almost exclusively in marine organisms. For example, β-glutamate has been detected in a marine eubacterium, *Alteromonas luteoviolacea* (17), but at much lower concentrations than found in marine methanogens. *Prochloron didemnii*, a prokaryotic algae symbiont, produces 3-(*N*-methylamino)glutaric acid (3-methyl-β-glutamate) (24) and a red sea sponge, *Fasciospongia cavernosa*, produces N-acylated 2-methylene β-alanine (25). The functions of the last two β-amino acids in those cells are not known. The unique structure of $N^{\epsilon}$-acetyl-β-lysine may reflect one or more biosynthetic pathways exclusive to the methanogenic archaebacteria.

When *Ms. thermophila* was adapted to NaCl concentrations ranging from 0.05 to 1.5 M, the intracellular levels of L-α-glutamate and $N^{\epsilon}$-acetyl-β-lysine were sufficiently high to compensate for the osmotic potential created by the NaCl in the medium. In addition, no other low molecular weight organic compounds were at high enough concentrations, based on HPLC or NMR detection, to contribute significantly as compatible solutes. The results indicate that L-α-glutamate is the predominant solute in medium of low osmotic strength, whereas $N^{\epsilon}$-acetyl-β-lysine becomes the major species in response to a high osmotic potential. The values for intracellular levels of $N^{\epsilon}$-acetyl-β-lysine in both *Ms. thermophila* and *Mg. cariaci* grown in 0.5 M NaCl were comparable, consistent with its role as a compatible solute in the latter cells as well. $N^{\epsilon}$-Acetyl-β-lysine was also detected at high levels by NMR and HPLC in other methanogens, including *Methanococcus deltae* and *Methanohalophilus* sp. Its detection in four distinct phylogenetic families suggests that it may be a ubiquitous response of these archaebacteria to high external solute concentration.

In some eubacterial systems the accumulation of glutamate is associated with an increase in intracellular $K^+$ levels in response to hypertonic conditions (26). *Methanosarcina* spp. have been reported to accumulate high intracellular $K^+$ levels (19, 20) and, at physiological pH, negatively charged glutamate (and β-glutamate for *Mg. cariaci*) may partially neutralize the net $K^+$ charge. However, in medium that contains a high solute concentration, the accumulation of charged species such as $K^+$ or glutamate is limited by their adverse effect on cell protein and the membrane electrochemical potential ($\Delta\Psi$). Therefore, an alternative osmolyte is required that is less deleterious to metabolic functions. $N^{\epsilon}$-Acetyl-β-lysine was synthesized only in response to high extracellular solute concentrations in *Ms. thermophila* and was the predominant species in *Mg. cariaci* when no physiological osmolytes such as betaine were provided in the medium. This β-amino acid derivative is zwitterionic at physiological pH; therefore, its intracellular accumulation would presumably have a less perturbing effect on cell metabolism than a charged species such as glutamate (26). In addition, the fact that this compound is an amino acid derivative suggests that, like glycine betaine in *Escherichia coli* (27), $N^{\epsilon}$-acetyl-β-lysine is less likely to be used as a precursor for biosynthesis. This would enable the cell to accumulate a high intracellular concentration of $N^{\epsilon}$-acetyl-β-lysine that functions to balance osmotic pressure.

Results of this study indicated that osmoregulatory mechanisms in *Ms. thermophila* were integrated in such a way that this species could respond optimally to osmotic signals. At low solute concentrations, $N^{\epsilon}$-acetyl-β-lysine synthesis and, presumably, betaine transport were repressed. As the solute concentration was increased, in the absence of extracellular glycine betaine, glutamate and eventually $N^{\epsilon}$-acetyl-β-lysine were synthesized and increased in proportion to the solute concentration. However, if *Ms. thermophila* or *Mg. cariaci* were grown in the presence of glycine betaine, then synthesis of $N^{\epsilon}$-acetyl-β-lysine was inhibited and glycine betaine accumulated in the cells. The ability to regulate biosynthesis of compatible solutes and repress the biosynthesis of $N^{\epsilon}$-acetyl-β-lysine in lieu of betaine transport indicates that these archaebacteria possess regulatory mechanisms that enable them to use the most bioenergetically conservative pathway for survival.

We thank Mr. Negash Belay for suggestions on adapting the culture of *Mg. cariaci* to growth on minimal medium, Dr. Donald Monahan for technical advice concerning the HPLC analyses, and Barbara Bichl and Keng-Tee Chew for technical assistance with *Ms. thermophila* studies. This work has been supported by National Institutes of Health Grant GM 33643 (to M.F.R.) and U.S. Department of Energy Grant DE-FG03-86ER13498 (to R.P.G.).

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