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Detection of the Osmoregulator Betaine in Methanogens

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Trimethyl glycine (glycine betaine) was detected by ^{13}C nuclear magnetic resonance spectroscopy at high intracellular concentrations in several methanogens (*Methanogenium cariaci*, "*Methanogenium anulus*" AN9, *Methanohalophilus zhilinae*, *Methanohalophilus mahii*, and *Methanococcus voltae*) grown on marine media containing yeast extract. ^{13}C labeling studies with *Methanogenium cariaci* suggested that the betaine which accumulated inside the cells was not synthesized de novo but was transported in from the medium. Proof of such a transport system was provided by growing *Methanogenium cariaci* on yeast-free medium supplemented with betaine. Under these conditions, betaine was the dominant osmoregulator.

Organisms that grow in high salt concentrations have evolved a variety of ways to maintain cell turgor. Many eubacteria concentrate small organic solutes such as polyols, amino acids, and sugars. The most halotolerant organisms tend to accumulate substituted amino acids such as glycine betaine (4, 8). Archaebacteria are known to have high K^+ levels which may act as osmoregulators in these organisms (5, 10). Recently, relatively low levels of betaine were found in several halophilic archaebacteria, including several *Natronococcus* and *Halobacterium* species (7). In these organisms the betaine is not free in the supernatant but is associated with the membrane fraction.

We now report the use of ^{13}C nuclear magnetic resonance (NMR) spectroscopy to detect betaine in the clarified lysates of several marine methanogens grown in rich media. ^{13}C NMR spectroscopy has been used in the past to detect low-molecular-weight solutes at high intracellular concentrations in intact cells and in cell extracts from marine and halophilic organisms (for example, glycerol in *Dunaliella salina* and glucosylglycerol in a *Synechococcus* sp. [2, 6]). Recently, this technique has detected β-glutamate (β-aminoglutaric acid) as a major soluble species in thermophilic methanococci (9). In the present study we showed that the betaine is not synthesized de novo in these methanogens, but must arise from uptake of betaine or a closely related precursor (e.g., choline or glycine) from the yeast extract, by incubating *Methanogenium cariaci* cells with [$^{13}C_2$]acetate.

"*Methanogenium anulus*" AN9 (OGC 50) (G. M. Maestrojuan, L. Xun, D. R. Boone, L. Zhang, and R. A. Mah, submitted for publication), *Methanogenium cariaci* JR1 (OGC 49), *Methanohalophilus zhilinae* WeN5 (OGC 62), *Methanohalophilus mahii* SLP (OGC 68), and *Methanococcus voltae* PS (OGC 70) were obtained from the Oregon Graduate Center Collection of Methanogenic Archaeobacteria. "*Methanogenium anulus*" AN9, *Methanohalophilus zhilinae*, and *Methanohalophilus mahii* were grown in MH medium, which is the same as MS medium (1) but with 87.75 g of NaCl, an additional 5 g of $MgCl_2 \cdot 6H_2O$, and 1.5 g of KCl added per liter. *Methanococcus voltae* was grown in a mixture MH and MS media (1:2). *Methanohalophilus zhilinae* was grown in MHA medium, which is the same as MH medium but with the calcium omitted; after equilibration with a 7:3 mixture of N_2 and CO_2, MHA medium was dispensed in vessels with an N_2 gas phase. The catabolic substrate was 40 mM trimethylamine for the *Methanohalophilus* strains and 100 mM formate for "*Methanogenium anulus*" and *Methanococcus voltae*. MS, MH, and MHA media contain 2 g each of Trypticase peptones and yeast extract per liter. *Methanogenium cariaci* was grown on H_2-CO_2 (80:20 [vol/vol]) in a medium (pH 7.0) consisting of 3.1 mM KH_2PO_4, 1.26 mM K_2HPO_4, 7.5 mM NH_4Cl, 4 mM KCl, 512 mM NaCl, 12.2 mM sodium acetate, 23.8 mM $NaHCO_3$, 0.33 mM $CaCl_2$, 0.003 mM resazurin, 0.001 mM sodium selenate, 0.001 mM sodium tungstate, 22.1 mM $MgCl_2$, 1.2 mM Na_2S, 1.2 mM cysteine hydrochloride, 10 ml of a vitamin mix (12) and 10 ml of a trace mineral elixir (3) per liter, and either 1 g of yeast extract (Difco Laboratories) per liter or 10 mM betaine (Sigma Chemical Co.) added.

Anaerobic cultures were harvested by centrifugation, and the pellets were either frozen and lyophilized or used directly for preparation of ethanol extracts as described previously (9). The supernatant from the incubation of the cell pellet with 70% ethanol was lyophilized and then suspended in NMR buffer consisting of 10 mM potassium phosphate, 0.1 mM EDTA, and 50% D_2O; this buffer was at pH 7.2, unless otherwise noted. ^1H-decoupled ^{13}C NMR spectra (75.4 MHz) of ethanol extracts of the different methanogens in 5-mm tubes were obtained with the following spectral parameters: 16,502-Hz sweep width, 54,080 data points, 35° pulse angle, 1.6-s acquisition time, and 4-Hz line broadening. For unenriched samples 10,000 to 14,000 transients were accumulated; 7,800 transients were acquired for samples from cells incubated with [$^{13}C_2$]acetate. Chemical shifts were measured relative to that of dioxane (67.4 ppm).

The natural-abundance ^{13}C spectrum of an ethanol extract of 0.5 g of *Methanohalophilus mahii* grown in MH medium is shown in Fig. 1A. The resonances at 54.1, 66.9, and 169.7 ppm were relatively intense, with the peak at 54.1 ppm about three times as intense as that at 66.9 ppm. The chemical shift of the peak at 54.1 ppm was consistent with that of a trimethyl ammonium group, while that at 66.9 ppm was consistent with a CH_2N group. The peak at 169.7 ppm was quite sensitive to pH and was assigned as a free carboxylate group (the carboxylate carbon is not as intense as the methylene carbon because it does not have a directly bonded proton and so will have a low nuclear Overhauser enhancement). The pattern of these three carbon resonances sug-

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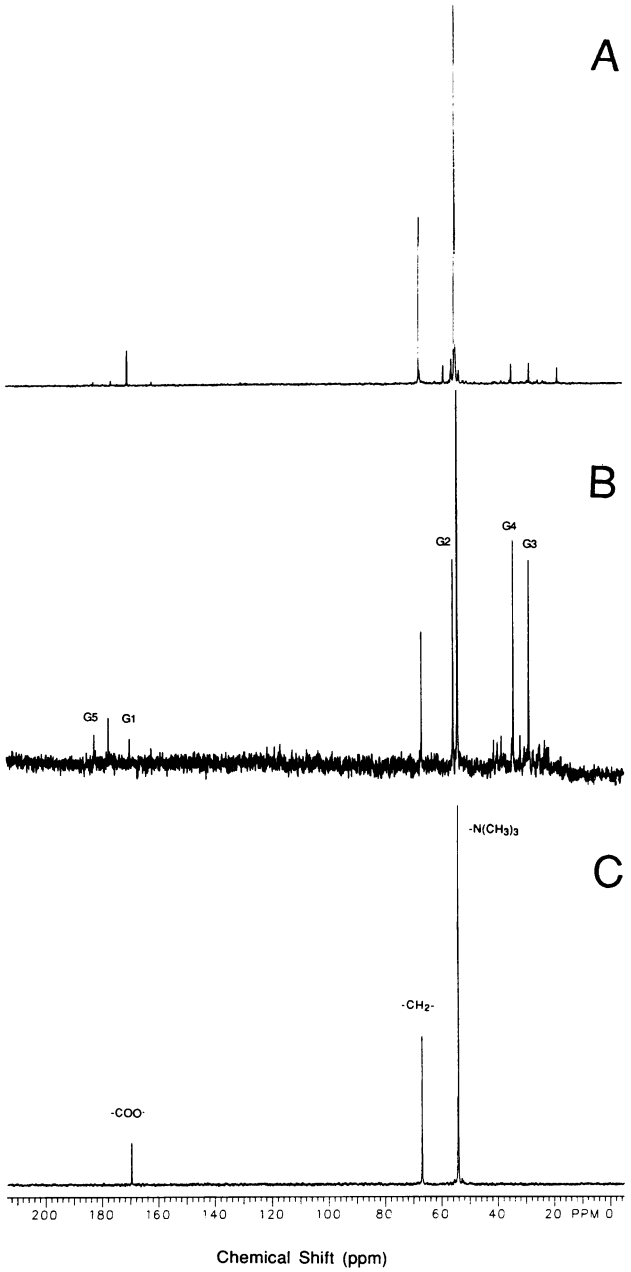

FIG. 1. Natural-abundance ^{13}C NMR spectra (75.4 MHz) of ethanol extracts of *Methanohalophilus mahii* (pH 8.2) (A), *Methanococcus voltae* (pH 9.1) (B), and authentic glycine betaine (50 mM) (C). Resonances belonging to betaine and glutamate (the number after G refers to the carbon number) are indicated.

gested that the unknown compound in these extracts was glycine betaine. This was confirmed by comparing the extract with a sample of authentic betaine (Fig. 1C). These three carbons dominated the spectrum and indicate that betaine is the major soluble molecule in *Methanohalophilus mahii*. Much lower levels of glutamate (55.4 [C-2], 27.7 [C-3], and 34.1 [C-4] ppm) and alanine (17.7 [C-3] and 57 [C-2] ppm) were also detected. Betaine also was found in cell extracts of *Methanococcus voltae* (Fig. 1B). In these cells the betaine concentration is comparable to that of glutamate (compare the intensity of the CH_2 group of betaine with those of C-3 and C-4 of glutamate). A large number of other methanogens were examined with ^{13}C NMR spectroscopy for the occurrence of this compound in the cell supernatant (Table 1). It was detected only in those organisms (including *Methanogenium* spp., *Methanohalophilus* spp., and one *Methanococcus* sp.) isolated from high-salinity environments and subsequently grown in a rich (e.g., containing yeast extract) medium. In these cells betaine levels were comparable to or greater than free glutamate levels, consistent with the idea that betaine and glutamate function as osmoregulators (which usually occur at an intracellular concentration of 0.5 to 2 M [11]). This contrasts remarkably with the levels of betaine found in other archaebacteria, in which intracellular levels were 10 to 20 mM and in which the betaine was not free but was complexed with negatively charged phospholipids, in particular phosphatidylglycerol phosphate (7).

TABLE 1. Occurrence of betaine in ethanol extracts of methanogens[a]

Methanogen	Yeast (g/liter)[b]	Occurrence of betaine
Methanobrevibacter smithii PS	1	−
Methanococcus thermolithotrophicus SN1	1	−
Methanococcus voltae PS	2	+
Methanogenium cariaci JR1	1	+
"*Methanogenium anulus*" AN9	2	+
Methanohalophilus mahii SLP	2	+
Methanohalophilus zhilinae WeN5	2	+
Methanosarcina barkeri Fusaro	1	−
Methanosphaera stadtmanae MCB-3	1	−

[a] Glutamate occurred in every extract.
[b] Amount of yeast extract added to the growth medium.

The methanogens which had betaine were grown in a medium with yeast extract. While the betaine could be the result of de novo synthesis by these cells, it (or a closely related precursor) could also be transported into the cells from components of the yeast extract. Many eubacteria have specific transport systems for betaine and related molecules. *Methanogenium cariaci* requires acetate for growth, implying that it does not have CO dehydrogenase activity (D. Robertson, N. Belay, and M. F. Roberts, unpublished results). Therefore, material synthesized by the cells must be derived from acetate as the basic C_2 unit, with the addition of CO_2 to make the C_3 unit pyruvate, etc. If the betaine is synthesized de novo by the methanogens (for instance, if it is an oxidation product from choline or derived from methylating glycine), two or more of its carbons should be labeled with ^{13}C and observed as multiplets when the cells are supplemented with [$^{13}C_2$]acetate. If the betaine is derived from the yeast extract and transported into the cell, then the betaine carbons should show no enrichment over natural-abundance background levels and should appear as singlets at significantly lower intensities than glutamate and other molecules synthesized by the cells. The natural-abundance ^{13}C spectrum of an extract from *Methanogenium cariaci* is shown in Fig. 2A. The resonances from the three carbons from betaine were quite intense. Other identified resonances in the 20- to 70-ppm region belonged to glutamate and β-glutamate (39.2 [C-2 and C-4] and 47.7 [C-3]). The extract from [$^{13}C_2$]acetate-labeled cells showed a large increase in the intensity and multiplet structure for the β-glutamate and glutamate carbons in this region compared with the natural-abundance spectrum (Fig. 2B). In contrast, the betaine carbons in this sample were all singlets and were present at

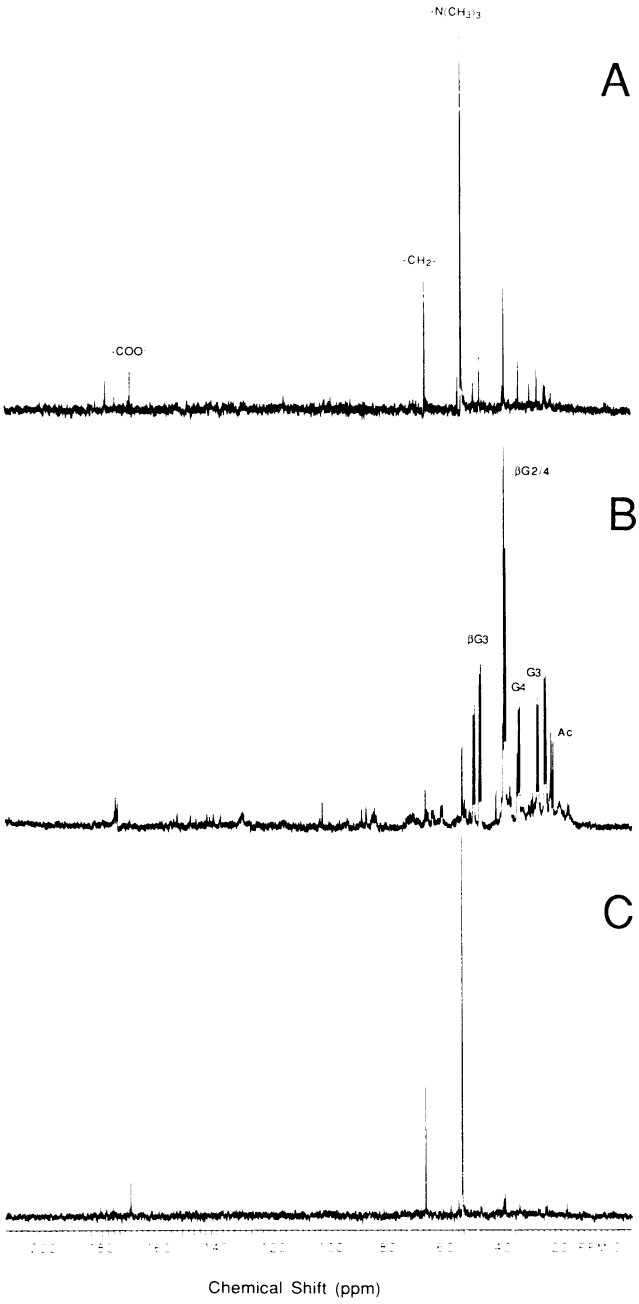

FIG. 2. ^{13}C NMR spectra (75.4 MHz) of ethanol extracts of *Methanogenium cariaci* cells grown on H_2-$^{12}CO_2$ and [^{12}C]acetate (A), grown on the same medium with the acetate replaced by 10 mM [$^{13}C_2$]acetate (B), and grown on unenriched medium with 10 mM betaine (C). Resonances corresponding to the carbons of betaine, β-glutamate (βG, with the number indicating the carbon), and glutamate (G) are labeled. Some residual [$^{13}C_2$]acetate (Ac) was also detected.

levels consistent with what was observed in the natural-abundance spectrum. This indicates that betaine is not formed from metabolism of the [$^{13}C_2$]acetate but is probably derived from the yeast extract. To prove this, the medium for growth of *Methanogenium cariaci* was supplemented with 10 mM betaine instead of the yeast extract. The ^{13}C spectrum of an ethanol extract of these cells is shown in Fig. 2C. Under these growth conditions, betaine was the dominant soluble species in the cells. In contrast, exogenous 10 mM choline and glycine added to the growth medium did not lead to the appearance of betaine in ethanol extracts of *Methanogenium cariaci*. This implies that the transport system is highly specific and is consistent with the fact that betaine is taken up from the medium and is not synthesized de novo by either oxidation of choline or methylation of glycine.

The results of this work show that several methanogens have the capacity to transport betaine or precursors of that compound into the cell when grown with high salt concentrations. When the cells are in a rich medium, betaine accumulation dominates the soluble-molecule pool, although methanogens also synthesize de novo other species (including both β-glutamate and glutamate) in response to high salt levels.This suggests that these archaebacteria have evolved several means of maintaining osmotic balance, rather than only high ion levels as suggested previously (5, 10).

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