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Composition, Variation, and Dynamics of Major Osmotic Solutes in *Methanohalophilus* Strain FDF1

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***Methanohalophilus* strain FDF1, a member of the halophilic genus of methanogens, can grow over a range of external NaCl concentrations from 1.2 to 2.9 M and utilize methanol, trimethylamine, and dimethyl sulfide as substrates for methanogenesis. It produces the osmolytes glycine betaine, β-glutamine, and N^{ε}-acetyl-β-lysine with increasing external NaCl, but the relative ratio of these zwitterions depends primarily on the methanogenic substrate and less on the external osmolarity. When the cells are grown on methanol in defined medium, accumulation of glycine betaine predominates over the other zwitterionic solutes. The cells also synthesized a carbohydrate which was not detected in cells grown on trimethylamine. This negatively charged compound, identified as α-glucosylglycerate from the ^{13}C and ^{1}H chemical shifts, does not act as an osmoregulatory solute in the salt range 1.4 to 2.7 M in this methanogen as evidenced by its invariant intracellular concentration. $^{13}CH_3OH$-pulse/$^{12}CH_3OH$-chase experiments were used to determine half-lifes for these organic solute pools in the cells. L-α-Glutamate showed a rapid loss of heavy isotope, indicating that L-α-glutamate functions as a biosynthetic intermediate in these cells. Measurable turnover rates for both β-glutamine, which acts as an osmolyte, and α-glucosylglycerate suggest that they function as metabolic intermediates as well. Molecules which function solely as osmolytes (glycine betaine and N^{ε}-acetyl-β-lysine) showed a slower turnover consistent with their roles as osmotic solutes in *Methanohalophilus* strain FDF1.**

Methanogens are strict anaerobes able to produce methane from H_2 and CO_2 and in some cases from formate, acetate, methanol, and methylamine (1, 3, 6, 7). *Methanohalophilus* species use methanol and mono-, di-, and trimethylamine as substrates for methanogenesis (10, 11, 21). They are also among the most halotolerant of the methanogenic archaebacteria, with some species able to grow in up to 4.3 M NaCl (21). Until recently, little was known about the occurrence of organic solutes in these organisms. Nuclear magnetic resonance (NMR) spectroscopy (18) has been used to identify organic solutes which can act as osmolytes in diverse methanogens. These include L-α-glutamate, β-glutamate (15, 17), glycine betaine (9, 16), N^{ε}-acetyl-β-lysine (19), and β-glutamine (9). With the exception of β-glutamate, all these compounds are synthesized and maintained at moderately high levels by *Methanohalophilus* strain FDF1. Only the zwitterionic molecules appear to serve as osmoregulatory solutes in these cells. Carbohydrates, which are frequently used to balance osmotic pressure in eubacteria and eukaryotes, were not detected at high concentrations in *Methanohalophilus* strain FDF1 grown on trimethylamine (9) or in any of the other methanogens examined by NMR (18).

In the present work, extracts of *Methanohalophilus* strain FDF1 were compared for cells grown in methanol and trimethylamine. Both carbon compounds are reduced by the cells to methane (using methyltransferases which transfer the methyl group to coenzyme M, which is subsequently reduced to methane by the methylreductase) (7). In the absence of H_2, these compounds are also oxidized to CO_2 to provide reducing equivalents for the cells (7). The difference in these two substrates is that ammonium ion is generated in utilization of trimethylamine. The present work indicates that the amounts and identities of organic solutes change with the methanogenesis substrate. These changes are discussed in terms of osmolyte characteristics and the physiological changes caused by the switch in methane substrate.

MATERIALS AND METHODS

Methanohalophilus strain FDF1 (OGM 59) was grown in a basal medium under an N_2-CO_2 atmosphere (4:1) as described before (9). The substrates (trimethylamine and methanol) and $Na_2S \cdot 9H_2O$ were sterilized individually by autoclaving and added to the medium at final concentrations of 20, 100, and 1 mM, respectively. NaCl and NH_4Cl were added to the basal medium at the indicated concentrations. The medium pH was adjusted to 7.2 before sterilization. All glassware was acid cleaned before use. Cells were grown at 37°C and harvested by centrifugation at 6,000 × *g* for 15 min. Cell pellets that contained approximately 10^{11} cells were extracted twice by heating for 5 min at 65°C in 1 ml of 70% (vol/vol) ethanol-water. Pooled extracts were centrifuged at 5,000 × *g* for 5 min, filtered through 0.2-μm-pore-size polytetrafluoroethylene membrane filters (Gelman Sciences), and lyophilized for subsequent analysis (19).

Pulse-chase experiments were performed with cells grown on basal medium (100 ml per bottle) containing 2 M NaCl with 30 mM $^{13}CH_3OH$. When the cells reached a density indicated by an optical density at 540 nm of 0.4, 150 mM unenriched CH_3OH was added to each bottle to dilute the ^{13}C-labeled substrate. Cultures were harvested at different times (0, 0.5, 1, 2, 4, and 24 h) after the addition of the excess CH_3OH, and the intracellular solutes were extracted as above and lyophilized.

^{13}C NMR spectra were acquired at 75.4 MHz on a Varian Unity 300 spectrometer. Spectral accumulation parameters included the following: 16,000 Hz sweep width, 32,000 points, 5.8-μs pulse (35-degree flip angle), 1.6 s between

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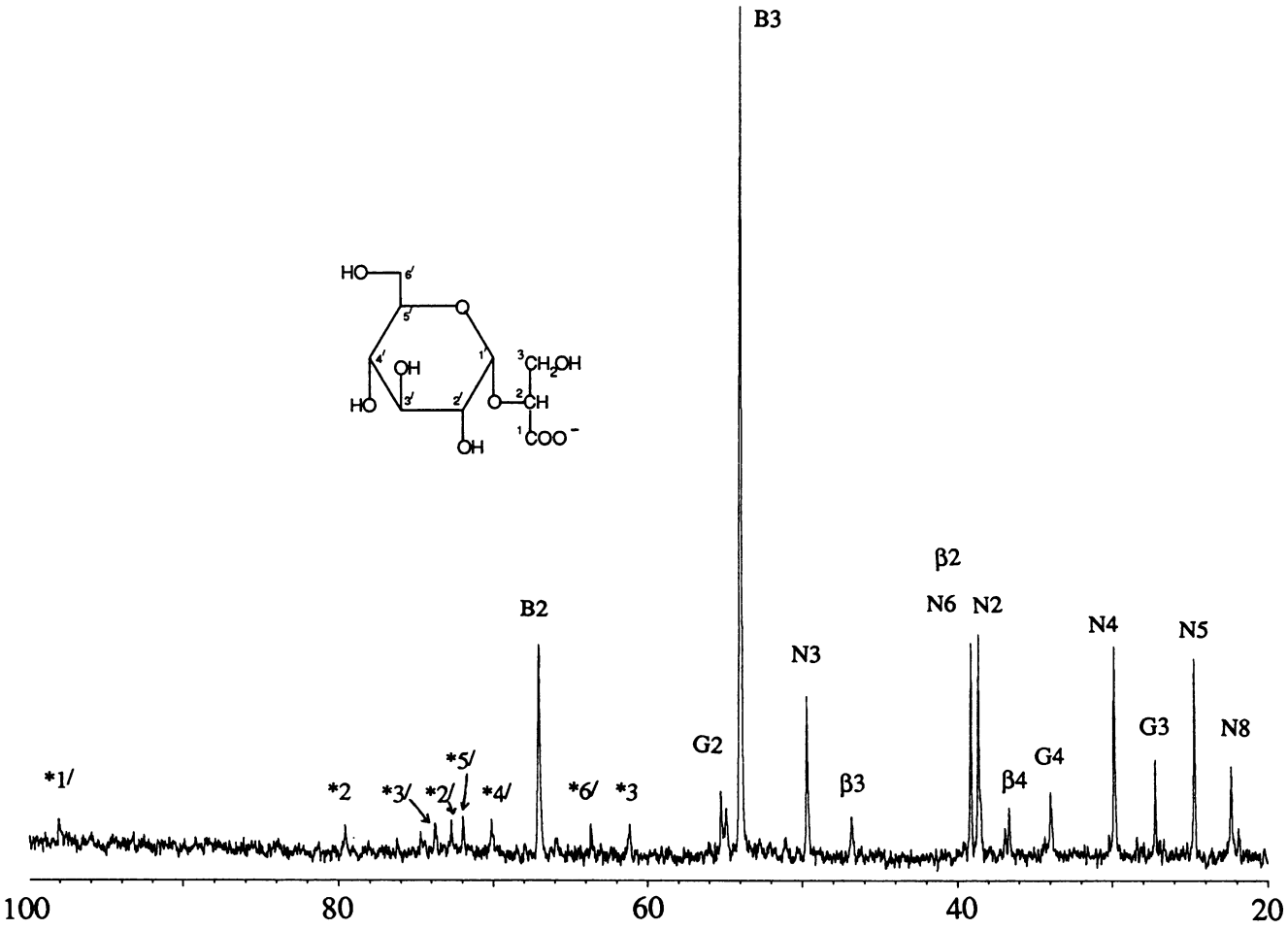

FIG. 1. ^{13}C-NMR spectrum (75.4 MHz) of an ethanol extract of *Methanohalophilus* strain FDF1 grown in 1.4 M NaCl with methanol as the substrate for methanogenesis. Numbered carbons are identified for each solute: B, (glycine) betaine; N, N^{ε}-acetyl-β-lysine; β, β-glutamine; G, L-α-glutamate. The carbon resonances of the carbohydrate which appears upon growth on methanol are labeled by an asterisk (*); those belonging to the glucose ring are identified by a prime sign adjacent to the carbon number.

pulses, and ^{1}H WALTZ-16 modulated decoupling. All free induction decays were processed with a 4-Hz line broadening before Fourier transformation. An internal standard of 0.1 ml of 150 mM sodium acetate provided 15 μmol of natural abundance (1.1%) ^{13}C signal for quantitation of amounts of solutes in unenriched extracts. This reference (and individual carbons) was normalized for nuclear Overhauser effect and spin-lattice relaxation time differences from the other CH and CH_2 carbons to be quantified. Intracellular concentrations of organic solutes were calculated from the micromoles of each solute, determined from the ^{13}C-NMR spectrum, the total number of cells used to prepare the extract (typically 2×10^{11} cells), and the cell volume (2.4×10^{-16} liter per cell) determined previously (9). Errors in determining the micromoles of each solute in the natural abundance spectra were typically 10 to 15% based on averaging the intensities of each protonated carbon in a given molecule. Absolute intracellular concentrations may have a slightly larger error, since the cell volume depends on an accurate estimate of total number of cells (based on the culture optical density). Nevertheless, ratios of components as a function of extracellular NaCl are very accurately estimated by this methodology. Resonances in samples with ^{13}C enrichment from $^{13}CH_3OH$ were integrated, and the intensities of natural abundance peaks were subtracted from this value. Integrated intensities were calculated for spectra of extracts of nearly identical numbers of cells, using an external capillary containing acetate as a standard.

RESULTS AND DISCUSSION

The ^{13}C-NMR spectra of extracts of *Methanohalophilus* strain FDF1 grown in defined medium are rich in organic solutes. Many of the compounds present in the cells when grown on methanol (Fig. 1) were the same as those observed in cells grown on trimethylamine (9). The chemical shifts

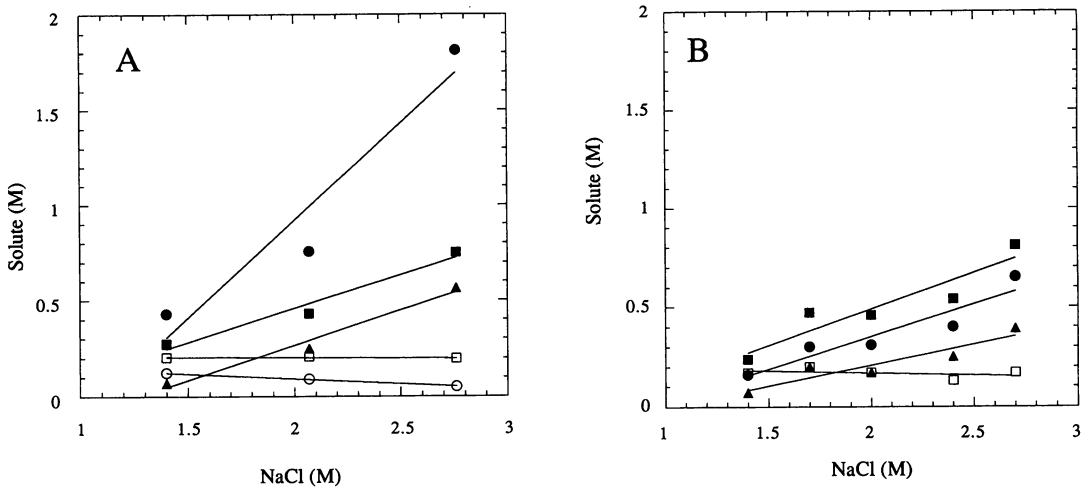

FIG. 2. Intracellular concentrations of organic osmolytes in extracts of *Methanohalophilus* strain FDF1 cells grown on methanol (A) or trimethylamine (B) as a functional of external NaCl. Symbols: ●, glycine betaine; ■, N^{ε}-acetyl-β-lysine; ▲, β-glutamine; □, L-α-glutamate; and ○, L-α-glucosylglycerate. The data in panel B are taken from reference 9.

(parts per million from dioxane at 67.4 ppm) of carbons with directly bonded protons for compounds observed in either case include glycine betaine (66.9, C-2; 54.1, C-3), N^{ε}-acetyl-β-lysine (39.2, C-2; 50.0, C-3; 30.3, C-4; 25.1, C-5; 39.5/39.6, C-6; 22.3, C-8), β-glutamine (39.0, C-2; 47.3, C-3; 37.2, C-4), and L-α-glutamate (55.4, C-2; 27.7, C-3; 34.1, C-4). A major difference when the cells are grown in methanol as opposed to trimethylamine was the appearance of eight new carbon resonances with directly bonded protons (indicated by asterisks in Fig. 1) as well as another carboxylate resonance (the carboxylate region is not included in the spectral region shown). The ^{13}C chemical shifts of this newly appearing compound were consistent with reported values for α-glucosylglycerate (8), whose structure is shown in Fig. 1. The glucosyl resonances occur at 97.8 (C-1′), 72.7 (C-2′), 73.7 (C-3′), 70.0 (C-4′), 72.1 (C-5′), and 61.1 (C-6′) ppm; the glycerate resonances occur at 178.0 (C-1), 79.5 (C-2), and 63.7 (C-3) ppm. This anionic compound is related to glucosylglycerol, a compound synthesized by cyanobacteria in response to salt (2). Confirmation of the structure as glucosylglycerate was provided by comparing ^{13}C chemical shifts of an acid-hydrolyzed extract with the chemical shifts of glucose and L-glycerate and ^{1}H COSY connectivities of the unhydrolyzed extract (data not shown). Enzymatic analysis of free glucose in normal and acid-hydrolyzed extracts by using a glucose oxidase assay also indicated that D-glucose was a component of the carbohydrate.

The amounts of the different organic solutes were measured as a function of external NaCl in cells grown with methanol as the substrate for methanogenesis. ^{13}C natural abundance NMR was used to quantify amounts of each of the osmolytes, which were then compared with data for cells grown on trimethylamine (9). The results are shown in Fig. 2. The zwitterionic species, glycine betaine, β-glutamine, and N^{ε}-acetyl-β-lysine, increased with increasing NaCl. In contrast, L-α-glutamate levels were constant over this salt range, both in cells grown in methanol (data presented here) and those grown on trimethylamine (9). Carbohydrates are often used as osmolytes and are the predominant species in slightly halotolerant cells (12, 20). In at least one case (*Ectothiorhodospira* species), a carbohydrate (trehalose) becomes a preferred organic osmolyte under nitrogen limitation conditions (5, 20). In these organisms, the glucose dimer trehalose is synthesized in response to osmotic stress, along with glycine betaine and the novel osmolyte ectoine. Both ectoine and glycine betaine contain nitrogens. When *Ectothiorhodospira* species is grown under nitrogen-limiting conditions, the trehalose concentration is increased fourfold (5, 20). Thus, there is precedence for a compound such as glucosylglycerate acting as an osmolyte in *Methanohalophilus* strain FDF1 grown on methanol. As shown in Fig. 2A, in the NaCl concentration range examined (1.4 to 2.7 M), glucosylglycerate was not functioning as an osmoregulatory solute. In fact, its intracellular concentration decreased slightly at the highest NaCl level. Both anionic solutes in *Methanohalophilus* strain FDF1, glucosylglycerate and glutamate, occurred at comparable concentrations and remained constant or decreased as a function of external NaCl. In *Methanohalophilus* strain FDF1 grown on trimethylamine, glutamate was also constant over a wide (1.4 to 2.7 M) NaCl range (9), while intracellular K^{+} (which was three to four times the glutamate) increased roughly twofold over the same range (1.7 to 2.7 M NaCl). The synthesis and accumulation of glucosylglycerate by the cells is not sufficient to offset cation increases with external NaCl. Clearly,

TABLE 1. Solute distribution in *Methanohalophilus* strain FDF1 grown in 1.4 M NaCl on defined medium with methanol as the substrate and various levels of NH_4Cl

Solute	Fraction of major organic solute detected by ^{13}C-NMR[a]		
	4 mM NH_4C	20 mM NH_4Cl	40 mM NH_4Cl
Glycine betaine	0.44	0.40	0.37
N^{ε}-Acetyl-β-lysine	0.29	0.30	0.33
β-Glutamine	0.04	0.05	0.07
L-α-Glutamate	0.10	0.14	0.10
α-Glucosylglycerate	0.13	0.11	0.13

[a] Major organic solutes detected by ^{13}C-NMR spectroscopy include glycine betaine, N^{ε}-acetyl-β-lysine, β-glutamine, L-α-glutamate, and glucosylglycerate. Very minor amounts of other species represent <0.01 of this total fraction and were not included in determining the fractional distribution of each of these species.

there must be inorganic anions which neutralize the cation charge inside the cells. The accumulation of glucosylglycerate in cells grown on methanol must be in response to some physiological response other than increased osmotic pressure.

At each NaCl concentration tested, the total amounts of osmolytes were slightly higher for cells grown on methanol than for the cells grown on trimethylamine. The combined level of organic osmolytes in cells grown on methanol was typically 40 to 60% of the extracellular NaCl osmolarity. N^{ε}-Acetyl-β-lysine was the dominant osmolyte at all NaCl concentrations for trimethylamine-grown cells, while glycine betaine was the overwhelming choice of the cells grown with methanol. In 2 M NaCl, the ratio of betaine to N^{ε}-acetyl-β-lysine was ~2:1 with methanol and about 1:1 with trimethylamine as the substrate for methanogenesis.

A major difference in cell growth on trimethylamine and methanol is the release of free NH_4^+ in situ when the amine is the substrate for methanogenesis. Glycine betaine contains a single nitrogen, and β-glutamine and N^{ε}-acetyl-β-lysine each contain two nitrogens. To compare nitrogen effects on observable organic solute concentrations in the cell, we prepared extracts from cells grown on high and low NH_4Cl. The fractional organic solute composition in extracts of cells grown on methanol in 8% NaCl with 4 to 40 mM NH_4^+ is shown in Table 1. The fraction of individual compounds changed with external NH_4^+ in a way that was consistent with alterations in nitrogen availability. Both N^{ε}-acetyl-β-lysine and β-glutamine increased relative to glycine betaine at the highest NH_4Cl level. In cells grown in 4 mM NH_4Cl, the ratio of betaine to N^{ε}-acetyl-β-lysine was 1.5; this decreased to 1.3 and 1.1 in the 20 and 40 mM NH_4Cl cultures, respectively. The ratio of glycine betaine to β-glutamine also decreased with increasing external NH_4Cl, going from 11 to 5.3, respectively, for 4 and 40 mM NH_4Cl cultures. Thus, the decrease in glycine betaine relative to N^{ε}-acetyl-β-lysine and β-glutamine when cells are grown in trimethylamine compared with growth on methanol is in the same direction as the spectral changes seen in cells grown in methanol, with higher external NH_4Cl.

At 1.4 M NaCl, the changes in osmolyte ratios with methanogenic substrate were more dramatic than the changes induced by varying the external NH_4Cl concentration. If the intracellular NH_4^+ concentration is low (regardless of the 20 mM level in the normal medium), utilization of trimethylamine for methane production would generate relatively high NH_4^+ levels inside the cell. Metabolism of methanol would not generate this NH_4^+ in the cells. Thus, with a lower intracellular NH_4^+, the cells would be biased toward accumulating zwitterions with fewer nitrogens. There was no significant change in the level of the carbohydrate or glutamate with the different external NH_4^+ levels tested. This suggests that the synthesis of glucosylglycerate is not a direct response to altered intracellular ammonium levels, an effect noted with carbohydrate osmolytes in other bacterial cells (5, 20). It must be emphasized that synthesis of osmotic solutes by nonphotosynthetic microorganisms requires a substantial input of energy, of which there is no unlimited supply. The limiting nitrogen level for FDF1 methanol-grown cultures has not been determined. It is quite possible that the NH_4^+ level tested was above this limit and that under these growth conditions, nitrogen availability is of minor importance. Thus, the α-glucosylglycerate might still vary with external NaCl, but only under much lower nitrogen levels.

Another possible explanation for the increased levels of glycine betaine in cells grown in methanol is a difference in methyltransferase activity. Other ^{13}C labeling evidence (14) has indicated that glycine is the precursor of glycine betaine in *Methanohalophilus* strain FDF1. The methyl donor for converting this to glycine betaine is not known at present. If *S*-adenosylmethionine is used as the direct methyl donor, differences in producing this chemical species ultimately from the methyl groups of methanol or trimethylamine could bias cells grown on methanol to increase the relative amount of this material. A more detailed investigation will require isolating the appropriate biosynthetic enzymes and examining glycine betaine production in vitro.

Measuring the turnover of molecules in cells can be used to provide information on whether or not they have multiple functions. For example, osmolytes have been shown to exhibit slow turnover rates, while molecules which are also involved in biosynthesis are rapidly metabolized by cells (13, 18). The half-life of organic solutes was monitored in *Methanohalophilus* strain FDF1 with a $^{13}CH_3OH$-pulse/$^{12}CH_3OH$-chase experiment. Incubation of cells with

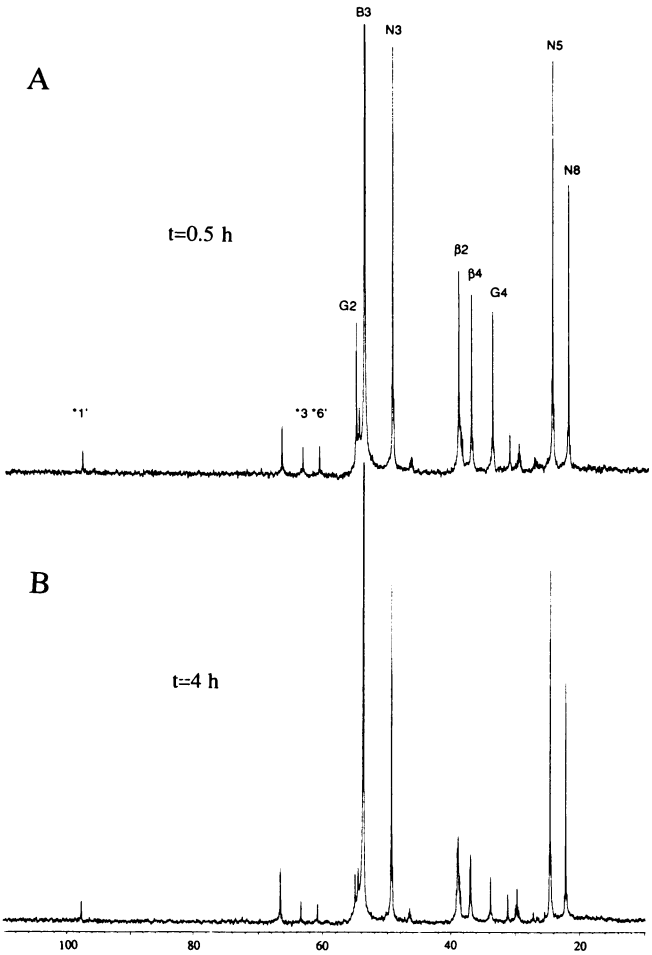

FIG. 3. ^{13}C-NMR spectra (75.4 MHz) of ethanol extracts of *Methanohalophilus* strain FDF1 grown in defined medium with 2 M NaCl on 30 mM $^{13}CH_3OH$ until the optical density at 540 nm equalled 0.4 and then mixed with 150 mM $^{12}CH_3OH$ to dilute the ^{13}C label and harvested at 0.5 h (A) and 4 h (B) after the introduction of the $^{12}CH_3OH$. The identities of different carbons in the spectrum are labeled as in Fig. 1, with the carbon number in the carbon indicated. The external capillaries have been removed in these spectra.

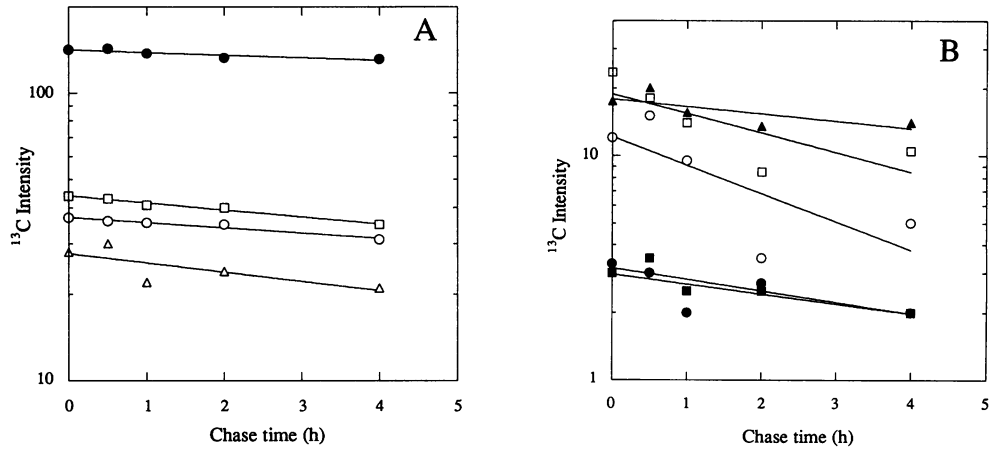

FIG. 4. Intensities of ^{13}C-enriched (from $^{13}CH_3OH$) carbons in extracts of *Methanohalophilus* strain FDF1 as a function of incubation time with $^{12}CH_3OH$. (A) Glycine betaine $N(CH_3)_3$ (●); C-3 (□), C-5 (○), and C-8 (△) of N^{ε}-acetyl-β-lysine; (B) C-4 of β-glutamine (▲); C-2 (□) and C-4 (○) of α-glutamate; glucose C-6 (●) and glycerate C-3 (■) of L-α-glucosylglycerate.

$^{13}CH_3OH$ selectively labels carbons which are generated from the methyl group of acetate (14). The $^{12}CO_2$ in the headspace of the bottles was used in conjunction with the $^{13}CH_3$ unit provided from the enriched methanol to synthesize [2-^{13}C]acetyl units, which were subsequently fixed into cell carbon. The $^{13}CH_3COX$ labeled C-2 and C-4 in glutamate and β-glutamine, the $N(CH_3)_3$) of glycine betaine, C-3, C-5, and C-8 of N^{ε}-acetyl-β-lysine, and C-1′, C-6′, and C-3 of α-glucosylglycerate. A detailed discussion of this labeling pattern of the osmolytes has been presented elsewhere (14). The α-glucosylglycerate labeling pattern was consistent with a standard gluconeogenic pathway for carbohydrate biosynthesis, in which the glucose moiety is formed from the condensation of three carbon units, derived from [3-^{13}C] pyruvate. Thus, glucose C-1′ and C-6′ and the glycerate C-3 were enriched in ^{13}C (similar labeling of carbohydrates has been documented in *Methanospirillum hungatei* [4]). In all these organic solutes, unlabeled carbons appeared as multiplets if they were adjacent to an incorporated ^{13}C.

TABLE 2. Turnover rates and half-life values for ^{13}C-labeled solutes in *Methanohalophilus* strain FDF1 determined in a $^{13}CH_3OH$-pulse/$^{12}CH_3OH$-chase experiment

Solute	Carbon	Pool turnover rate[a] (h^{-1})	$t_{1/2}$[b] (h)
Glycine betaine	C-3	0.022	32
N^{ε}-Acetyl-β-lysine	C-3	0.056	12
	C-5	0.042	16
	C-8	0.074	9.3
β-Glutamine[c]	C-4	0.076	9.1
L-α-Glutamate	C-2	0.200	3.5
	C-4	0.291	2.8
Glucosylglycerate	C-1′	0.105	6.6
	C-6′	0.101	6.8
	C-3	0.117	5.9

[a] The total pool turnover rate for each labeled carbon is generated from an exponential fit of the ^{13}C intensity as a function of time after the dilution of $^{13}CH_3OH$ with $^{12}CH_3OH$.

[b] The half-life represents the time after the $^{12}CH_3OH$ dilution for the ^{13}C-specific labeling introduced by metabolism of $^{13}CH_3OH$ to decrease by 50%.

[c] The β-glutamine C-2 overlaps with the N^{ε}-acetyl-β-lysine C-2 and C-6 and after intensity losses has significant error because of the overlap; hence, it was not used to measure a β-glutamine turnover rate.

Methanohalophilus strain FDF1 cells were grown on 30 mM $^{13}CH_3OH$ to an optical density at 540 nm of 0.4 and then diluted with 150 mM $^{12}CH_3OH$. When $^{12}CH_3OH$ was added to the medium to dilute the ^{13}C-labeled substrate, spectral changes became evident as a function of time. Figure 3 shows spectra for cells initially ^{13}C labeled and then incubated for 0.5 and 4 h with $^{12}CH_3OH$. By 4 h, glutamate C-2 and C-4, β-glutamine C-2 and C-4, and glucosylglycerate C-1′, C-6′, and C-3 enriched carbons have clearly decreased relative to ^{13}C-labeled glycine betaine [$N(CH_3)_3$] and N^{ε}-acetyl-β-lysine (C-3, C-5, and C-8) carbons. The integrated intensities for each of these labeled carbons as a function of time after the addition of $^{12}CH_3OH$ (calculated by subtracting out the natural abundance label estimated from the intensity of the other carbons in each molecule which are not ^{13}C labeled by the methanol carbon) are shown in Fig. 4. The log of the residual ^{13}C intensity in each solute decreased linearly with $^{12}CH_3OH$ incubation time. This first-order turnover rate was slowest for glycine betaine and N^{ε}-acetyl-β-lysine and fastest for glutamate. β-Glutamine and glucosylglycerate pool turnover rates lay in between these values, suggesting that both of these species can be metabolized by *Methanohalophilus* strain FDF1. From the turnover data, the half-life for loss of 50% ^{13}C label ($t_{1/2}$) in each solute pool could be estimated. The first-order turnover rates and half-life values, summarized in Table 2, show that there are three classes of solute pools: (i) L-α-glutamate, which as an important biosynthetic intermediate is rapidly utilized by the cells; (ii) glucosylglycerate and β-glutamine, which have intermediate turnover times; and (iii) N^{ε}-acetyl-β-lysine and glycine betaine, whose pools exhibit little turnover during the chase phase. Clearly, β-glutamine is not only an osmolyte; it also functions as an intermediate in cell metabolism. The relatively rapid turnover of α-glucosylglycerate suggests that whatever else it may do, it acts as a biosynthetic intermediate in these cells. Differences in label turnover within a given molecular pool are also observed. In N^{ε}-acetyl-β-lysine, the β-lysine C-3 and C-5 have slower turnover rates than the acetyl methyl carbon (C-8). This implies that the side-chain acetyl group can be removed and the β-lysine moiety reacetylated with a nonenriched acetyl group.

In summary, the substrate used for methanogenesis has a pronounced effect on the ratio of organic solutes accumulated in *Methanohalophilus* strain FDF1. The effect is consistent with metabolism of trimethylamine generating a locally high intracellular concentration of NH_4^+ which is then used preferentially to synthesize solutes with higher nitrogen content. Studies of the turnover of these solute pools showed that glycine betaine and N^{ε}-acetyl-β-lysine behave as compatible solute osmolytes because they showed little short-term turnover in the pulse-chase experiment. β-Glutamine, while it functions as an osmolyte, can also be metabolized by the cells.

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REFERENCES

1. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43:**260–296.
2. **Borowitzka, L. J., S. Demmerle, M. A. Mackay, and R. S. Norton.** 1980. Carbon-13 nuclear magnetic resonance study of osmoregulation in a blue-green alga. Science **210:**650–651.
3. **Daniels, L., R. Sparling, and G. D. Sprott.** 1984. Assimilatory reduction of sulfate and sulfite by methanogenic bacteria. Biochim. Biophys. Acta **768:**113–163.
4. **Ekiel, I., I. C. P. Smith, and G. D. Sprott.** 1983. Biosynthetic pathways in *Methanospirillum hungatei* as determined by ^{13}C nuclear magnetic resonance. J. Bacteriol. **156:**316–326.
5. **Galinski, E. A., and R. Herzog.** 1990. The role of trehalose as a substitute for nitrogen-containing compatible solutes (*Ectothiorhodospira halochloris*). Arch. Microbiol. **153:**607–613.
6. **Jones, W. J., D. P. Nagle, and W. B. Whitman.** 1987. Methanogens and the diversity of archaebacteria. Microbiol. Rev. **51:** 135–177.
7. **Keltjens, J. T., and C. van der Drift.** 1986. Electron transfer reactions in methanogens. FEMS Microbiol. Rev. **39:**259–303.
8. **Kollman, V. H., J. L. Hanner, R. E. London, E. G. Adame, and T. E. Walker.** 1979. Photosynthetic preparation and characterization of ^{13}C-labeled carbohydrates in *Agmenellum quadruplicatum*. Carbohydr. Res. **73:**193–202.
9. **Lai, M. C., K. R. Sowers, D. E. Robertson, M. F. Roberts, and R. P. Gunsalus.** 1991. Distribution of compatible solutes in the halophilic methanogenic archaebacteria. J. Bacteriol. **173:**5352–5358.
10. **Mathrani, I. M., and D. R. Boone.** 1985. Isolation and characterization of a moderately halophilic methanogen from a solar saltern. Appl. Environ. Microbiol. **50:**140–143.
11. **Mathrani, I. M., D. R. Boone, and R. A. Mah.** 1988. Isolation and characterization of four halophilic methanogens. Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, I 74.
12. **Reed, R. H., L. J. Borowitzka, M. A. Mackay, J. A. Chudeck, R. Foster, S. R. C. Warr, D. J. Moore, and W. D. P. Stewart.** 1986. Organic solute accumulation in osmotically stressed cyanobacteria. FEMS Microbiol. Rev. **39:**51–56.
13. **Roberts, M. F., B. S. Choi, D. E. Robertson, and S. Lesage.** 1990. Free amino acid turnover in methanogens measured by ^{15}N NMR spectroscopy. J. Biol. Chem. **265:**18207–18212.
14. **Roberts, M. F., M. C. Lai, and R. P. Gunsalus.** Submitted for publication.
15. **Robertson, D. E., S. Lesage, and M. F. Roberts.** 1989. β-Aminoglutaric acid is a major soluble component of *Methanococcus thermolithotrophicus*. Biochim. Biophys. Acta **992:**320–326.
16. **Robertson, D., D. Noll, M. F. Roberts, J. Menaia, and D. R. Boone.** 1990. Detection of the osmoregulator betaine in methanogens. Appl. Environ. Microbiol. **56:**563–565.
17. **Robertson, D., M. F. Roberts, N. Belay, K. O. Stetter, and D. R. Boone.** 1990. Occurrence of β-glutamate, a novel osmolyte, in marine methanogenic bacteria. Appl. Environ. Microbiol. **56:** 1504–1508.
18. **Robertson, D. E., and M. F. Roberts.** 1991. Organic osmolytes in methanogenic archaebacteria. Biofactors **3:**1–9.
19. **Sowers, K., D. E. Robertson, D. Noll, R. P. Gunsalus, and M. F. Roberts.** 1990. N^{ε}-Acetyl-β-lysine: an osmolyte synthesized by methanogenic archaebacteria. Proc. Natl. Acad. Sci. USA **87:**9083–9087.
20. **Truper, H. G., and E. A. Galinski.** 1990. Biosynthesis and fate of compatible solutes in extremely halophilic phototropic eubacteria. FEMS Microbiol. Rev. **75:**247–254.
21. **Zhilina, T. N.** 1986. Methanogenic bacteria from hypersaline environments. Syst. Appl. Microbiol. **7:**216–222.