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Effects of Osmolyte Precursors on the Distribution of Compatible Solutes in *Methanohalophilus portucalensis*

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The halophilic methanogen *Methanohalophilus portucalensis* synthesizes three distinct zwitterions, β-glutamine, N^{ε}-acetyl-β-lysine (NAβLys), and glycine betaine, as osmolytes when it is grown at high concentrations of external NaCl. The selective distribution of these three species was determined by growing cells in the presence of osmolyte biosynthetic precursors. Glycine betaine is formed by the stepwise methylation of glycine. Exogenous glycine (10 mM) and sarcosine (10 mM), although internalized, do not bias the cells to accumulate any more betaine. However, exogenous *N,N*-dimethylglycine (10 mM) is available to the appropriate methyltransferase and the betaine generated from it suppresses the synthesis of other osmolytes. Precursors of the two zwitterionic β-amino acids (β-glutamate for β-glutamine and α-lysine and diaminopimelate for NAβLys) have only small effects on β-amino acid accumulation. The largest effect is provided by L-α-glutamine, suggesting that nitrogen assimilation is a key factor in osmolyte distribution.

Methanohalophilus portucalensis (formerly known as *Methanohalophilus* strain FDF1) is among the most halophilic of the methanogens, growing in NaCl concentrations of 1.2 to 4.4 M (6). It utilizes hydrogen and carbon dioxide, methanol, acetate, methylamine, and trimethylamine as substrates for methanogenesis and growth. Like all methanogens examined to date, *M. portucalensis* synthesizes a number of low-molecular-weight organic compounds as compatible solutes in response to extracellular-NaCl concentrations. The accumulation of these compounds maintains equivalent levels of osmotic pressure in intracellular and extracellular environments (6, 9). *M. portucalensis* synthesizes and accumulates the zwitterions β-glutamine (6), N^{ε}-acetyl-β-lysine (NAβLys) (12), and glycine betaine (6) as the major intracellular organic solutes along with smaller amounts of the organic anions L-α-glutamate and glucosylglycerate (6, 9). The production of β-glutamine at high concentrations of NaCl as a compatible solute is thus far unique to this organism.

A major question is why the organism synthesizes and accumulates three different zwitterions to balance external osmotic pressure. Clues to understanding the mechanisms for specific osmolyte regulation can be provided by identifying the physiological conditions that affect the balance of osmolytes. We used one-dimensional ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy, as well as two-dimensional NMR techniques, to determine the distribution of compatible solutes, to monitor ^{13}C uptake to confirm biosynthetic pathways, and to identify new solutes that accumulate under various growth conditions with specific osmolyte precursors.

MATERIALS AND METHODS

Chemicals. [^{13}C]methanol and [$^{13}C_2$]glycine (99% ^{13}C) were obtained from Cambridge Isotopes. All exogenous compounds used in cell culture, with the exception of choline (obtained from Matheson, Coleman, and Bell Manufacturing), were obtained from Sigma. The *o*-phthalaldehyde used for high-performance liquid chromatography (HPLC) analysis was obtained from Pierce. D_2O (99.9%) used in NMR analyses was obtained from Sigma.

Cell growth and sample preparation. *M. portucalensis* was grown in basal media containing the following: NaCl as required; $MgCl_2 \cdot 6H_2O$, 3 g/liter; KCl, 2 g/liter; $CaCl_2 \cdot 2H_2O$, 0.1 g/liter; K_2HPO_4, 4 g/liter; NH_4Cl, 1.5 g/liter; cysteine · HCl, 5 g/liter; $NaHCO_3$, 4 g/liter; WWW vitamin elixir (6), 10 ml/liter; trace element solution (6), 10 ml/liter; and resazurine, 2 ml (prepared under an H_2-CO_2 [4:1] atmosphere)/liter. The medium was adjusted to a pH of 7.2 prior to sterilization by autoclaving. Methanol and $Na_2S \cdot 9H_2O$ were autoclaved individually and added to the medium to final concentrations of 100 and 1 mM, respectively. ^{13}C-labeled methanol was added to a final concentration of 50 mM with the subsequent addition of 50 mM unlabeled substrate. Any exogenous compounds were added prior to sterilization to a final concentration of 10 mM. Cells were incubated at 37°C with shaking, and growth was monitored by measuring the optical density at 660 nm. Cells were harvested by centrifugation and extracted with ethanol as previously documented (5, 6).

NMR analysis. ^{1}H NMR spectra (500 MHz) were acquired on a Varian Unity 500 spectrometer with a 5-mm broadband probe. Samples were dissolved in 0.4 to 0.5 ml of D_2O. Spectral acquisition parameters included a 4-kHz sweep width, 19,200 datum points, a 1.0-s recycle delay, and up to 256 transients. Free-induction decays were processed with 0.5-Hz line broadening. Proton resonances were integrated and quantified with an internal standard of 12 μmol of imidazole. ^{1}H WALTZ-decoupled ^{13}C natural-abundance NMR spectra (125.7 MHz) of the same samples were acquired on the Varian Unity 500 spectrometer with a 5-mm broadband probe. Acquisition parameters included a 25-kHz sweep width, 32,768 datum points, a 36.6° pulse angle, a 1.0-s recycle delay, and typically 10,000 transients for natural-abundance spectra. Generally, 5,000 transients were collected for samples with ^{13}C labeling. Free-induction decays were processed with 4-Hz line broadening. Resonances for carbons with directly bonded protons were integrated and compared to a 6-μmol dioxane or a 12-μmol imidazole standard added to the extract to determine the amount of each solute. Two-dimensional {^{1}H}^{13}C-heteronuclear multiple quantum coherence (HMQC) experiments were also used to confirm the identities of several ^{13}C resonances. The two-dimensional {^{1}H}^{13}C-HMQC datum acquisition and processing parameters included 128 transients and 128 increments, 448 datum points, and a 0.050-s delay time.

HPLC analysis. Reverse-phase HPLC was used to confirm the identities and concentrations of certain osmolytes, most notably β-glutamine concentrations for total osmolyte calculations. The ^{1}H resonances of β-glutamine are particularly difficult to integrate; therefore, HPLC was utilized in addition to NMR. Ethanol-extracted samples were derivatized with equal volumes of *o*-phthalaldehyde to generate fluorescent amino acid adducts as described by Gardner and Miller (2) and analyzed on a C_{18} Microsorb reverse-phase column with dual Rainin pumps and elution at 1 ml/min. Chromatograms were analyzed with Dynamax software. The mobile phase used was a 100-min linear gradient starting with a sodium acetate buffer and ending with methanol. Observed amino acids (with elution times indicated) included glutamic acid (14 min), β-glutamic acid (18 min), β-glutamine (28 min), and NAβLys (42 min). Isoleucine (62 min) was used as an internal standard to quantify solutes.

RESULTS AND DISCUSSION

Effects of precursors on betaine accumulation. In an attempt to determine the conditions and mechanisms which gov-

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TABLE 1. ^{13}C and ^{1}H chemical shifts of solutes observed in *M. portucalensis*

Compound	^{13}C chemical shifts, ppm (^{1}H chemical shifts, ppm)							
	C-1	C-2 (H-2)	C-3 (H-3)	C-4 (H-4)	C-5 (H-5)	C-6 (H-6)	C-7	C-8 (H-8)
Betaine	169.7	66.9 (3.90)	54.1 (3.23)					
NAβLys	178.8	39.2 (2.45)	50.0 (3.47)	30.3 (1.57)	25.1 (1.66)	39.5 (3.17)	174.8	22.3 (1.9)
β-Glutamine	178.9	39.0 (2.55)	47.3 (3.75)	37.2 (2.70)	175.3			
β-Glutamate	178.5	39.2 (2.54)	47.7 (3.75)	39.2 (2.54)	178.5			
α-Glutamate	175.6	55.4 (3.70)	27.7 (2.10)	34.1 (2.35)	182.3			
α-Glutamine		31.5 (2.15)	27.0 (3.78)	54.8 (2.45)				
Glucosylglycerate	99.7 (5.0)	72.7 (3.5)	73.8 (3.8)	70.0 (3.4)	71.9 (3.75)	62.0 (3.65)	178.1	80.5 (4.15)
Inositol	72.4 (3.53)	73.5 (4.05)	72.4 (3.53)	73.7 (3.59)	75.7 (3.27)	73.7 (3.59)		

ern the selection and synthesis of compatible solutes in *M. portucalensis*, cells were grown in the presence of a variety of exogenous compounds selected on the basis of their relationship as either intermediates or precursors of proposed osmolyte biosynthetic pathways. If internalized, each compound could affect the production of one of the three primary compatible solutes. Each culture was grown to an A_{660} of ~1.0, yielding cells that are approaching the end of the exponential growth phase and therefore providing the greatest cell mass before entering stationary phase. Cells were centrifuged, and the washed pellet was extracted with ethanol. The osmolyte concentrations were determined by ^{13}C natural-abundance NMR spectroscopy. Only the resonances corresponding to carbon atoms with directly attached protons were considered in calculating relative concentrations. Errors in determining the osmolyte concentrations (indicated on bar graphs) were calculated as the standard deviations in micromoles of solute from each natural-abundance ^{13}C resonance for the same solute. The ^{13}C and ^{1}H shifts of organic osmolytes observed, both those synthesized de novo and those internalized from the medium, are summarized in Table 1.

Any physiological effects of the added precursors (10 mM) on *M. portucalensis* growth were assessed by monitoring the cell doubling time of the organism in medium with 12% NaCl. Optical density measurements (at 660 nm) were taken periodically throughout cell growth. Control cultures exhibited a lag of typically 80 to 100 h. As shown in Table 2, most of the exogenous solutes had no effect on this lag, the doubling time, or the final cell yield (most cultures reached stationary phase with an A_{660} of ~1). If these solutes were internalized and used for osmolyte production, such results would suggest that synthesis and accumulation of osmolytes is not growth limiting for the extreme halophile *M. portucalensis*.

Glycine betaine is the dominant osmolyte in *M. portucalensis* grown with methanol or trimethylamine as a substrate (6, 9). It is also preferentially accumulated under conditions of low levels of nitrogen (9). Betaine synthesis in these cells has been proposed to occur by methylation of glycine via methyltransferase activity; the glycine in these cells is produced from serine (8). To generate betaine, the glycine is methylated, presumably stepwise, to form sarcosine, *N*,*N*-dimethylglycine (DMG), and finally betaine. In an effort to enhance betaine accumulation by *M. portucalensis*, cells were grown at a fixed concentration of NaCl (12%), with each of these intermediates added to the medium to a final concentration of 10 mM. Exogenous glycine betaine and proposed precursors had no effect on cell growth (Table 2); however, they might still have affected the intracellular distribution of osmolytes by enhancing betaine accumulation. The effect of each additive on zwitterionic osmolyte production in this organism is illustrated in Fig. 1, where the mole fractions of the three zwitterions are compared to amounts in cells grown under normal conditions with methanol as the substrate for methanogenesis. Betaine accumulation was greatly increased in the presence of DMG and betaine itself. These cells internalized and/or synthesized enough betaine to account for over 90% of the internal organic osmolyte pool. In the case of betaine, internalization was clearly the preferred method of osmolyte accumulation. *M. portucalensis* grown on CH_3OH in the presence of 10 mM betaine had an osmolyte pool overwhelmingly dominated by betaine as quantified by natural-abundance ^{13}C NMR spectroscopy (Fig. 1). However, ^{13}C spectra of an extract of a culture grown on $^{13}CH_3OH$ supplemented with unlabeled betaine showed a distribution of ^{13}C-labeled osmolytes similar to that of cells grown in $^{13}CH_3OH$ without exogenous material (Fig. 2A). The ^{13}C-labeled betaine was present at a concen-

TABLE 2. Effects of exogenous solutes (10 mM) on *M. portucalensis* growth

Additive	Lag time (h)	Doubling time (h)
Control	80–100	24
Betaine	100–110	23
DMG	100–120	35
Sarcosine	90–100	33
Glycine	100–110	31
Choline	100–110	25
Inositol	20–30	44
α-Glutamate	100–110	25
β-Glutamate	100–110	27
α-Glutamine	0	60
Diaminopimelate	120–130	18
Lysine	150–160	22

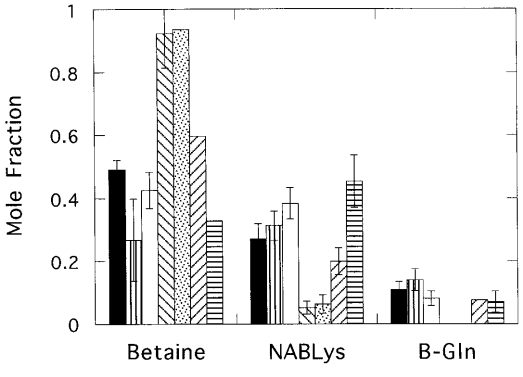

FIG. 1. Effects of betaine precursors (10 mM added to the medium) on the distribution of intracellular solutes in *M. portucalensis*. ■, control; ▥, glycine; □, sarcosine; ▧, DMG; ▨, betaine; ▨, choline; ▤, inositol.

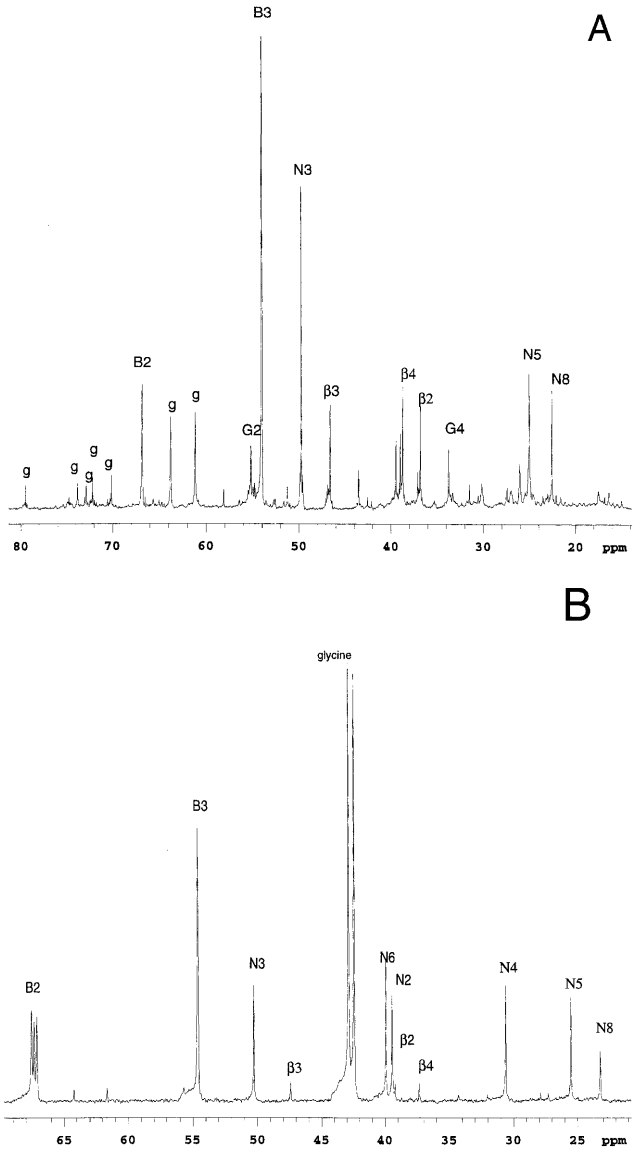

FIG. 2. (A) ^{1}H-decoupled ^{13}C NMR (125.7 MHz) spectrum of an ethanol extract of *M. portucalensis* grown on $^{13}CH_3OH$ in the presence of 10 mM betaine. Under these conditions, only labeled carbons are observed. Resonances are identified for each osmolyte (B, betaine; N, NAβLys; β, β-glutamine; g, glucosylglycerate carbon; G, α-glutamate with the carbon number. (B) ^{1}H-decoupled ^{13}C NMR (125.7 MHz) spectrum of an ethanol extract of *M. portucalensis* grown on methanol in the presence of $^{13}C_2$-glycine (10 mM). The doublet at 42.8 ppm is from the glycine C-2; the doublet surrounding the natural-abundance singlet of the betaine C-2 at 67.8 ppm indicates ^{13}C-label incorporation at that carbon.

tration slightly higher than that of NAβLys. This indicates that increased net betaine accumulation was due to cell internalization and not betaine synthesis.

Accumulation of intracellular betaine from DMG or external betaine suppressed production of glucosylglycerate and β-glutamine (those solutes were undetectable by ^{13}C NMR spectroscopy). However, sarcosine or glycine addition to the medium had no significant effect on the distribution of zwitterionic osmolytes in *M. portucalensis*. One possible explanation is that neither glycine nor sarcosine was internalized by the cells. This possibility was examined by adding 10 mM [$^{13}C_2$]glycine to the medium. If any of the external doubly labeled glycine was imported (detected by a glycine carbon doublet at 42 ppm) and converted to betaine, there would be a ^{13}C-^{13}C doublet flanking the natural-abundance singlet for the betaine C-2 at 67 ppm. As shown in Fig. 2B, some glycine was, in fact, internalized and converted to betaine, since the ^{13}C doublet for C-2 of doubly labeled glycine betaine was clearly seen in the spectrum of an ethanol extract from cells grown in the presence of this exogenous compound. Normally, there is no NMR-detectable glycine in ethanol extracts of *M. portucalensis*. However, the doublet at 42.8 ppm corresponded to C-2 of glycine. A rough idea of the concentration of [^{13}C]glycine can be derived by dividing the ^{13}C intensity of glycine C-2 by 90 (since the precursor was 99% enriched and natural abundance was 1.1%) and comparing it to the betaine $N(CH_3)_3$ intensity divided by 3 (these carbons are not enriched by ^{13}C), keeping in mind that the intracellular concentration of betaine at 12% NaCl was found to be 0.78 M (9). This result indicates that the intracellular [^{13}C]glycine in these cells was 5 to 15 mM, a value indicating that the exogenous glycine had equilibrated across the cell membrane. This concentration of unlabeled glycine in the cells was not detected in the natural-abundance ^{13}C NMR spectrum. ^{13}C label was also clearly incorporated into betaine as indicated by the splitting pattern of the glycine C-2 resonance at 67.8 ppm (Fig. 2B). An idea of the relative amounts of labeled-to-unlabeled material can be provided by integrating the glycine C-2 region at 67.8 ppm and dividing the intensity of enriched ^{13}C satellite resonances by 90 and comparing this number to the center singlet, which represents natural-abundance betaine. Only 2.4% of the betaine pool was enriched. Exogenous glycine was internalized, but it did not provide the major pool of glycine used to generate glycine betaine. This may indicate (i) substrate channeling, (ii) compartmentalization in the cell, or (iii) the presence of endogenous pools of glycine far in excess of the internalized [$^{13}C_2$]glycine. In ^{13}C natural-abundance spectra of ethanol extracts of *M. portucalensis*, there is no detectable intensity at ~42 ppm. This finding allows us to put an upper limit of 50 mM on endogenous glycine. While small intracellular pools of glycine (and sarcosine) may exist, they do not appear to be in equilibrium with the [$^{13}C_2$]glycine; otherwise, a larger percentage of the betaine would have been ^{13}C labeled.

The methylation scheme for betaine synthesis in other organisms involves the stepwise addition of methyl groups catalyzed by two methyltransferases. A similar scheme is likely to be operational in *M. portucalensis* (4). The first two methylation reactions, i.e., glycine conversion to sarcosine and sarcosine conversion to DMG, are carried out in *M. portucalensis* in such a fashion that endogenous glycine and sarcosine are not in equilibrium with the external solutes that are internalized. The remaining methylation step, conversion of DMG to betaine, readily uses the exogenous DMG. The observation that increasing the internal concentration of this solute enhanced betaine synthesis and accumulation strongly suggests that this intermediate is what regulates betaine accumulation. An excess of this solute appears to direct the cells to use betaine as the primary osmolyte and to suppress accumulation of both NAβLys and β-glutamine.

Previous ^{13}C-labeling studies suggested that choline was not a precursor for glycine betaine. As a control, choline was examined for its effect on zwitterionic osmolyte production in *M. portucalensis*. In many aerobic organisms and in *Methanobacterium thermoautotrophicum* ΔH, choline can be oxidized to form betaine (1). Cells incubated with 10 mM choline accumulated slightly more betaine than the control but not to levels outside the range of experimental errors. No significant choline (easily distinguished from betaine by its C-1 at 71.8 ppm) was detected in the extracts. However, if the exogenous choline

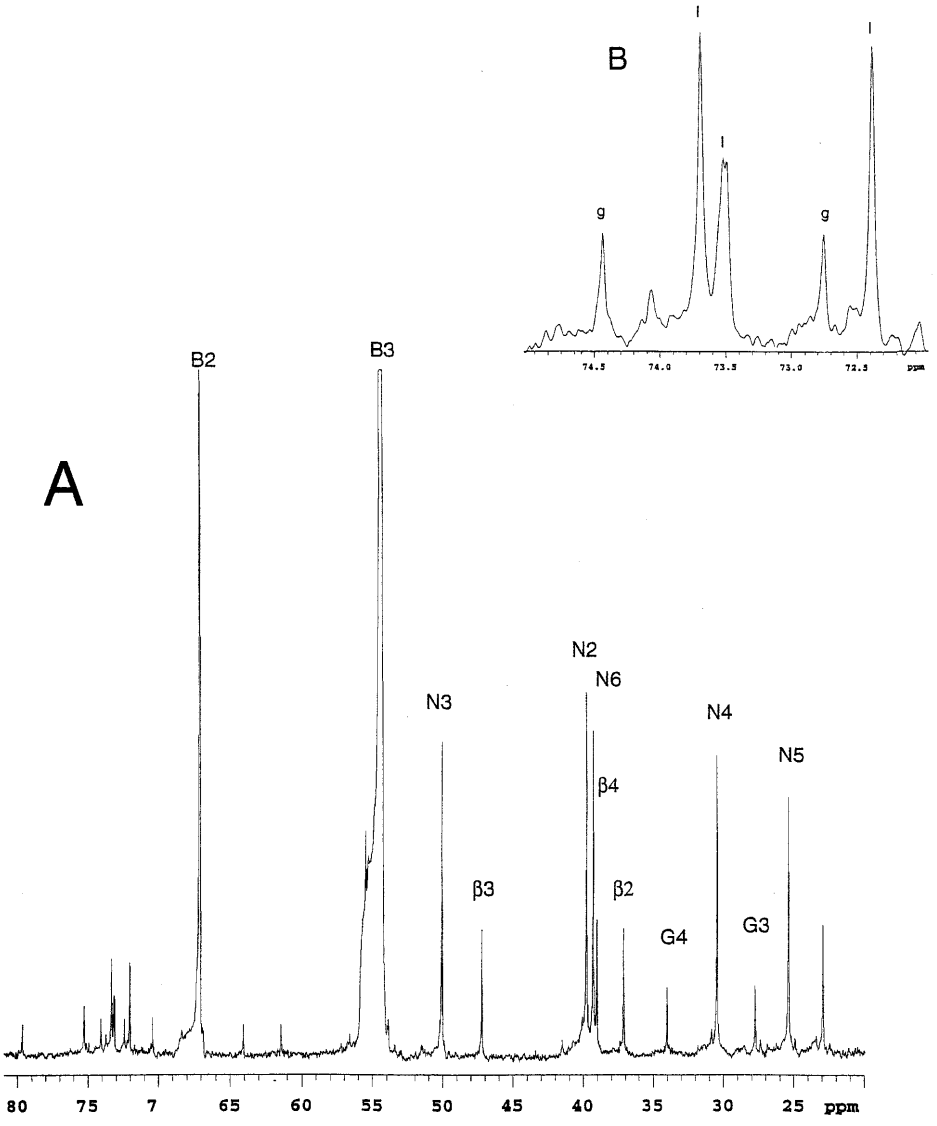

FIG. 3. (A) ^{1}H-decoupled ^{13}C spectrum of an ethanol extract of *M. portucalensis* grown on methanol in the presence of 10 mM choline. The inset (B) shows an enlargement of the 72- to 75-ppm region that contains resonances for *myo*-inositol (I). Resonances identified by carbon number belong to the following: g, glucosylglycerate; B, betaine; N, NAβLys; β, β-glutamine; G, α-glutamate.

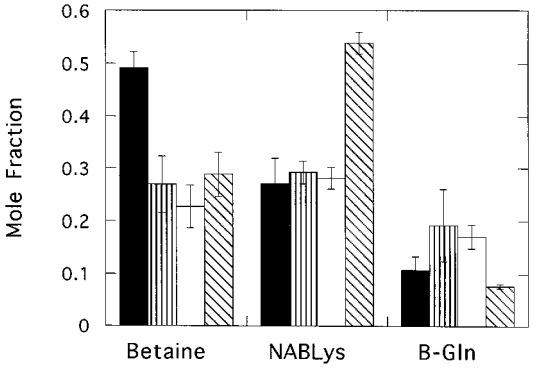

FIG. 4. Effects of possible β-glutamine precursors (10 mM added to the medium) on the distribution of compatible solutes in *M. portucalensis*. ■, control; ▥, β-glutamate; □, α-glutamate; ▧, α-glutamine.

were to equilibrate inside the cells, its natural abundance would be too low to detect in the ^{13}C spectrum. More interestingly, under these conditions, cells synthesized and accumulated *myo*-inositol, a solute not previously detected in extracts

TABLE 3. ^{13}C-label uptake from $^{13}CH_3OH$ by *M. portucalensis*

A_{660}	^{13}C solute/total solute[b]			
	Betaine	β-Glutamine	NAβLys	Glucosylglycerate
1.03	0.040	0.005	0.036	
1.26	0.069	0.071	0.060	0.067
1.48	0.62	0.093	0.358	0.292

[a] Cells were grown to an A_{660} of 0.6 on nonlabeled methanol and then incubated with $^{13}CH_3OH$ until the indicated A_{660} value was reached.

[b] ^{13}C uptake was measured by comparing the intensities of carbons labeled by $^{13}CH_3OH$ in a particular solute to the total amounts of each solute determined from a ^{1}H NMR spectrum.

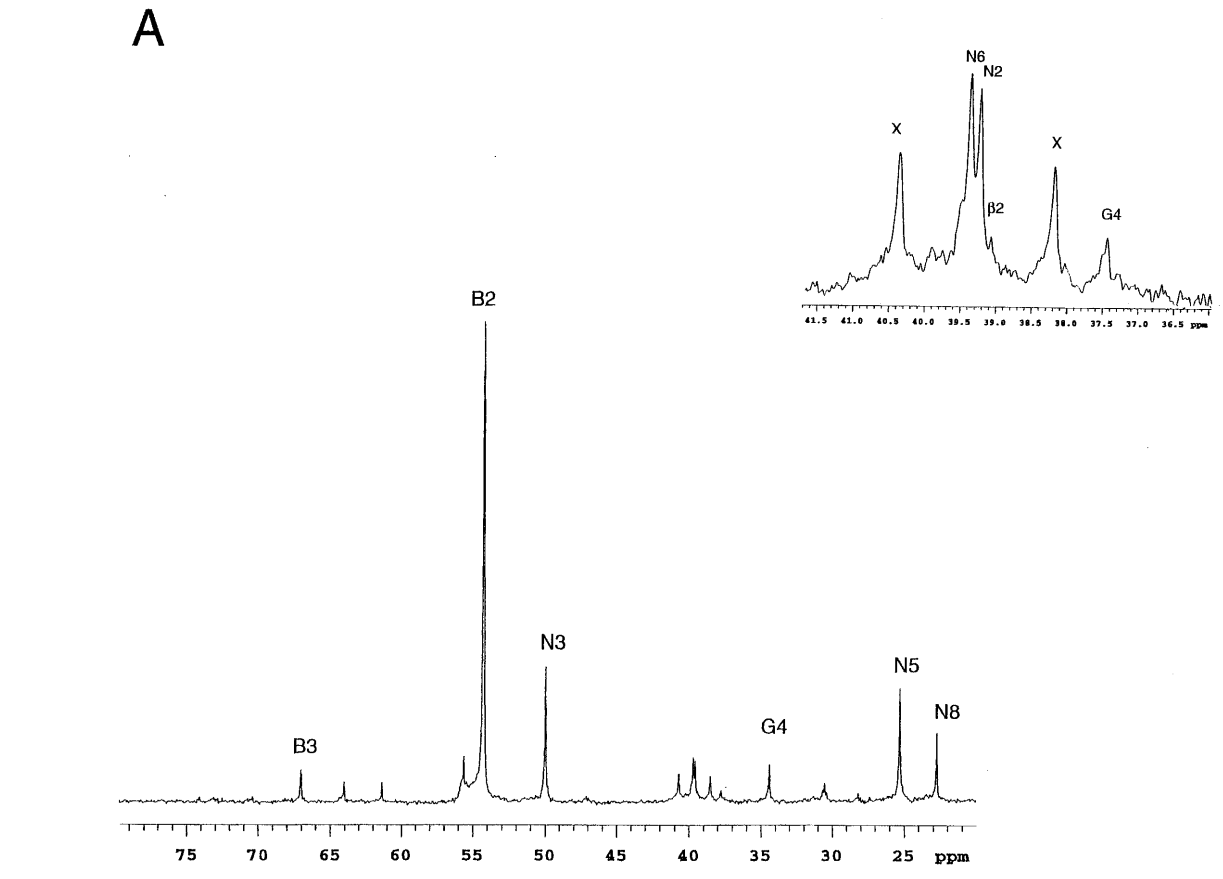

FIG. 5. (A) ^{1}H-decoupled ^{13}C NMR (125.7 MHz) spectrum of an ethanol extract of *M. portucalensis* after incubation with $^{13}CH_3OH$ until the A_{660} reached 1.4. Previously unidentified resonances are shown in the inset. (B) Contour plot of an HMQC experiment on the same sample shown in panel A. F1 is the ^{13}C chemical shift; F2 is the ^{1}H chemical shift. Resonances are labeled according to osmolyte and carbon number. B, betaine; N, NAβLys; G, α-glutamate; X, unknown; β, β-glutamine.

of this organism (Fig. 3). The biosynthesis of inositol in all organisms is by conversion of glucose-6-phosphate to inositol-1-phosphate and hydrolysis of the inositol phosphate to inositol (7). It is unclear how the presence of choline affects this process. If cells were grown in the presence of 10 mM inositol, low levels of inositol, corresponding roughly to a small decrease in betaine accumulation, were internalized by the cells. Interestingly, the exogenous inositol decreased the lag phase for cell growth. Thus, *M. portucalensis* may potentially use inositol for osmotic balance.

β-Glutamine: possible biosynthetic pathways? The biosynthetic pathway of β-glutamine in *M. portucalensis* in all likelihood involves the action of glutamine synthetase on β-glutamate (8), although β-glutamate is a poor substrate for known glutamine synthetase enzymes (3). β-Glutamate is synthesized and used as an osmolyte by a wide variety of methanogens (11), although NMR-detectable concentrations are not accumulated in *M. portucalensis*. For the quantities of cells used for these ethanol extracts, an organic solute would need to be present at ≥50 mM inside the cells to be reliably detected in the natural-abundance spectra of the extracts. The observation that β-glutamate was not detected indicates that its intracellular concentration is less than 50 mM. The synthetic path for β-glutamate has not been deduced in this or other methanogens

(10). Possible routes include aminomutase activities acting on α-glutamate or α-glutamine. *M. portucalensis* cells were grown in the presence of both α- and β-glutamate isomers, and α-glutamine. The effects of these three exogenous compounds on organic zwitterions in cells grown in 12% NaCl are shown in Fig. 4. Each of these solutes depressed intracellular betaine roughly 50%. To compensate for the decrease in this osmolyte, the cells clearly internalized and accumulated low levels of the exogenous solute. The two glutamate isomers also increased the amount of β-glutamine twofold, with no effect on NAβLys levels. The addition of L-α-glutamine to the medium had a surprising effect, both on cell growth and on osmolyte production. The presence of L-α-glutamine abolished the lag phase but slowed cell growth (the doubling time increase was ~2.5-fold). The organic osmolyte distribution was affected but quite differently from when the medium was supplemented with either glutamate isomer. Betaine decreased, but the β-glutamine level was only marginally affected. The loss of betaine was paralleled by an increase in NAβLys. An explanation for this result may be that internalized α-glutamine increased the available nitrogen in the cell and that this increase in nitrogen is critical to the balance between betaine and NAβLys.

Equivalent levels of enhancement of β-glutamine accumulation by the two glutamate isomers may be consistent with biosynthesis of β-glutamate from α-glutamate by an aminomutase activity. If this were the case, the α- and β-glutamate isomers should show similar labeling patterns and ^{13}C uptake rates. Cultures of *M. portucalensis* were grown on unlabeled substrate to an A_{660} of ~0.6 and then incubated with $^{13}CH_3OH$. Aliquots were removed periodically, and extracts were prepared. ^{13}C NMR spectroscopy was used to quantify the amounts of label uptake in different carbons, while ^{1}H NMR spectroscopy was used to quantify the total amount of each solute. Cells grown on $^{13}CH_3OH$ synthesized α-glutamate as well as β-glutamine, with label incorporated in C-2 and C-4 (8). These cultures also produced α-glutamine labeled at the C-2 and C-4 resonances (indicating that α-glutamine was synthesized from α-glutamate via glutamine synthetase). However, there was a difference in the labeling of the α-glutamate versus that of the β-glutamine pools (Table 3). Levels of ^{13}C label uptake were similar for betaine C-3, NAβLys C-3, C-5, and C-8 and for α-glutamate C-2 and C-4. Glucosylglycerate carbons were also rapidly labeled. However, there was no label at early time points in β-glutamine carbons. ^{1}H spectra of these short-time incubations of cells with $^{13}CH_3OH$ indicated that β-glutamine was present at normal concentrations (roughly 0.2 mol fraction of the zwitterionic osmolyte pool). The lack of ^{13}C labeling of β-glutamine while α-glutamate was labeled indicates that the two solutes were not in equilibrium. α-Glutamate cannot be directly on the pathway to β-glutamine. Eventually, β-glutamine C-2 and C-4 were labeled. However, the lag in ^{13}C uptake into β-glutamine indicated that the direct precursor was not one of the observable solutes.

Some new carbons appeared under these labeling conditions (Fig. 5A). An HMQC experiment showed that the two resonances at 39.5 and 39.2 ppm belonged to C-6 and C-2 of NAβLys (Fig. 5B) (these shared a slight enrichment of ^{13}C possibly due to a small amount of scrambling into lysine synthesis), leaving unidentified ^{13}C resonances at 40.5 and 38.3 ppm (the resonance at 37.5 ppm in this longer ^{13}C incubation represented label in the C-4 of β-glutamine). Since there were no new resonances in the ^{1}H spectrum, these carbons must have represented low concentrations of highly enriched solutes (though not citric acid cycle intermediates, since these were checked to see if they overlapped with the two unknown resonances).

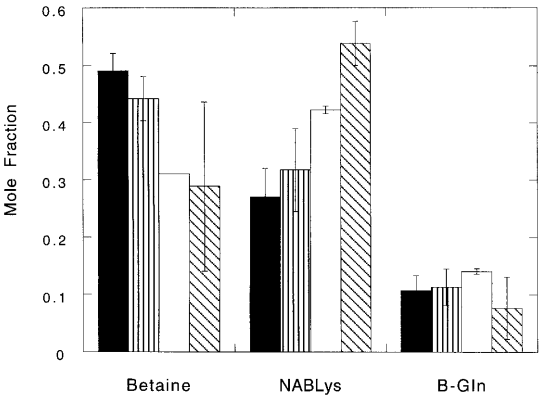

FIG. 6. Effects of possible NAβLys precursors (10 mM added to the medium) on the distribution of compatible solutes in *M. portucalensis*. ■, control; ▥, α-lysine; □, diaminopimelate; ▧, α-glutamine.

Modulation of NAβLys levels. In *M. portucalensis*, lysine is synthesized via the diaminopimelate pathway (8). This amino acid is then assumed to be converted to β-lysine by a lysine aminomutase activity (12). The β-amino acid is then acetylated to form NAβLys. In an attempt to enhance the synthesis of NAβLys, cells were grown in the presence of 10 mM α-lysine and 10 mM diaminopimelate. Both additives increased the lag times for cell growth but had little effect on doubling times once the cultures began to grow. The effects of these solutes on the distribution of organic osmolytes are shown in Fig. 6. The presence of lysine had virtually no effect on the distribution of compatible solutes, nor was lysine itself observed in the ^{13}C spectra of these extracts. This finding suggests that if lysine is internalized, it cannot be the limiting intermediate in the NAβLys biosynthetic pathway. However, the presence of diaminopimelate, a precursor of α-lysine, markedly increased the accumulation of NAβLys, enough so that in cells grown in 12% NaCl, NAβLys levels were greater than those of betaine. Availability of this intermediate in the lysine pathway can increase accumulation of NAβLys at the expense of glycine betaine. The observation that α-lysine itself had no effect might suggest that this cationic amino acid is not significantly internalized, or if it is internalized, perhaps it is catabolized rather than used for NAβLys biosynthesis.

Interestingly, the largest enhancement in NAβLys accumulation occurred when the cells were grown in the presence of 10 mM α-glutamine (Fig. 6). This result suggests that the more important factor in zwitterionic osmolyte selectivity is not the availability of carbon sources but the availability of nitrogen donors. Further investigation of the enzymes involved in the biosynthetic path of NAβLys may be crucial to understanding how nitrogen assimilation is linked to osmolyte levels.

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