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Biosynthetic Pathways of the Osmolytes N^{ε}-Acetyl-β-Lysine, β-Glutamine, and Betaine in *Methanohalophilus* Strain FDF1 Suggested by Nuclear Magnetic Resonance Analyses

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***Methanohalophilus* strain FDF1 synthesizes β-glutamine, betaine, and N^{ε}-acetyl-β-lysine as osmoprotective agents when the cells are grown in high external concentrations of NaCl. Nuclear magnetic resonance spectroscopic analyses of $^{13}CH_3OH$-$^{12}CO_2$ label incorporation by the cells provide information on the biosynthetic pathways of these organic osmolytes. The labeling studies indicate that *Methanohalophilus* strain FDF1 produces glutamate and β-glutamine via a partial oxidative Krebs pathway. ^{13}C labeling of betaine is consistent with methylation of glycine generated from serine (via serine hydroxymethyltransferase). The labeling pattern for N^{ε}-acetyl-β-lysine is consistent with the synthesis of its precursor α-lysine occurring by the diaminopimelate pathway in these cells.**

Methanohalophilus species are among the most halophilic of the methanogenic archaebacteria. A variety of organic solutes, including the β-amino acids β-glutamine and N^{ε}-acetyl-β-lysine (11, 15), are used to reduce any differential osmotic pressure between the intracellular and extracellular environments in these cells. These cells also synthesize glycine betaine de novo (11). Since these compatible solutes accumulate in response to external NaCl levels, there must be regulatory mechanisms linked to osmotic pressure. Before study of the regulation, the enzymes and/or other proteins responsible for synthesis of these species must be isolated. This requires knowledge of the biosynthetic pathways used by these cells in forming these osmolytes. Previously, ^{13}C nuclear magnetic resonance (NMR) spectroscopy has been used to identify compounds which can act as osmolytes in a diverse range of methanogens (11, 16–21). This methodology can also be used to discriminate between different biosynthetic pathways if specifically labeled ^{13}C species can be incorporated into the cells. Specific [1-^{13}C]acetate or [2-^{13}C]acetate incorporation into amino acids, lipid phytanyl chains, and sugars in *Methanospirillum hungatei* (4, 5) and into an unusual cyclic PP_i diester in *Methanobacterium thermoautotrophicum* (6) provided biosynthetic information for those compounds in those particular methanogens. In the present work, in situ labeling with [2-^{13}C]acetate is used to propose biosynthetic pathways for L-α-glutamate, betaine, N^{ε}-acetyl-β-lysine, and β-glutamine. The results indicate that *Methanohalophilus* strain FDF1 synthesizes glutamate (and related compounds) via a partial oxidative Krebs or tricarboxylic acid pathway, and can be used to suggest biosynthetic pathways for the other major solutes in this organism which are consistent with the observed labeling scheme.

Methanogens vary in their incorporation and utilization of exogenously supplied acetate, and for some species (e.g., *Methanococcus thermolithotrophicus* [16]) [^{13}C]acetate may not be the best compound for generating specifically labeled molecules. The introduction of a specific ^{13}C label into *Methanohalophilus* strain FDF1 is facilitated by the ability of the cells to use a variety of substrates, including methanol and mono-, di-, and trimethylamine, for methanogenesis (12, 13). In the absence of H_2, a portion of the methyl groups of methanol are oxidized to CO_2 to provide reducing equivalents for the cells (9). The presence of N_2-$^{12}CO_2$ in the bottle head space will effectively dilute any $^{13}CO_2$ produced from the methanol. Therefore, the cells synthesize acetate by using $^{13}CH_3$- from methanol and ^{12}CO- from the CO_2 gas. The [2-^{13}C]acetyl unit generated is then converted to [3-^{13}C]pyruvate, incorporating $^{12}CO_2$ from the medium. These species then act as precursors for all the organic molecules in *Methanohalophilus* strain FDF1.

To generate the [2-^{13}C]acetate, *Methanohalophilus* strain FDF1 (OGM 59) was grown at 37°C in a basal medium containing 12% NaCl with 30 mM $^{13}CH_3OH$ under an N_2-CO_2 atmosphere (4:1) (11, 15). The medium pH was adjusted to 7.2 before sterilization by autoclaving. Under these conditions, the cells synthesize acetate by using the $^{13}CH_3$- from methanol and ^{12}CO- from the CO_2 gas. Once cells were grown on this ^{13}C-labeled medium to an optical density at 540 nm of ~0.4, they were harvested by centrifugation at 6,000 × *g* for 15 min. Cell pellets, containing approximately 10^{11} cells (calculated from the original cell suspension volume and the cell count per milliliter determined with a Petroff-Hausser counting chamber under a phase-contrast microscope [11]), 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 (21).

^{13}C NMR spectra were acquired at 125.7 MHz on a Unity 500 spectrometer. Spectral accumulation parameters included a 25,000 Hz sweep width, 65,536 datum points, a 6-μs pulse, 1.3 s between pulses, and ^1H WALTZ-16 modulated decoupling. All free-induction decays were processed with a 4-Hz line broadening prior to Fourier transformation. ^{13}C chemical shifts were referenced with respect to dioxane at 67.4 ppm.

The major organic solutes in the ethanol extract of meth-

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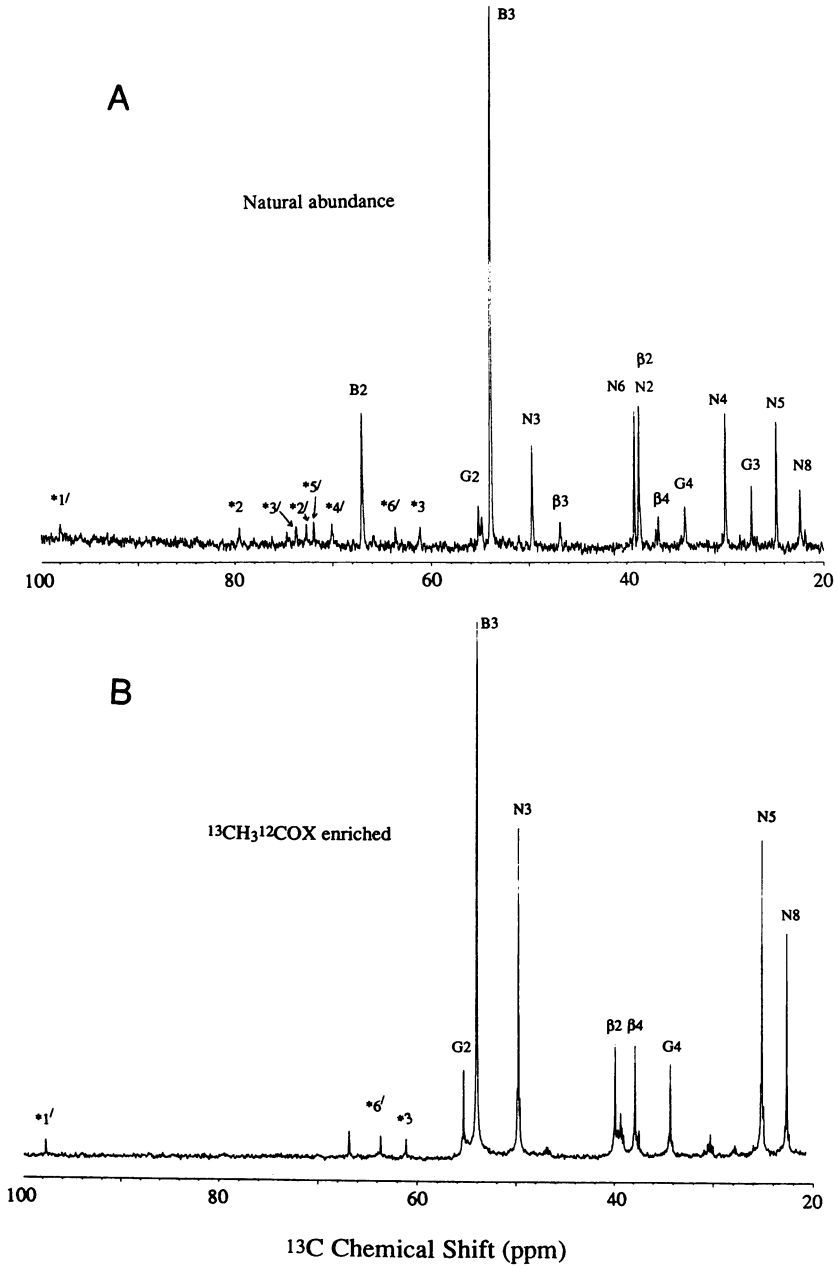

FIG. 1. ^{13}C NMR spectra (125.7 MHz) of extracts of *Methanohalophilus* strain FDF1. (A) Natural abundance; (B) growth with $^{13}CH_3OH$ and CO_2 (which generates [2-^{13}C]acetyl units for labeling cell carbon). Each resonance is identified as to the particular solute molecule (B, betaine; G, L-α-glutamate; β, β-glutamine; *N*, N^{ε}-acetyl-β-lysine; *, α-glucosylglycerate (the numbers with primes refer to carbons of the glucose ring; for details of the identification of this species, see reference 15) and the carbon number in the particular solute molecule.

anol-grown *Methanohalophilus* strain FDF1 are betaine, N^{ε}-acetyl-β-lysine, β-glutamine, and L-α-glutamate. These compounds are easily identified in the ^{13}C NMR spectrum of an ethanol extract prepared from cells grown without a source for ^{13}C enrichment (Fig. 1A). Observable ^{13}C chemical shifts for carbons with directly bonded protons arise from 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 and 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). As shown in Fig. 1B, when the cells are grown in the presence of $^{13}CH_3OH$ and $^{12}CO_2$ and then extracted, the ^{13}C NMR spectrum shows a definite selectivity in which carbons are labeled by the [2-^{13}C]acetyl unit ($^{13}CH_3{}^{12}COX$). L-α-Glutamate is clearly labeled at C-2 and C-4; C-3 (27.7 ppm) is not ^{13}C labeled, but it is a multiplet because of ^{13}C-^{13}C splitting from the adjacent labeled carbons. This low-intensity multiplet at around 28 ppm can be seen more distinctly in Fig. 2C. β-Glutamine, which is synthesized by these cells in response to high external concentrations of NaCl, shows a labeling pattern similar to that of glutamate: C-2 (which overlaps with N^{ε}-acetyl-β-lysine C-2) and C-4 are enriched

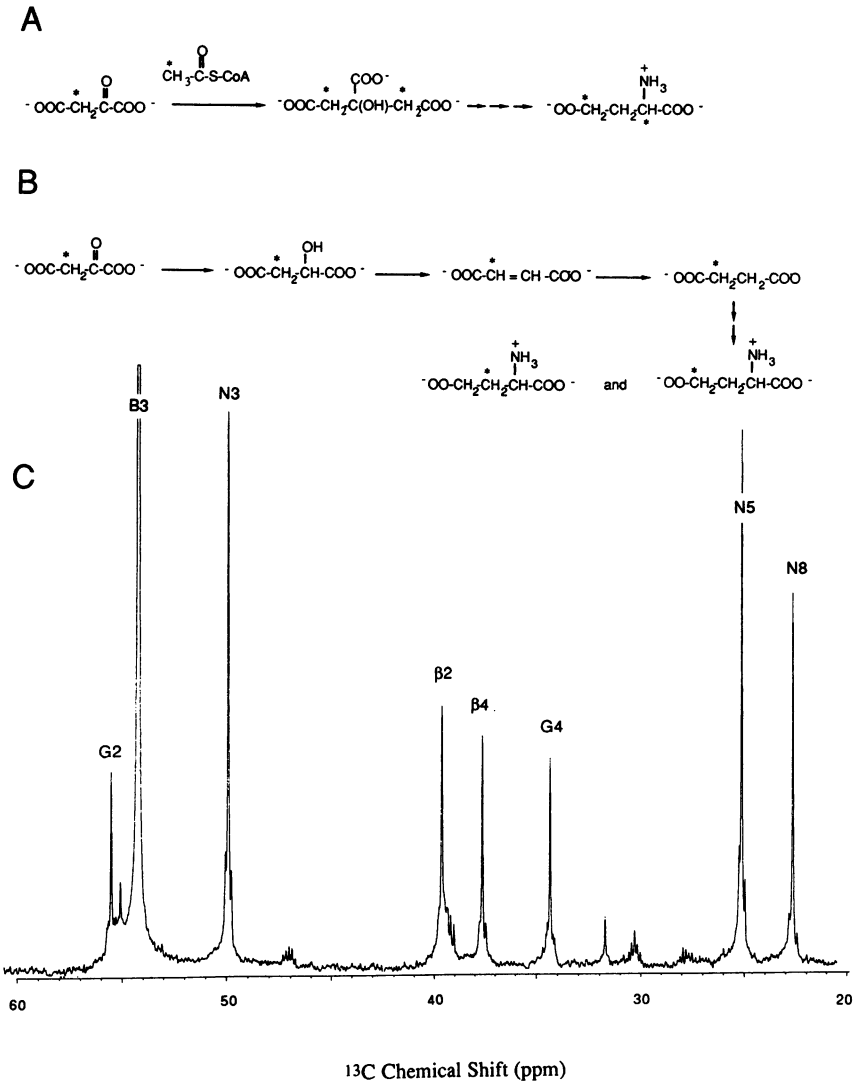

FIG. 2. ^{13}C labeling of glutamate and β-glutamine predicted from a partial oxidative (A) or reductive (B) tricarboxylic acid pathway in cells under N_2-CO_2 with $^{13}CH_3OH$ as the substrate for methanogenesis. Asterisks in pathways, ^{13}C label introduced from $^{13}CH_3OH$. (C) Expansion of the ^{13}C NMR spectrum (125.7 MHz) of the labeled sample, with ^{13}C-enriched resonances for betaine (B3), N^{ε}-acetyl-β-lysine (N3, N5, and N8), β-glutamine (β2 and β4), and L-α-glutamate (G2 and G4) indicated (the number after each letter indicates which carbon of the molecule is labeled).

singlets, while C-3 (47.3 ppm) is much lower in intensity and is observed as a multiplet. Even though C-2 overlaps with N^{ε}-acetyl-β-lysine carbons, other evidence indicates that it is the enriched carbon detected at ~39 ppm. If the C-2 of β-glutamine were not enriched, C-3 would be a doublet (surrounding a singlet for any ^{13}C at C-3 not adjacent to other enriched ^{13}C nuclei) from coupling to C-4. Instead, the C-3 of β-glutamine is visible as a small five-line multiplet. This implies that C-3 is coupled to more than one ^{13}C, i.e., that C-2 as well as C-4 is ^{13}C enriched. The only way this labeling pattern can arise is if *Methanohalophilus* strain FDF1 synthesizes glutamate by using citrate as a symmetric intermediate. Methanogens do not possess a complete Krebs cycle. Instead, they fall into two groups: (i) those with a partial reductive Krebs pathway (e.g., *Methanobacterium thermoautotrophicum* [7] and *Methanococcus thermolithotrophicus* [15]) and (ii) those with a partial oxidative Krebs pathway (e.g., *Methanosarcina* spp. [23]). As shown in Fig. 2A, a partial oxidative Krebs pathway would selectively enrich C-2 and C-4 of glutamate (and presumably β-glutamine if it is synthesized directly from L-α-glutamine, from L-α-glutamate followed by amidation of the side chain carboxylate, or from another compound derived from L-α-glutamate). In contrast, a partial reductive Krebs pathway would selectively enrich C-2 and C-3 of glutamate (though not in the same molecule). Experimentally, both glutamate and β-glutamine C-2 and C-4 are selectively enriched with ^{13}C (Fig. 2C); hence, *Methanohalophilus* strain FDF1 utilizes a partial oxidative Krebs pathway for synthesis of glutamate and related compounds. *Methanohalophilus* spp. belong to the order of *Methanomicrobiales*, family of *Methanosarcinae*, and thus it is not surprising that *Methanohalophilus* strain FDF1 also uses a partial oxidative Krebs pathway to produce glutamate. The ^{13}C labeling of β-glu-

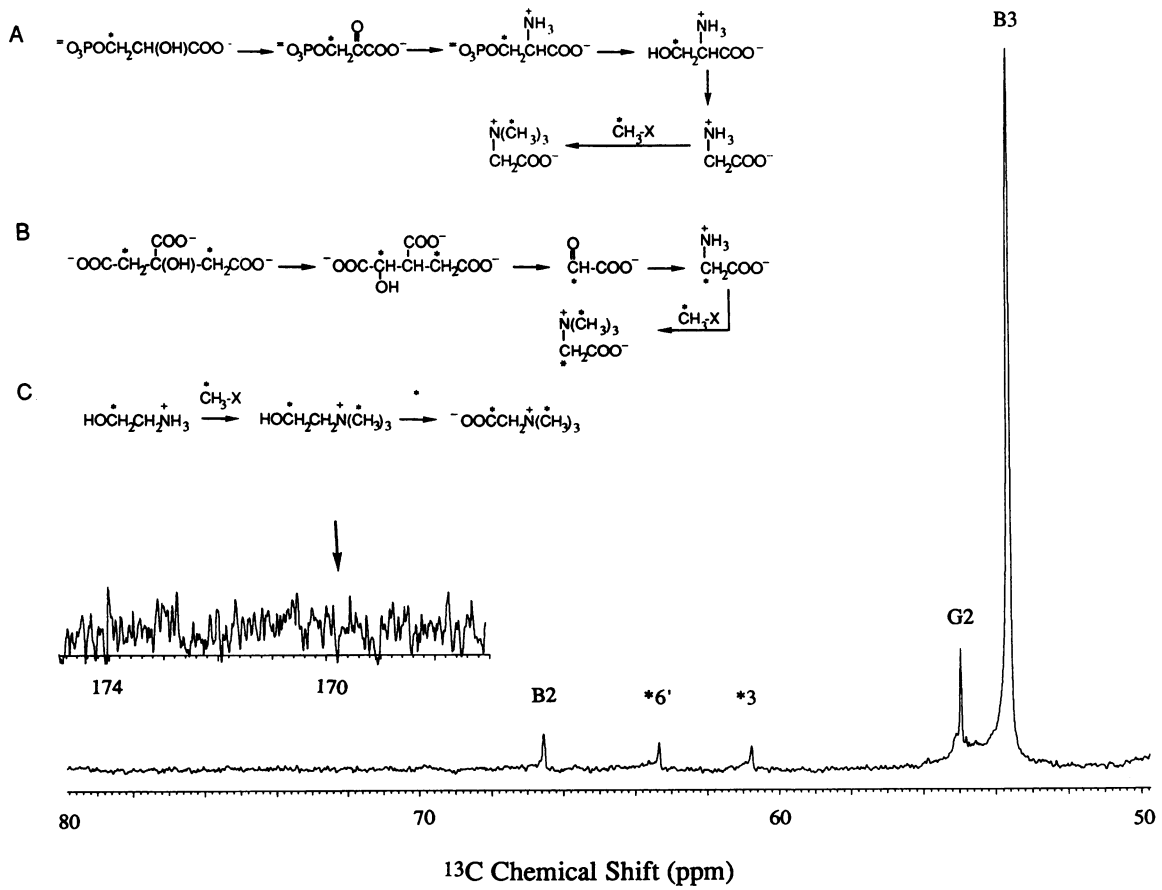

FIG. 3. ^{13}C labeling of betaine in cells under N_2-CO_2 with $^{13}CH_3OH$ as the substrate for methanogenesis by different pathways: methylation of glycine produced from serine (A), methylation of glycine produced from glyoxylate (in turn produced from isocitrate) (B), and methylation and dehydrogenation of ethanolamine (derived by decarboxylation of serine) (C). Below panel C are shown expansions of the ^{13}C NMR spectrum (125.7 MHz) of the labeled sample showing the betaine carboxylate region (an arrow for B1 at ~170 ppm shows where the resonance should be) and the betaine CH_2 (B2 at 66.9 ppm) and CH_3 (B3 at 54.1 ppm) resonances. Also shown are the labeled glucosylglycerate C-6' (*6') and C-3 (*3) and glutamate C-2 (G2). The vertical scale of the carboxy region has been expanded 15 times to detect any labeled carboxylate groups.

tamine is consistent with its synthesis from glutamate, either directly via an aminomutase activity and glutamine synthetase (β-glutamate has previously been shown to be a substrate, albeit a poor one, for glutamine synthetase [10]) or indirectly from a compound derived from a common intermediate in the α-glutamate pathway or a product of glutamate metabolism. Previous NMR investigations of β-glutamate synthesis in *Methanococcus thermolithotrophicus* have indicated that the β-glutamate in that organism is not directly derived from α-glutamate but is linked to an insoluble precursor (16, 17). Thus, while the NMR analyses do not unambiguously define the biosynthetic route for β-glutamine, they place a constraint on any novel synthetic schemes which may be proposed in the future. The key result of the ^{13}C labeling studies is the *Methanohalophilus* strain FDF1 utilizes a partial oxidative Krebs pathway for synthesis of glutamate. Biosynthesis of the other osmolytes (glycine betaine and N^{ε}-acetyl-β-lysine) can be examined in view of this oxidative partial tricarboxylic acid path.

Previous work using ^{15}N labeling indicated that glycine betaine was indeed synthesized by *Methanohalophilus* strain FDF1 (11). There are two possible strategies for synthesis of betaine in anaerobic cells (Fig. 3). The first involves methylation of glycine, which can itself be produced in several ways. In the first glycine pathway (Fig. 3A), glycine is generated from serine via serine hydroxymethyltransferase. Serine, synthesized from 3-phosphoglycerate, will be ^{13}C labeled at C-3 from $^{13}CH_3{}^{12}COX$. Removal of C-3 by the transferase generates unlabeled glycine. The glycine is then methylated by an intermediate derived from $^{13}CH_3OH$ (perhaps *S*-adenosylmethionine or a methyltransferase similar to those that transfer CH_3- from methanol to a corrinoid prosthetic group and eventually to coenzyme M [9]). Hence, the product betaine is ^{13}C labeled only in the *N*-methyl carbons. In the second glycine pathway (Fig. 3B), [2-^{13}C] glycine is generated from isocitrate via the glyoxylate cycle. Since *Methanohalophilus* strain FDF1 does indeed contain enzymes from this half of the Krebs cycle, this represents a distinct possibility, and, in fact, a similar pathway for synthesis of betaine from glycine has been observed in halophilic phototrophic eubacteria (22). The betaine produced with this scheme for glycine synthesis will be labeled at C-2 as well as the *N*-methyl carbons. In the second strategy for betaine synthesis (Fig. 3C), [2-^{13}C]ethanolamine is the key precursor and is generated as proposed for the family Chenopodiaceae (2, 8). In that scheme, the ethanol-

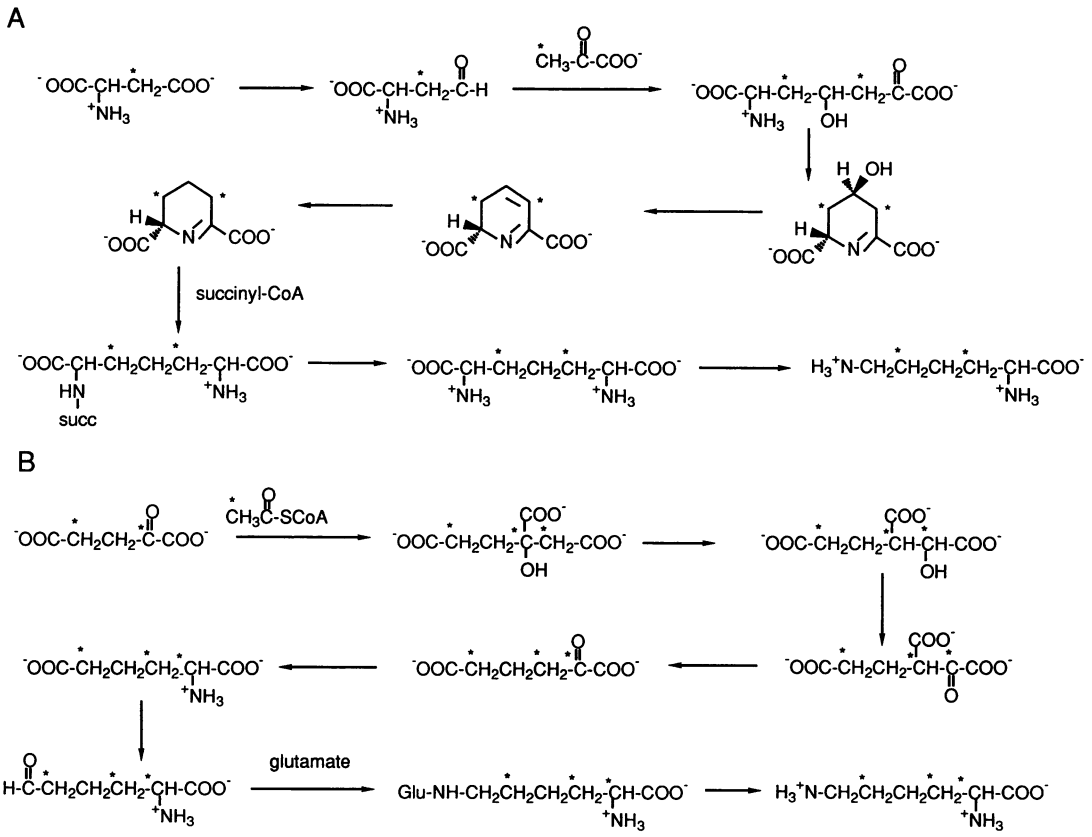

FIG. 4. ^{13}C labeling of lysine in cells under N_2-CO_2 with $^{13}CH_3OH$ as the substrate for methanogenesis via the diaminopimelate pathway (A) and the aminoadipic acid pathway (B). CoA, coenzyme A. Asterisks in pathways, ^{13}C label introduced from $^{13}CH_3OH$.

amine is generated from serine by decarboxylation. The ethanolamine is then methylated ($^{13}CH_3$- via a methyltransferase) to produce choline with ^{13}C enrichment at C-2 as well as the *N*-methyl groups. The choline is then dehydrogenated to yield glycine betaine with both C-2 and the *N*-methyl carbons enriched. Betaine synthesis in cyanobacteria has been shown to proceed via the latter pathway (24). In all three possible pathways, the *N*-methyl groups of betaine would be ^{13}C labeled, while C-2 is labeled in two of the three synthetic routes. Experimentally, one sees that only the *N*-methyl carbons of betaine (54.1 ppm) are selectively labeled in cells incubated with $^{13}CH_3OH$-$^{12}CO_2$ (Fig. 3C). The C-2 at 66.9 ppm is much lower in intensity, consistent with natural abundance levels of ^{13}C in this compound. The lack of ^{13}C labeling of the betaine C-2 which occurs at ~67 ppm unambiguously excludes the glyoxylate and ethanolamine pathways. The remaining pathway, methylation of glycine, produced from serine, is consistent with the ^{13}C labeling experiment; hence, it is a good candidate for the betaine biosynthetic pathway in *Methanohalophilus* strain FDF1.

Another zwitterionic solute synthesized by *Methanohalophilus* strain FDF1 in response to external NaCl is N^{ε}-acetyl-β-lysine. This species shows selective ^{13}C enrichment of C-3, C-5, and C-8, which all appear as singlets. The multiplet structures of C-2 (39.2 ppm), C-4 (30.3 ppm), and C-6 (39.5 ppm overlapping with β-glutamine C-4) indicate that they are adjacent to enriched carbons. Confirmation that C-2 and C-6 are not enriched with ^{13}C was also obtained from an analysis of the ^{1}H spectrum of the extract (data not shown). The protons attached to these carbons do not show the large splitting which would be caused by directly bonded ^{13}C. There are two routes used by bacteria for synthesis of lysine: (i) the diaminopimelate (common in bacteria, some fungi and algae, and higher plants) and (ii) the aminoadipic acid (often used by fungi and some algae) pathways. The $^{13}CH_3OH$-$^{12}CO_2$ labeling patterns predicted by each of these pathways are shown in Fig. 4, assuming that N^{ε}-acetyl-β-lysine is derived from α-lysine. The α-lysine could be subsequently rearranged to form β-lysine by a lysine 2,3-aminomutase and acetylated on the side chain nitrogen, or acetylation of the side chain nitrogen of α-lysine could occur prior to the aminomutase reaction. In this instance, the first of these sequences converting α-lysine to N^{ε}-acetyl-β-lysine is suggested to occur, on the basis of observations with related aminomutase activities in other eubacterial cells. N^{ε}-acetyl-α-lysine is not a substrate for the lysine aminomutase which has been isolated and characterized from *Clostridium* strain SB4 (14). In a similar fashion, when *Pseudomonas* spp. catabolize L-α-lysine, they form the β-amino acid first and then acetylate the side chain nitrogen (3). The acetyl methyl group (C-8) of N^{ε}-acetyl-β-lysine would be ^{13}C labeled in either case. The lysine backbone formed by the diaminopimelate route would be selectively labeled at C-3 and C-5, while operation of the aminoadipate route would label lysine at C-2, C-3, and C-5. The ^{13}C NMR spectra clearly show that the lysine backbone of N^{ε}-acetyl-β-lysine is labeled only at C-3 and C-5, implicating the diaminopimelate pathway as the route of lysine synthesis in this methanogen. The occurrence of enzymes of the diaminopimelate pathway

has been documented in *Methanobacterium thermoautotrophicum* (1). A similar pathway for formation of N^{ε}-acetyl-β-lysine from lysine has also been deduced by ^{13}C specific labeling in *Methanogenium cariaci*; however, in those cells a partial reductive Krebs cycle is used to form glutamate, generating a molecule with ^{13}C label incorporation at C-3 or C-4, and hence an N^{ε}-acetyl-β-lysine pool with ^{13}C incorporated at C-3, C-4, and C-8 (17) is produced.

In summary, ^{13}C specific labeling studies of *Methanohalophilus* strain FDF1 have provided details on the biosynthesis of organic solutes in this cell. Perhaps most notably, these studies illustrate that this particular methanogen utilizes an oxidative incomplete Krebs cycle for generation of α-ketoglutarate. This is the second methanogen for which such a path has been documented. Most methanogens have evolved to utilize a reductive pathway. Interestingly, the two species which use an oxidative partial Krebs cycle, *Methanosarcina barkeri* and *Methanohalophilus* strain FDF1, are the most diverse in terms of methanogenic substrates. This diversity also carries over into how *Methanohalophilus* strain FDF1 accumulates organic solutes in response to osmotic stress. Exogenous betaine has been shown to be transported into *Methanohalophilus* strains Z7401 and FDF2 from the medium and it suppresses biosynthesis of other zwitterionic osmolytes; in strain Z7401 it can also be synthesized de novo (11). Several other methanogens, including *Methanosarcina thermophila* TM1 (21) and *Methanogenium cariaci* (18), transport betaine into the cell and accumulate it in response to external NaCl, but they are incapable of synthesizing it from simple one-carbon or two-carbon precursors (e.g., CO_2 or CH_3COX). The high-salt-concentration environment of *Methanohalophilus* spp. may have led to selection of both synthetic and transport systems for this key compatible solute. The other zwitterionic osmolyte synthesized by the cells is N^{ε}-acetyl-β-lysine. Production of this solute is suggested to follow the same scheme as that deduced for *Methanogenium cariaci* (17). N^{ε}-Acetyl-β-lysine is also an intermediate in the catabolism of L-α-lysine by *Pseudomonas* spp. (3). Under anaerobic conditions, those organisms degrade lysine by first converting it to β-lysine and then performing *N*-acetylation of the side chain nitrogen as a prelude to other degradative steps. In fact, N^{ε}-acetyl-β-lysine can serve as a substrate for growth of *Pseudomonas* spp. In contrast to *Pseudomonas* spp., methanogens use the β-lysine derivative in a completely different manner. Other evidence (15, 17) has shown that, once synthesized, N^{ε}-acetyl-β-lysine is not significantly metabolized by the methanogens. Hence, N^{ε}-acetyl-β-lysine is a true compatible solute used to combat osmotic stress in methanogens.

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