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Acetate and CO_2 Assimilation by *Methanothrix concilii*†

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Biosynthetic pathways in *Methanothrix concilii*, a recently isolated aceticlastic methanogen, were studied by ^{13}C-nuclear magnetic resonance spectroscopy. Labeling patterns of amino acids, lipids, and carbohydrates were determined. Similar to other methanogens, acetate was carboxylated to pyruvate, which was further converted to amino acids by various biosynthetic pathways. The origin of carbon atoms in glutamate, proline, and arginine clearly showed that an incomplete tricarboxylic acid cycle operating in the oxidative direction was used for their biosynthesis. Isoleucine was synthesized via citramalate, which is a typical route for methanogens. As with *Methanosarcina barkeri*, an extensive exchange of the label between the carboxyl group of acetate and CO_2 was observed. Lipids predominantly contained diphytanyl chains, the labeling of which indicated that biosynthesis proceeded through mevalonic acid. Labeling of the C-1,6 of glucose from [2-^{13}C]acetate is consistent with a glucogenic route for carbohydrate biosynthesis. Except for the different origins of the methyl group of methionine, the metabolic properties of *Methanothrix concilii* are closely related to those of *Methanosarcina barkeri*.

Since acetic acid is the major precursor of methane generated in anaerobic digestors (12, 16), there is considerable interest in the study of aceticlastic methanogens (17). These methanogens produce methane almost exclusively from the methyl group of acetate (9, 15, 15a). Most of the studies to date have centered around *Methanosarcina barkeri* (17) because of the unavailability of other pure cultures. However, rod-shaped aceticlastic methanogens, classified in the genus *Methanothrix*, have been described recently (7, 9, 15a, 20) and appear to be widely distributed in anaerobic environments (7, 20). Since *Methanothrix* spp. have a higher affinity for acetate (K_s = 0.7 to 1.1 mM) (9, 15a) than do *Methanosarcina* spp. (K_s = 5.0 mM) (17), they could play a vital role in the digestion of sludge with low acetate concentrations.

On the basis of oligonucleotide sequence determination, the phylogenetic position of *Methanothrix soehngenii* is relatively far from that of other methanogens, being most closely related to *Methanosarcina barkeri* (18). The remoteness of *Methanothrix* spp. from other methanogens and their ecological relevance of other methanogens prompted us to study, by nuclear magnetic resonance (NMR) methods, the major biosynthetic pathways for the assimilation of carbon precursors—acetate and bicarbonate—in *Methanothrix concilii* GP6, a recent axenic isolate (15a). As shown previously (5) ^{13}C-NMR spectroscopy provides an efficient tool for biosynthesis studies, especially when labeled precursors are readily available.

MATERIALS AND METHODS

Growth. Stock cultures of *Methanothrix concilii* GP6 (deposited as NRC 2989 in the National Research Council Collection) were maintained as described earlier (15a). For growth with labeled substrates, Aa medium was prepared as described earlier (15a) with the exceptions that acetate, Na_2CO_3, and $FeSO_4$ were omitted, the pH before reduction with cysteine-Na_2S was adjusted to 6.8, and 100 ml of prereduced medium was dispensed into 250-ml Erlenmeyer flasks modified to accept a serum bottle closure under 100% N_2 gas phase. After autoclaving (121°C for 15 min) all flasks were supplemented with 6 mg of $FeSO_4$ per liter, 4 mM $NaHCO_3$, and finally 17 mM [1-^{13}C]acetate or [2-^{13}C]acetate. All of these supplements were added as filter-sterilized stock solutions made anaerobic by storage under N_2. By similar methods, other flasks received 4 mM $NaH^{13}CO_3$ and 17 mM cold acetate. Six flasks of each of the three labeled substrates were inoculated with 1% (vol/vol) stock culture that was concentrated 10 times (15a) in Aa medium devoid of acetate, Na_2CO_3, and $FeSO_4$. At the end of incubation (35°C for 10 days) the cells were harvested, fractionated, and prepared for NMR analysis as described earlier (5) except for the method of obtaining carbohydrates. The membrane-wall fraction, which sedimented from French pressure cell lysates at 12,000 × *g* for 30 min, was depleted of lipid and hydrolyzed for 6 h in 1 M HCl at 110°C in a sealed ampoule. Afterwards, amino acids were removed by passing the hydrolysate through a column of Dowex 50W-X8 resin in the H^+ form.

Source of radioisotopes. [1-^{13}C]acetate was from Stohler Isotope Chemicals, and [2-^{13}C]acetate and $NaH^{13}CO_3$ were from Merck, Sharp and Dohme Canada, Ltd. The ^{13}C-labeled compounds were at 90% enrichment levels, and their purity was checked by NMR spectroscopy before use.

NMR measurements. NMR spectra were recorded with a Bruker CXP-300 and AM-500 spectrometers operating at 75.47 and 125 MHz for ^{13}C. Pulses of 45° were used, and the recycling time was 2 s. We acquired 5,000 to 20,000 scans. Amino acid samples were run in ^2H_2O solution at pH 0.8, and lipids were run in C^2HCl_3/methanol (5:1). Other details of measurements were described before (5).

RESULTS

Methanothrix concilii uses acetate as the only carbon source for methanogenesis (15a), with methane almost exclusively formed from the methyl group and CO_2 formed from the carboxyl (15a):

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \quad G^{\circ\prime} = -36 \text{ kJ/mol}$$

This energy-yielding reaction (4) accounts for most of the acetate metabolized, since only 5% or less of the acetate is used for biosynthesis (15a). At the early stage of growth, small concentrations of CO_2 (optimum, 1.8 mM) are neces-

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TABLE 1. Distribution of ^{13}C label in amino acids of *Methanothrix concilii* grown in the presence of [1-^{13}C]acetate (c), [2-^{13}C]acetate (m), and bicarbonate (b)

Amino acid	C-α	C-β	C-γ	C-δ	C-ε	Other
Alanine	c[a]	m				
Serine	c	m				
Aspartic acid	c	m				
Threonine	c	m	b (c)			
Glutamic acid	m	c	m			
Arginine	m	c	m	c		
Proline	m	c	m	c		
Leucine	m	c	c	m, m		
Valine	c	c	m, m			
Isoleucine	m	c	c	m		C-γ':m
Phenylalanine		m		m	b	C-ζ:b
Tyrosine		m		m	b	
Lysine		m	b (c)	m	c	
Methionine		m	b		m	

[a] About 25% of labeled bicarbonate goes into the positions predominantly originating from [1-^{13}]acetate.

sary (15a), and the pool of CO_2 is later supplemented by decarboxylation of acetate. Because acetate is mostly converted to methane and CO_2 and since the initial bicarbonate pool is diluted by CO_2 originating from acetate, large initial amounts of labeled acetate (1 g) and bicarbonate (0.2 g) were necessary to obtain sufficient material for NMR studies. Because of further difficulties in obtaining sufficient cellular material (ca. 1.1 to 1.2 g [dry weight] of cells per mol of acetate), our studies have concentrated on amino acid biosynthesis.

Amino acids. Results of the distribution of label in the amino acids obtained from [1-^{13}C]acetate, [2-^{13}C]acetate, and [^{13}C]bicarbonate are summarized in Table 1. Signals were mostly identified on the basis of earlier results obtained with *Methanospirillum hungatei* (5).

In addition to predominant labeling, a high degree of scrambling was observed, its character being the same for all amino acids. There is a considerable incorporation of the label from bicarbonate into positions expected to be labeled from the C-1 of acetate. From the relative intensities of ^{13}C-NMR signals it can be calculated that about 70% of the label from bicarbonate goes into positions expected to be labeled from CO_2, about 25% goes into those labeled predominantly from the C-1 of acetate, and below 5% goes into those labeled from the C-2 position of acetate. Also, some incorporation of the label from [1-^{13}C]acetate into "CO_2 positions" was observed, and this can be explained by the decarboxylation of the acetate. The extent of such scrambling is dependent on the total pool of CO_2 in the headspace; in our experiments with [1-^{13}C]acetate, the CO_2 positions were labeled at the level of 60 to 65%.

Glutamate, proline, and arginine. The labeling pattern of these amino acids obtained from cells grown on [2-^{13}C]acetate is shown in Fig. 1B. For comparison, similar spectra for amino acids from *Methanospirillum hungatei* and *Methanosarcina barkeri* are shown in Fig. 1A and C, respectively. It can be seen that there is a major difference in labeling of glutamic acid, proline, and arginine between *Methanosarcina barkeri* and *Methanothrix concilii* on the one hand and *Methanospirillum hungatei* on the other. In *Methanospirillum hungatei* there is a complete scrambling of the label between C-β and C-γ in glutamate (signals at 24.90 and 29.50 ppm, respectively), which is consistent with the reductive direction of the tricarboxylic acid cycle (randomization of the label at the level of succinate). In both *Methanosarcina barkeri* and *Methanothrix concilii* the C-2 position of acetate labels exclusively C-α and C-γ (signals at 52.62 and 29.50 ppm) but not C-β. This is expected for the oxidative direction of the tricarboxylic acid cycle. Labeling patterns of proline and arginine are consistent with their biosynthesis from glutamate: in both *Methanothrix concilii* and *Methanosarcina barkeri* the C-γ is labeled from the C-2 position of acetate (signals at 23.40 and 23.78 ppm for proline and arginine, respectively) as they originate from the C-γ of glutamate. Both C-β signals are missing (which are present in *Methanospirillum hungatei* at 28.36 and 26.97 ppm for proline and arginine, respectively) since C-β of glutamate is not labeled.

Isoleucine. The origin of carbons in isoleucine is the same as in *Methanospirillum hungatei* (5), which clearly shows that isoleucine is synthesized via the citramalate pathway, typical for methanogenic bacteria (3, 6).

Methionine. There is one signal at 13.88 ppm in the spectrum of *Methanothrix concilii* grown in the presence of [2-^{13}C]acetate which is not present in spectra of *Methanosarcina barkeri* and *Methanospirillum hungatei*. Its chemical shift is close to what is expected for the end methyl group of methionine. To be certain about this assignment, the amino acid mixture was separated on thin-layer chromatography plates to determine whether that signal at 13.88 ppm was still present in the fraction containing methionine. The result was positive and especially interesting since in other methanogens the methyl group of methionine is derived from CO_2 (5, 8).

Other amino acids. All other amino acids were labeled in a manner consistent with the operation of the most common biosynthetic routes—the same as in *Methanospirillum hungatei* (5).

Lipid chains. ^{13}C-NMR spectra of the total lipids extracted from *Methanothrix concilii* give a chemical shift pattern characteristic of phytanyl chains (1, 5). Based on the presence of the signals of end-CH_3 groups at 22.75 and 22.84 ppm and of C-15 at 28.39 ppm, it can be concluded that lipids contain predominantly diphytanyl chains (as opposed to bidiphytanyl). Characteristic signals of bidiphytanyl chains (C-16 at 34.58 ppm) (2, 5) cannot be detected, so based on the quantitation of the signal to noise ratio in NMR spectra the amounts of C_{40} chains must be below 5% of those of diphytanyls.

The origin of carbon atoms in lipids is shown in Fig. 2. Similar to what was found in *Methanospirillum hungatei* (5) and other archaebacteria (2), every second carbon along the chain and branch-methyl groups are labeled from the methyl group of acetate, and the remaining carbons from the C-1 of acetate (although in this case we cannot exclude CO_2, since it is exchanged with the carboxyl group of acetate). Such labeling is consistent with biosynthesis proceeding via mevalonic acid as an intermediate.

Carbohydrates. ^{13}C-NMR spectra of neutral carbohydrates obtained from *Methanothrix concilii* grown on 3.0 g of [2-^{13}C]acetate contained major signals at 95.98, 92.18, 60.76, and 60.63 ppm. They correspond to C-1 and C-6 of α and β anomers of glucose (see reference 5 for comparison). The identity of the major compound was further verified by thin-layer chromatography (on silica gel plates, with the solvent system ethyl acetate-isopropanol-water [65:67:67, vol/vol/vol]). The carbohydrate fraction from *Methanothrix concilii* gave one major spot with an R_f value of 0.53, identical to that of glucose.

The intensities of other glucose signals appearing on the ^{13}C-NMR spectrum (measured as heights of the signals) were

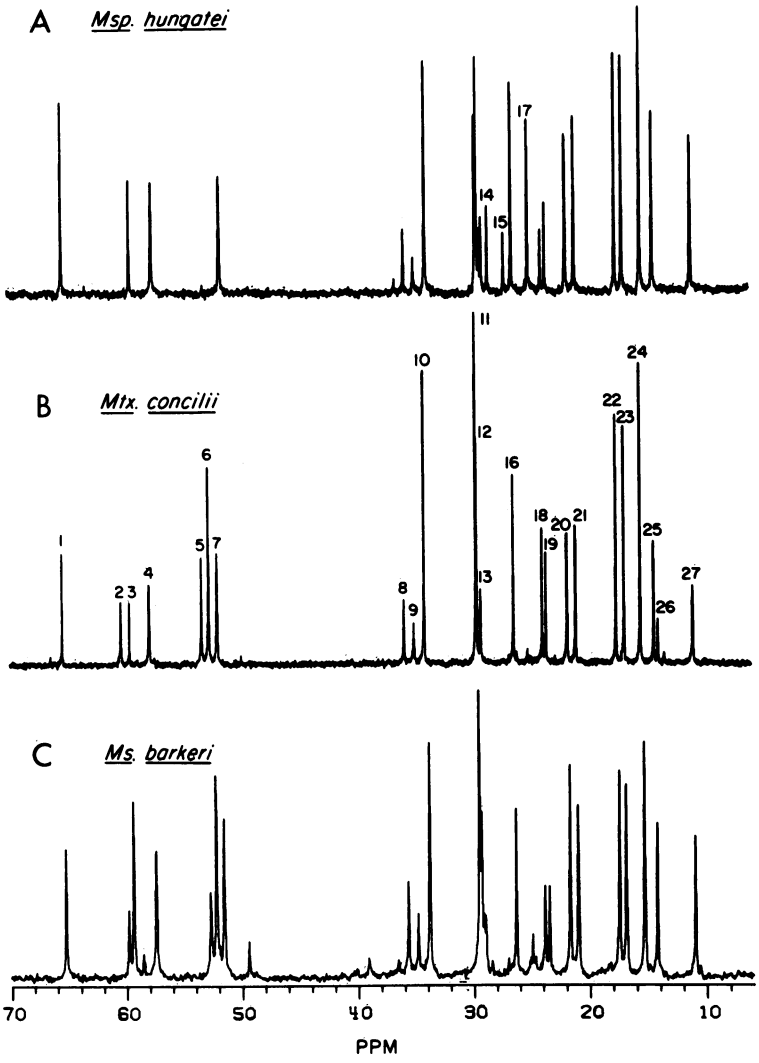

FIG. 1. High-field fragments of ^{13}C-NMR spectra (75.46 MHz) of hydrolyzed cytoplasmic proteins. Bacteria were grown on [2-^{13}C]acetate. Identification of the signals: 1, Thr, C-β; 2, Pro, C-α; 3, Ser, C-β; 4, Ile, C-α; 5, Arg, C-α; 6, Glu, C-α; 7, Leu, C-α; 8, Phe, C-β; 9, Tyr, C-β; 10, Asp, C-β; 11, Glu, C-γ; 12, Lys, C-δ; 13, Met, C-β; 14, Pro, C-β; 15, Arg, C-β; 16, Lys, C-β; 17, Glu, C-β; 18, Arg, C-γ; 19, Pro, C-γ; 20 and 21, Leu, C-δ; 22 and 23, Val, C-γ; 24, Ala, C-β; 25, Ile, C-γ'; 26, Met, C-ε; 27, Ile, C-δ. The spectrum for *Methanospirillum hungatei* (A) is taken from measurements done by Ekiel et al. (5), and that for *Methanosarcina barkeri* (C) is from unpublished data of I. Ekiel and G. D. Sprott.

determined to be between 3 and 6% of those from the C-1 and C-6 positions. This is consistent with conclusions obtained from amino acid and lipid labeling, where there was virtually no scrambling of the label originating from [2-^{13}C]acetate. The strong preference for labeling of C-1 and C-6 in glucose can be explained by biosynthesis via a glucogenic pathway similar to what was previously suggested for other methanogens (5, 11).

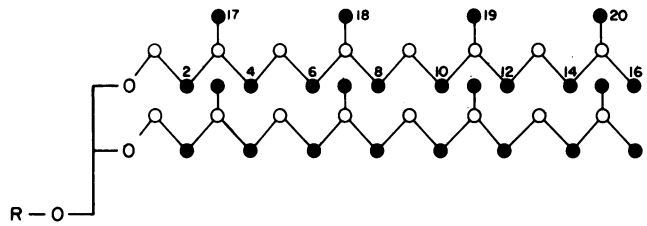

FIG. 2. Labeling pattern of lipid chains. Origin of carbon atoms: ●, methyl group of acetate; ○, carboxyl group of acetate and bicarbonate.

DISCUSSION

With the exception of methionine, the labeling of all amino acids synthesized from acetate and bicarbonate was exactly the same for *Methanothrix concilii* and *Methanosarcina barkeri*, since the labeling was fully consistent with the major biosynthetic pathways used by *Methanosarcina barkeri* (19). Labeling of the methyl group of methionine from the C-2 position of acetate as found for *Methanothrix concilii* is consistent with methyltransferase reaction between homocysteine and methyltetrahydrofolate, where serine would serve as the C-1 precursor. This contrasts with methionine biosynthesis in *Methanobacterium thermoautotrophicum* (8), *Methanospirillum hungatei* (5), and *Methanosarcina barkeri* (Fig. 1), where the methyl group of methioinine is derived from CO_2.

Our NMR results strongly indicate that the major biosynthetic reaction in *Methanothrix* spp. is the formation of pyruvate from acetate and CO_2 as found for those methanogens capable of reducing CO_2 to CH_4 (5, 8, 19). The labeling patterns and the manner of scrambling of label indicate that acetate is predominantly decarboxylated, forming CO_2 and CH_4; the CO_2 derived from acetate is in equilibrium with the bicarbonate pool with the resultant labeling of CO_2 positions by the C-1 of [1-^{13}C]acetate. These results are consistent with the involvement of a CO dehydrogenase in the acetate to $CH_3 - X + CO_2$ reactions, as in *Methanosarcina barkeri* (4), and with the finding that *Methanothrix soehngenii* has an active CO dehydrogenase (13). It is possible that the requirement for an external source of CO_2 during the early stages of *Methanothrix concilii* culture growth (15a) is due to the initial inability of the bacteria to generate sufficient CO_2 for biosynthetic purposes.

It was reported before that *Methanothrix concilii* can neither grow nor form methane from CO_2 and H_2 (15a). Similarly, *Methanothrix soehngenii* cannot reduce CO_2 with hydrogen (20). Results obtained in this work show that there is no significant (>5%) flow of the label from CO_2 into positions labeled from the methyl group of acetate. Also, no flow from the C-1 to the C-2 of acetate was observed. This means that *Methanothrix concilii* does not synthesize acetate solely from CO_2, in contrast to *Methanosarcina barkeri*, which can use CO_2/H_2 alone for both biosynthesis and methanogenesis (10).

Most methanogenic bacteria use the reductive direction of an incomplete tricarboxylic acid pathway for making glutamate and the amino acids derived from glutamate (5, 8; I. Ekiel and G. D. Sprott, unpublished data). The only exception so far is found in *Methanosarcina barkeri*, which uses an incomplete oxidative tricarboxylic acid pathway (19). As shown by Stackebrandt et al. (18), *Methanosarcina* and *Methanothrix* species are more closely related to each other than to the other methanogens, supporting their classification as members of the family *Methanosarcinaceae*. The similarity in the way they use the incomplete tricarboxylic acid cycle for making glutamate, as demonstrated in this work, gives further support for such a classification.

Another feature common to *Methanosarcina* spp. and *Methanothrix* spp. is the similarity in the way the label is scrambled. Similar to *Methanosarcina barkeri* grown on acetate (4), there was a strong scrambling between the carboxyl group of acetate and CO_2 in *Methanothrix concilii*. In *Methanosarcina barkeri*, this isotopic exchange was interpreted as a partial reaction in the methanogenic pathway from acetate (4), so it is possible that aceticlastic methanogens share a common mechanism of methane synthesis.

It is well established that the major lipids of archaebacteria are diphytanylglycerol diethers or dibiphytanyldiglycerol tetraethers, with different proportions of these two types of chains in different species (14). Our finding that the polar lipids of *Methanothrix concilii* have predominantly (or exclusively) diphytanyl chains places *Methanothrix concilii* close to *Methanothrix soehngenii* and *Methanosarcina barkeri*, since both were reported to contain only diether lipids (14).

On the basis of its morphology and substrate specificity for growth and methane production, strain GP6 was classified as a member of the genus *Methanothrix* (15a). Although further studies on the phylogenetic relatedness of *Methanothrix concilii* to other methanogens are needed, the data presented on the similarities of biosynthetic pathways in strain GP6 and *Methanosarcina* spp. support the classification of strain GP6 as a *Methanothrix* sp. in the family *Methanosarcinaceae*.

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