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Metabolic Pathways in *Methanococcus jannaschii* and Other Methanogenic Bacteria†

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Eleven strains of methanogenic bacteria were divided into two groups on the basis of the directionality (oxidative or reductive) of their citric acid pathways. These pathways were readily identified for most methanogens from the patterns of carbon atom labeling in glutamate, following growth in the presence of [2-^{13}C]acetate. All used noncyclic pathways, but members of the family *Methanosarcinaceae* were the only methanogens found to use the oxidative direction. *Methanococcus jannaschii* failed to incorporate carbon from acetate despite transmembrane equilibration comparable to other weak acids. This organism was devoid of detectable activities of the acetate-incorporating enzymes acetyl coenzyme A synthetase, acetate kinase, and phosphotransacetylase. However, incorporation of [1-^{13}C]-, [2-^{13}C]-, or [3-^{13}C]pyruvate during the growth of *M. jannaschii* was possible and resulted in labeling patterns indicative of a noncyclic citric acid pathway operating in the reductive direction to synthesize amino acids. Carbohydrates were labeled consistent with glucogenesis from pyruvate. Leucine, isoleucine, phenylalanine, lysine, formate, glycerol, and mevalonate were incorporated when supplied to the growth medium. Lysine was preferentially incorporated into the lipid fraction, suggesting a role as a phytanyl chain precursor.

^{14}C-labeling and enzyme analysis has shown that *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri* assimilate the carbon of acetate via incomplete TCA (tricarboxylic acid) pathways which differ in their direction of operation. The pathway functions in a reductive direction to generate glutamate in *M. thermoautotrophicum* (5, 13, 36), whereas an oxidative direction is used in *M. barkeri* (33).

^{13}C nuclear magnetic resonance (NMR) methods have confirmed and extended these results to provide pathway information for the synthesis of most of the amino acids of *Methanospirillum hungatei* (7), *Methanococcus voltae* (6), and *Methanosaeta concilii* (9). The basic premise required for the NMR method to succeed is that during growth on CO_2-H_2, the methanogen incorporates ^{13}C labels, usually [1-^{13}C]- or [2-^{13}C]acetate. Long-term labeling is permitted because of the noncyclic nature of the biosynthetic pathways, often leading to relatively low scrambling of label in the cell metabolites. (The extent of scrambling, as used in this article, is the degree of incorporation of exogenous label into carbon positions in the cell metabolites where label is unexpected based on the central metabolic pathways in use.) Despite biosynthetic dilution of [^{13}C]acetate, known to occur in methanogens capable of synthesizing acetyl coenzyme A from two CO_2 molecules (32), sufficient label is still incorporated to obtain good NMR spectra starting with as little as 50 mg (dry weight) of ^{13}C-enriched cells (ca. 100-ml cultures). It is possible, therefore, to use the ^{13}C-NMR method to rapidly screen these bacteria for reductive versus oxidative pathways, to ascertain from scrambling levels whether pathways are cyclic, and to exclude pathways inconsistent with the labeling patterns.

Methanococcus jannaschii is a methanogen isolated from a sample collected from the base of a white smoker chimney (19). The bacterium grew most rapidly at 83°C, at a pH near 6.0, and a Na^+ concentration of about 0.5 M. CO_2 plus H_2 was the only substrate found to support growth (19). The metabolic pathways in *M. jannaschii*, and most other methanogens, have not been reported. Here we determine that assimilation of [^{13}C]pyruvate by *M. jannaschii* provides an effective means to monitor metabolism by ^{13}C-NMR, and compare the pathways in *M. jannaschii* to those in several other methanogens labeled by [^{13}C]acetate.

MATERIALS AND METHODS

Growth of methanogens. Organisms were obtained from the sources described by Sprott et al. (30). Growth was in 100-ml aliquots of medium dispensed in 1-liter capped bottles which were shaken at 120 rpm. Incubation was at 35°C except for *M. jannaschii*, grown at 65°C, and *M. thermoautotrophicum*, grown at 62°C. *Methanobacterium thermolithotrophicum* labeled by [2-^{13}C]acetate was a gift from R. Sparling, University of Manitoba. Defined medium JM (17) was used for most methanogens; exceptions were several defined media specifically designed for *Methanobacterium espanolae* (24), *M. concilii* (23), *M. jannaschii* (12), *M. voltae* (34), and *Methanothermus fervidus* (30), and complex Balch 3 medium (2) for *Methanosarcina mazei*. Substrates for growth were CO_2-H_2 (20/80, vol/vol) with the exceptions of *M. hungatei* (CO_2-H_2 plus acetate), *M. concilii* (acetate, N_2 gas phase), and *M. mazei* (methanol, N_2 gas phase). Cultures metabolizing CO_2-H_2 were pressurized daily to 70 kPa; *M. jannaschii* was pressurized twice daily and received 0.4 mM cysteine and 0.26 mM NaS · $9H_2O$ daily.

To label with ^{13}C-compounds, we omitted growth substrates from the media except for inclusion of the desired gas phase. Aqueous 20% (vol/vol) methanol (*M. mazei*, 125 mM) and filter-sterilized ^{13}C-compounds were added after auto-

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claving. Additions of sodium [^{13}C]acetate were 100 mg/100 ml, and sodium [^{13}C]pyruvate was added at 30 mg/100 ml.

Cell fractionation. In general, cells from a 100-ml culture were harvested and broken by French pressure cell treatment in 8 ml of 20 mM Tris-HCl (pH 7.5)–10 mM magnesium acetate–30 mM NH_4Cl–6 mM 2-mercaptoethanol buffer (7). Lysis in water (6 ml per pellet derived from a 100-ml cell culture) was used for *M. jannaschii* and *M. voltae*. Lysates incubated with DNase I (0.5 mg) and RNase I (0.5 mg) for 1 h at room temperature were then clarified by centrifugation at 12,000 × *g* for 30 min.

Proteins were precipitated from the supernatant with ethanol (70% final) at 4°C for 1 h and collected by centrifugation at 10,500 × *g* for 15 min. The protein pellets, washed once with 9 ml of 80% ethanol, were hydrolyzed with 6 N HCl in vacuo at 110°C for 48 h. Protein hydrolysates were dried in vacuo and dissolved in D_2O at pH 0.8.

Lysate pellets were extracted for lipid by the neutral Bligh and Dyer (3) method. Lipids were dried and dissolved in benzene-d_6/methanol-d_4 (7:2, vol/vol). Carbohydrates were recovered from the lipid-depleted cell residue by hydrolyzing it for 4 h at 110°C with 2 M H_2SO_4. The acidic extract was neutralized with $BaCO_3$ and clarified at 10,500 × *g* for 15 min, and the supernatant was passed through cation- and anion-exchange columns (7). Carbohydrates were lyophilized twice from water and resuspended in D_2O.

NMR analyses. Spectra were recorded with a Bruker AM 500 spectrometer operating at room temperature and 75 MHz. Tetramethylsilane was used as an internal chemical shift reference.

Distribution of weak acids. *M. jannaschii* cells were harvested anaerobically under CO_2-H_2 at 7,400 × *g* for 5 min and resuspended into 5-ml aliquots of fresh medium (7.5 to 9.0 mg [dry weight] of cells). Internal and external spaces and distribution of weak acids were determined as described previously (31) in packed cell pellets (7,400 × *g* for 10 min) following 15-min incubations at 62°C in the presence of ^{14}C-compounds. Penetrations were compared with total water space (gravimetric) for the following: glucose, 40 μM, 4.1 μCi/μmol; taurine, 3.0 μM, 56 μCi/μmol; inulin, 0.56 mg, 2 μCi/mg; and urea, 100 μM, 0.15 μCi/μmol. Sodium salts of weak acids were used at the following concentrations: 5,5-dimethyl-2,4-oxazolidinedione (DMO), 8.7 μM, 46 μCi/μmol; pyruvate, 0.4 mM, 0.25 μCi/μmol; acetate, 0.2 mM, 2.5 μCi/μmol; propionate, 32 μM, 6.3 μCi/μmol; and butyrate, 22 μM, 13.4 μCi/μmol. Cytoplasmic pH was calculated using a pK_a of 6.0 for DMO, extrapolated to 62°C from the data of Addanki et al. (1). Other pK_a values were obtained from Kaback (20) or from a biochemistry handbook.

Incorporation of ^{14}C-compounds. The ^{14}C-compound was added to 10 ml of growth medium at the concentration indicated just prior to inoculation with *M. jannaschii* (2%, vol/vol). Uptake was determined as the counts retained upon filtration of 0.5-ml samples on 0.45-μm-pore-size membrane filters washed with 5 ml of fresh growth medium.

To determine the distribution of ^{14}C, we labeled *M. jannaschii* by growth (2% inoculum) in 100 ml of medium containing [2-^{14}C]pyruvate (200 μM, 0.25 μCi/μmol), [U-^{14}C]glycerol (100 μM, 1.0 μCi/μmol), L-[U-^{14}C]lysine (50 μM, 0.2 μCi/μmol), or [2-^{14}C]mevalonate (2.13 μM, 47 μCi/μmol). Cells were harvested and washed twice with 8-ml aliquots of 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 6.0) containing 0.05 M KCl, 0.05 M $MgCl_2$, and 0.4 M sucrose. Cell fractions were obtained by treating sequentially with cold TCA (cell pool), ethanol and diethyl ether (lipid), and hot TCA (nucleic acid), leaving protein as the residue (14).

Enzyme assays. *M. jannaschii*, corresponding to 30 mg of dry weight in the late logarithmic growth phase, was harvested (4,400 × *g* for 10 min) under an atmosphere of H_2-CO_2 and washed with fresh growth medium in 15-ml Corex centrifuge tubes fitted with serum vial closures. Complete lysis was achieved by resuspending the cell pellet into 4 ml of anaerobic, 0.1 M Tricine-KOH buffer (pH 7.9) in the presence of 0.5 mg of DNase. The lysed suspension was clarified by centrifuging as before and transferred with an O_2-free syringe to a serum vial filled with H_2. The cell extract was stored on ice and used within 4 h. Protein was determined by the Coomassie blue method with bovine serum albumin as the standard (4). Buffers were prereduced by storage under H_2 in the presence of 0.1 mM $Na_2S \cdot 9H_2O$.

Acetyl coenzyme A synthetase (EC 6.2.1.1) and acetate kinase (EC 2.7.2.1) were assayed at 50°C in Tricine-KOH buffer, and phosphotransacetylase (EC 2.3.1.8) was assayed at 50°C in Tris-HCl (0.1 M, pH 7.3) by the method of Oberlies et al. (22). No oxidation to pink coloration was observed when resazurin was included to monitor anaerobiosis. Reaction mixtures containing cell extract with no activities were retested after spiking the assay mixture with the respective commercial enzyme.

Reproducibility. Experiments were repeated at least twice, and representative data are shown.

Materials. ^{14}C-compounds were uniformly labeled except for [3-^{14}C]propionate, [1-^{14}C]butyrate, [2-^{14}C]DMO, [2-^{14}C]pyruvate, [2-^{14}C]mevalonate (used as the dibenzoylethylenediamine salt), and [1,5-^{14}C]citrate. Radioactive compounds were purchased from Dupont Canada Ltd., Mississauga, Ontario, except for L-serine and L-malate (Amersham Canada Ltd., Oakville, Ontario). Sodium salts of [1-^{13}C]pyruvate (99 atom% ^{13}C), [2-^{13}C]pyruvate (99 atom%), [3-^{13}C]pyruvate (99.1 atom%), [1-^{13}C]acetate (99 atom%), [2-^{13}C]acetate (99 atom%), and L-[U-^{13}C]lysine (93 atom%) were products of MSD Isotopes, Montreal, Quebec, Canada. Acetyl coenzyme A synthetase (baker's yeast), acetate kinase (*Bacillus stearothermophilus*), and phosphotransacetylase (*Leuconostoc mesenteroides*) were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

TCA pathway in methanogens. Six genera of methanogens were tested, and five of these incorporated the [2-^{13}C]acetate label into only the C-3 and C-4 positions of glutamate, indicating a reductive TCA pathway (Table 1). In contrast, glutamate was labeled in positions C-2 and C-4 in the members of the family *Methanosarcinaceae* tested. The labeling patterns of the glutamate and aspartate families of amino acids from *M. mazei* were the same as reported previously for *M. barkeri* (9), suggesting that the oxidative TCA pathway may be a general phenomenon of *Methanosarcina* spp.

Biosynthesis in *M. jannaschii*. Label from [^{13}C]acetate was not incorporated into cell components at detectable amounts during the growth of *M. jannaschii*. Comparison of uptake of [^{14}C]pyruvate and [^{14}C]acetate into growing cells confirmed that acetate was not taken up appreciably (Fig. 1) and suggested that [^{13}C]pyruvate would be an appropriate alternate substrate for the NMR study.

Amino acids. Signals were assigned from earlier results with similar amino acid hydrolysates from *M. hungatei* (7). Hydrolysates derived from *M. jannaschii* grown on [2-^{13}C]-

TABLE 1. Directionality of partial TCA pathways in methanogenic bacteria, as determined by ^{13}C-NMR[a]

Methanogen	Strain	Directionality	Reference or source
Methanococcus jannaschii	JAL-1	Reductive	This study
Methanococcus voltae	PS	Reductive	6
Methanothermus fervidus	V245	Reductive	This study
Methanospirillum hungatei	GP1	Reductive	7
	JF1	Reductive	This study
Methanobrevibacter smithii	PS	Reductive	This study
Methanobrevibacter arboriphilus	DH1	Reductive	This study
Methanobacterium bryantii	M.o.H.	Reductive	6
	M.o.H.G.	Reductive	This study
Methanobacterium thermoautotrophicum	ΔH	Reductive	11, this study
Methanobacterium strain	G2R	Reductive	This study
Methanobacterium thermolithotrophicum	SN1	Reductive	27, this study
Methanobacterium espanolae	GP9	Reductive	This study
Methanogenium cariaci		Reductive	27
Methanosarcina barkeri	MS	Oxidative	9
Methanosarcina mazei	S6	Oxidative	This study
Methanosaeta concilii	GP6	Oxidative	9
Methanohalophilus sp.	FDF1	Oxidative	26

[a] Cells were labeled by growth in media containing [2-^{13}C]acetate, under an atmosphere of H_2-CO_2 (4:1, vol/vol). Exceptions were *M. mazei*, grown on [2-^{13}C]acetate plus 0.4% (vol/vol) unlabeled methanol in an N_2 atmosphere, and *M. concilii*, grown on [2-^{13}C]acetate in an N_2 atmosphere.

and [3-^{13}C]pyruvate indicated that the label was scrambled not more than 10 to 15% (Fig. 2). Part of this scrambling (degree not assessed) is expected via pyruvate synthesis from the natural abundance of $^{13}CO_2$ in the CO_2 supplied for growth. Scrambling was most extensive with [1-^{13}C]pyruvate labeling, in which most unassigned signals appear to be accounted for by scrambling at the level of pyruvate from carbon 1 into carbons 2 and 3. Although dilution of [^{13}C]pyruvate probably occurs from de novo synthesis of pyruvate from CO_2, this was insufficient to cause difficulty in detecting most signals excluding histidine and methionine.

Glutamate, proline, and arginine. Label from [3-^{13}C]pyruvate was randomized in these amino acids into positions C-3 and C-4, indicating a reductive TCA pathway (Fig. 2; Table 2). This pattern is comparable to the glutamate family of amino acids in *M. hungatei* labeled with [2-^{13}C]acetate (7). Similarly, [1-^{13}C]pyruvate labeled those carbon positions which in *M. hungatei* are derived from CO_2 via pyruvate synthesis from acetyl coenzyme A, whereas [2-^{13}C]pyruvate compared to labeling with [1-^{13}C]acetate in *M. hungatei* (7).

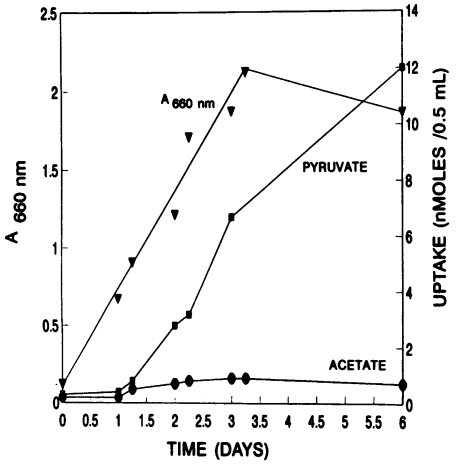

FIG. 1. Uptake of [2-^{14}C]pyruvate and [1,2-^{14}C]acetate during growth of *M. jannaschii*. Concentrations of pyruvate (1.60 μCi/μmol) and acetate (0.94 μCi/μmol) were 100 μM.

Aspartate, threonine, and methionine. Incorporation of the C-1, C-2, and C-3 of pyruvate into the first three carbon atoms of aspartate (Fig. 2; Table 2) shows clearly that aspartate is synthesized from pyruvate via the reductive TCA pathway. Threonine and methionine labeling patterns are consistent with their formation from aspartate. Methionine, however, was present in insufficient amount to detect most signals.

Isoleucine. The labeling patterns for isoleucine (Table 2) corresponded to those found for other methanogens (6–9), clearly indicating synthesis by the citramalate pathway.

Other amino acids. Labeling patterns for other amino acids shown in Table 2 were consistent with the biosynthetic routes common to most bacteria and reported for *M. hungatei* (7). Lysine was made by the diaminopimelic acid pathway. Histidine labeling was difficult to determine because of the small amounts of this amino acid present in the hydrolysates.

Carbohydrates. Hydrolysis of the lipid-depleted particulate cell material produced a monosaccharide fraction composed predominantly of glucose, galactose, and mannose. Growth in the presence of [3-^{13}C]pyruvate labeled the anomeric carbons (Fig. 3A) and C-6 positions (data not shown) of hexoses. Labeling with [2-^{13}C]pyruvate resulted in incorporation of ^{13}C into C-2 and C-5 of hexoses (Fig. 3B).

Lipids. The phytanyl chains of $C_{20,20}$-diether lipids were labeled by [2-^{13}C]pyruvate in positions 1, 3, 5, 7, 9, 11, 13, and 15, whereas [3-^{13}C]pyruvate was incorporated into positions 2, 4, 6, 8, 10, 12, 14, and 16 and the methyl groups at carbons 17, 18, 19, and 20. Macrocyclic diethers and tetraether lipid chains were labeled as expected from the above pattern.

Acetate assimilation. Incubation for 15 min at 62°C with various weak acids resulted generally in a distribution (in/out) of the compounds across the cell membrane indicative of the magnitude of the imposed pH gradient (Table 3) and of their relative pK_a values. Pyruvate, with the lowest pK_a, may have produced the lowest estimate of cytoplasmic pH

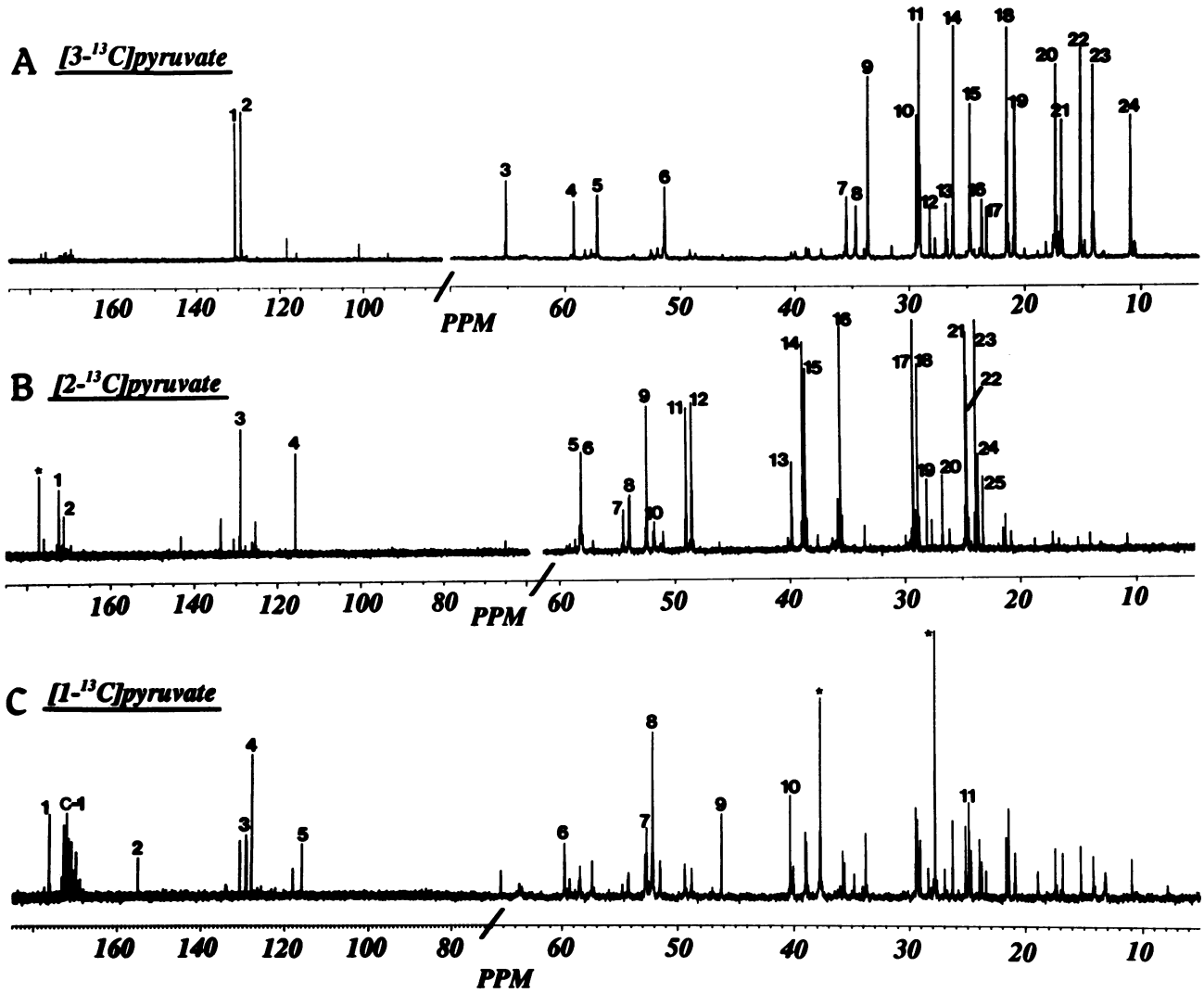

FIG. 2. ^{13}C-NMR spectra of amino acid hydrolysates from *M. jannaschii* grown on [^{13}C]pyruvate and CO_2-H_2. (A) [3-^{13}C]pyruvate; signals: 1, Tyr C-5′; 2, Phe C-5′; 3, Thr C-3; 4, Ser C-3; 5, Ile C-2; 6, Leu C-2; 7, Phe C-3; 8, Tyr C-3; 9, Asp C-3; 10, Glu C-4; 11, Lys C-5; 12, Pro C-3; 13, Arg C-3; 14, Lys C-3; 15, Glu C-3; 16, Arg C-4; 17, Pro C-4; 18 and 19, Leu C-5 and Leu 4-CH_3; 20 and 21, Val C-4 and Val 3-CH_3; 22, Ala C-3; 23, Ile 3-CH_3; 24, Ile C-5. (B) [2-^{13}C]pyruvate; signals: 1, Leu C-1; 2, Ile C-1; 3, Phe C-6′; 4, Tyr C-6′; 5, Thr C-2; 6, Val C-2; 7, Ser C-2; 8, Phe + Tyr C-2; 9, Lys C-2; 10, Met C-2; 11, Asp C-2; 12, Ala C-2; 13, Gly C-2; 14, Lys C-6; 15, Leu C-3; 16, Ile C-3; 17, Glu C-4; 18, Val C-3; 19, Pro C-3; 20, Arg C-3; 21, Glu C-3; 22, Ile C-4; 23, Leu C-4; 24, Arg C-4; and 25, Pro C-4. (C) [1-^{13}C]pyruvate; signals: 1, Glu C-5; 2, Tyr 7′ or 8′; 3 and 4, Phe 7′ and 8′; 5, Tyr 7′ or 8′; 6, Pro C-2; 7, Arg C-2; 8, Glu C-2; 9, Pro C-5; 10, Arg C-5; and 11, His C-3. *, unassigned major signals; C-1, unassigned carboxyl signals.

because of partial quenching of the pH gradient by the relatively high concentration of pyruvate used.

Acetate penetrated equally well compared to propionate and butyrate, giving a cytoplasmic pH typical of other methanogens (18). Since acetate freely penetrated the cells and CO_2 served as the sole carbon source for growth, the inability to incorporate exogenous acetate is predicted to result from an inability to generate acetyl coenzyme A for biosynthesis from acetate. Phosphotransacetylase, acetyl coenzyme A synthetase, and acetate kinase were not detected in assays with up to 0.42, 0.84, or 1.1 mg of protein, respectively. In all cases, the assay conditions were confirmed to be valid by registering a positive enzyme analysis upon the addition of the respective commercial enzyme.

Incorporation of other compounds. ^{14}C label from several hydrophobic amino acids, formate, and malate was incorporated into biomass during growth (Table 4). Lesser amounts of mevalonate, glycerol, and lysine were taken up (Tables 4 and 5). Fractionation of the cells revealed that pyruvate was distributed among proteins, nucleic acids, and lipids. Glycerol and mevalonate labels were found largely in the lipid fraction, as expected, whereas lysine was incorporated relatively more into lipids than into proteins. The possibility that lysine might serve as a phytanyl chain precursor was tested by growing cells (100-ml culture) in the presence of 10 mg of L-[U-^{13}C]lysine. Unfortunately, the low extent of uptake (Table 5) prevented detection of ^{13}C signals in the extracted lipid fraction. Similarly, no difference was detected in signal intensity from phytanyl chain carbons labeled by growth with 2.7 mM [2-^{13}C]- or [3-^{13}C]pyruvate

TABLE 2. Origin of carbon atoms from [^{13}C]pyruvate in amino acids synthesized by mixotrophic growth of *M. jannaschii* on CO_2-H_2 and pyruvate[a]

Amino acid	Amino acid carbon atom:						
	C-1[b]	C-2[b]	C-3[b]	C-4[b]	C-5[b]	C-6[b]	Other[b]
Ala, Ser	(1)[c]	2	3				
Gly	(1)[c]	2					
Asp, Thr	(1)[c]	2	3	U			
Phe, Tyr	(1)[c]	2	3				C-4′, (2)[c]; C-6′, 2 C-5′, 3; C-9′, (3)[c] C-7′, 1; C-8′, 1
Glu, Pro	U	1	2, 3	2, 3	1		
Arg	U	1	2, 3	2, 3	1		C-7, U
Leu	2	3	2	2	3		4-CH_3, 3
Val	(1)[c]	2	2	3			3-CH_3, 3
Ile	2	3	2	2	3		3-CH_3, 3
His	(3)[c]	(2)[c]	1	(1)[c]	2		C-7, (3)[c]
Lys	(1)[c]	2	3	U	3	2	
Met	(1)[c]	2	(3)[c]	U			S-CH_3, (U)[c]

[a] Grown on CO_2-H_2 with either [1-^{13}C]-, [2-^{13}C]-, or [3-^{13}C]pyruvate. This table is the composite of separate labelings with each ^{13}C label. U, unlabeled (CO_2).

[b] Source of the ^{13}C label from pyruvate carbon 1, 2, or 3 or from CO_2.

[c] Signal either not detected or assigned; the theoretical labeling patterns shown in parentheses are based on the pathways described in *M. hungatei* (7).

regardless of whether 5.4 mM L-lysine was included in the growth medium (data not shown).

Glucose, citrate, aspartate, and serine were not assimilated during growth. Lack of glucose assimilation suggests that glucose does not cross the cell membrane, supporting its use as an indicator of extracellular volume (Table 3) in *M. jannaschii*.

DISCUSSION

Metabolism in methanogens is known to proceed via citric acid cycle enzymes, which function in an incomplete pathway in either oxidative or reductive directions (Table 1) (13, 27, 31). Growth of most methanogens on H_2 plus CO_2 in media containing [2-^{13}C]acetate resulted in the incorporation of ^{13}C into cell carbon. Methanogens grown autotrophically or mixotrophically (CO_2-H_2 being one substrate) could be subdivided into those that require acetate for growth on CO_2-H_2 and synthesize amino acids relatively highly enriched in ^{13}C, those that are capable of autotrophic growth resulting in dilution of [^{13}C]acetate incorporation, and those such as *M. jannaschii* which grow autotrophically but fail to incorporate sufficient [^{13}C]acetate for detection.

^{13}C-NMR analyses of total protein hydrolysates from labeled cells readily detected signals derived, for example, from enrichment of the diagnostic α (oxidative) versus β (reductive) carbons of the glutamate family of amino acids (6, 7). Thermophilicity, halophilicity, or substrate utilization range does not appear to correlate with pathway directionality. However, strains of the same species or species of the same genus had, without exception, the same type of partial TCA pathway, albeit the sampling size is small for generalizations. On the basis of our present data, the oxidative-partial TCA pathway is characteristic of the family *Methanosarcinaceae* (Table 1).

Acetic acid diffused into cells of *M. jannaschii* and accumulated in response to the transmembrane pH gradient. Acetyl coenzyme A synthetase, acetate kinase, and phosphotransacetylase were not detected in extracts of *M. jannaschii*, explaining why the bacterium is defective in acetate assimilation. Since *M. jannaschii* grows autotrophically, it clearly has the capability of forming acetyl coenzyme A from CO_2. In contrast, acetate was assimilated from the growth medium in other methanogens (Table 1), indicating activation of acetate in these strains either by acetyl coenzyme A synthetase (22) or by the combination of acetate kinase and phosphotransacetylase (21).

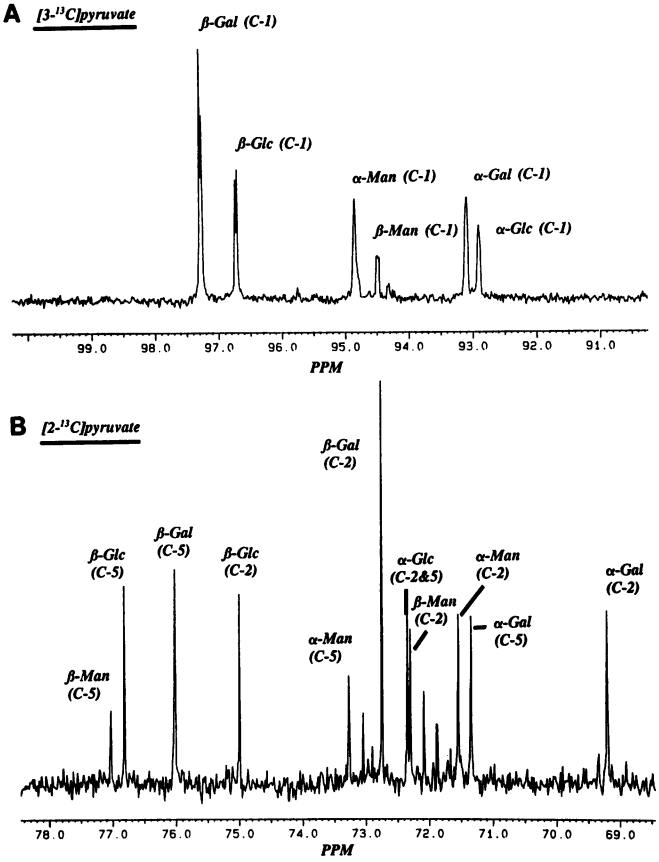

FIG. 3. ^{13}C-NMR spectra of the carbohydrate fraction from *M. jannaschii*. (A) Spectrum expanded to show the signals from anomeric carbons of monosaccharides labeled by [3-^{13}C]pyruvate. (B) Spectrum of monosaccharides following labeling by [2-^{13}C]pyruvate. Assignments are shown to the right of each signal.

It was possible to deduce the amino acid biosynthetic pathways found in *M. jannaschii* from ^{13}C labeling patterns when cells were grown on CO_2-H_2 and either [1-^{13}C]-, [2-^{13}C]-, or [3-^{13}C]pyruvate. Lysine was synthesized by the diaminopimelic acid route found in other methanogens (6, 9),

TABLE 3. Distribution of weak acids in *M. jannaschii* (62°C)

Organic acid	pH_o[a]	In/out	pH_i[a]	ΔpH
Pyruvic	5.84	4.65	6.51	0.67
Acetic	5.85	10.25	6.89	1.04
Propionic	5.83	12.44	6.97	1.14
Butyric	5.90	11.56	6.99	1.09
DMO	5.86	3.68	6.73	0.87

[a] pH_o, pH of the environment; pH_i, cytoplasmic pH.

TABLE 4. Incorporation of ^{14}C-compounds during growth of *M. jannaschii* on H_2-CO_2[a]

Compound	Initial concn (μM)	Sp act (μCi/μmol)	Uptake (nmol/mg)
Leu	100	0.45	114
Ile	100	1.7	73
Phe	100	3.3	34
Ser	100	2.1	0.72
Asp	50	2.1	0.33
Formate	67	0.58	89
Malate	130	0.45	10
Citrate	100	2.7	0.64
Glucose	20	4.1	0.97
Glycerol	100	2.6	1.34
Mevalonate	2.1	47	3.1

[a] Uptake of each ^{14}C-compound was assessed separately in serum bottles containing defined growth medium. Culture densities at the time of harvest were 0.53 to 0.68 mg of cells (dry weight) per ml.

whereas in eukaryotic cells and *Thermoproteus neutrophilus* (28), the 2-aminoadipate pathway is used. Isoleucine was made via the citramalate pathway found in other methanogens. The S-CH_3 group of methionine in *M. concilii* most likely originates from the C-3 of serine, perhaps via a tetrahydromethanopterin-dependent serine transhydroxymethylase similar to that found in *M. thermoautotrophicum* ΔH (15), since it is labeled by [2-^{13}C]acetate (9). However, this carbon is derived from CO_2 in several other methanogens (9), indicating that the CH_3 donor in these cases may derive its methyl group from a CO_2 reduced in the methanogenic pathway. In *M. jannaschii*, the latter reaction is likely, but the data are only preliminary, being complicated by scrambling of the [1-^{13}C]pyruvate label and the low amounts of methionine present in our extracts. Other pathways were the same as for *M. hungatei* (7).

M. jannaschii has less versatility than *M. voltae* to assimilate appreciable amounts of a range of exogenous substrates (6). The ability of *M. voltae* to actively transport most amino acids (6, 16) is apparently not a property common to all *Methanococcus* spp. In addition, *M. jannaschii* contrasts with many other methanococci in exhibiting an inability to grow on formate, although formate dehydrogenase activity is present (19) and formate is incorporated during growth on CO_2-H_2.

[^{14}C]lysine is assimilated from the medium into lipids and proteins, such that of the small amount of label taken up by *M. jannaschii*, nearly 80% appears in the lipid fraction (Table 5). This does not occur in *M. voltae*, in which most of the label was incorporated into protein (6). The possibility exists that mevalonate is partially synthesized from biosynthetic lysine in *M. jannaschii*, as found in *Halobacterium cutirubrum* (10). This was difficult to assess further, because of the small amounts of exogenous lysine taken up by *M. jannaschii*.

TABLE 5. Cell fractionation following incorporation of ^{14}C-compounds during growth of *M. jannaschii*[a]

Fraction	% Incorporated			
	Pyruvate	Glycerol	Lysine	Mevalonate
Cell pool	13.9 ± 1.4	56.6 ± 2.0	47.1 ± 1.7	2.9 ± 1.5
Lipid	25.4 ± 0.5	22.9 ± 9.4	31.2 ± 2.4	80.8 ± 3.0
Nucleic acid	24.9 ± 6.0	13.6 ± 7.0	6.5 ± 0.1	0.6 ± 0.3
Protein	35.9 ± 4.1	6.9 ± 0.4	15.5 ± 4.4	16.0 ± 3.9

[a] Uptake of the labels from the media during growth was 36% pyruvate, 1.7% glycerol, 0.6% lysine, and 33% mevalonate. Data are shown as the average of two independent growth experiments with standard deviations.

Labeling of amino acids during growth of *M. jannaschii* on [1-^{13}C]pyruvate (plus CO_2-H_2) resulted in a significant amount of label appearing in carbon positions strongly labeled by [2-^{13}C]pyruvate and [3-^{13}C]pyruvate (Fig. 2). This suggests scrambling at the level of pyruvate. A scheme to explain these results can be hypothesized based on the finding that anaerobic acetogenic bacteria catalyze the combination of a one-carbon intermediate generated from the carboxyl of pyruvate with coenzyme A and methyltetrahydrofolate via a corrinoid enzyme to form acetyl coenzyme A (25). If a similar reaction occurs in *M. jannaschii*, the carboxyl group of [1-^{13}C]pyruvate would, in part, be released to form $^{13}CO_2$ and $^{13}CH_3$-methanopterin. Dilution with unlabeled precursors and combination with coenzyme A could form acetyl coenzyme A, followed by the pyruvate synthase reaction to generate [2-^{13}C]pyruvate and [3-^{13}C]pyruvate. Synthesis of acetate from pyruvate has been detected in several *Methanococcus* spp., but repression of this activity by H_2 may prevent the operation of a futile cycle of pyruvate oxidation and synthesis (29, 35). Such repression by H_2 may be less pronounced in *M. jannaschii*.

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