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Pseudoauxotrophy of *Methanococcus voltae* for Acetate, Leucine, and Isoleucine

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Methanococcus voltae **is a methanogenic bacterium which requires leucine, isoleucine, and acetate for growth. However, it also can synthesize these amino acids, and it is capable of low levels of autotrophic acetyl coenzyme A (acetyl-CoA) biosynthesis. When cells were grown in the presence of $^{14}CO_2$, as well as in the presence of compounds required for growth, the alanine found in the cellular protein was radiolabeled. The percentages of radiolabel in the C-1, C-2, and C-3 positions of alanine were 64, 24, and 16%, respectively. The incorporation of radiolabel into the C-2 and C-3 positions of alanine demonstrated the autotrophic acetyl-CoA biosynthetic pathway in this bacterium. Additional evidence was obtained in cell extracts in which autotrophically synthesized acetyl-CoA was trapped into lactate. In these extracts, both CO and CH_2O stimulated acetyl-CoA synthesis. $^{14}CH_2O$ was specifically incorporated into the C-3 of lactate. Cell extracts of *M. voltae* also contained low levels of CO dehydrogenase, 13 nmol min^{-1} mg of $protein^{-1}$. These results further confirmed the presence of the autotrophic acetyl-CoA biosynthetic pathway in *M. voltae*. Likewise, $^{14}CO_2$ and [U-^{14}C]acetate were also incorporated into leucine and isoleucine during growth. During growth with [U-^{14}C]leucine or [U-^{14}C]isoleucine, the specific radioactivity of these amino acids in the culture medium declined, and the specific radioactivities of these amino acids recovered from the cellular protein were 32 to 40% lower than the initial specific radioactivities in the medium. Cell extracts of *M. voltae* also contained levels of isopropyl malate synthase, an enzyme that is specific to the leucine biosynthetic pathway, of 0.8 nmol min^{-1} mg of $protein^{-1}$. Thus, *M. voltae* is capable of autotrophic CO_2 fixation and leucine and isoleucine biosynthesis.**

Methanogenic bacteria are archaebacteria and strict anaerobes which obtain their energy for growth by producing methane from $H_2 + CO_2$, formate, methanol, methylamines, acetate and a few other simple organic compounds (23, 27). Many methane-producing bacteria can grow autotrophically. However, some lack the capacity for autotrophic growth and need to obtain organic carbon from their environment.

During autotrophic growth, *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri* are capable of the total synthesis of acetyl coenzyme A (acetyl-CoA) from two CO_2 molecules via a pathway resembling that described in *Clostridium thermoaceticum* (8, 9, 15, 31). Results of our studies have also demonstrated that the autotrophic acetyl-CoA biosynthetic pathway is present in *Methanococcus maripaludis*, which is a representative of the third major order of methanogens (19). Thus, it appears that this pathway is widely distributed among autotrophic methanogens. In all cases, CO dehydrogenase is a key enzyme in this pathway. Methanogens which are unable to grow autotrophically lack CO dehydrogenase and show a dependency on acetate as a carbon source (3, 23). The biosynthesis of acetyl-CoA and CO_2 reduction to methane share a common C_1 pool (21). The methyl group of the acetate is derived from methyltetrahydromethanopterin, an intermediate in methanogenesis (7, 14, 19, 30). Thus, the syntheses of acetate and methanogenesis are linked processes.

Methanococcus voltae requires leucine, isoleucine, and acetate for growth (24). The volatile fatty acids 2-methylbutyrate and isovalerate can substitute for isoleucine and leucine, respectively (24). Propionate can also substitute for isoleucine, and pantoyl lactone is stimulatory to growth (5, 6, 26). Omission of either one of the amino acids, the corresponding fatty acids, or acetate completely prevents growth (24). Here we report evidence that *M. voltae* is also capable of synthesizing these amino acids and acetate from CO_2 by the autotrophic acetyl-CoA pathway. Thus, these nutrients are required for growth, even though the cells retain the capacity for their biosynthesis. We call this phenomenon pseudoauxotrophy.

MATERIALS AND METHODS

Growth of bacteria and preparation of extracts. *M. voltae* and *M. maripaludis* were grown as described previously (13, 24, 25). When the cells were radiolabeled with $^{14}CO_2$, the concentrations of leucine and isoleucine in the medium were reduced to 0.76 mM, and the acetate concentration was reduced to 4.4 mM. *M. voltae* was preadapted for growth in low levels of leucine and isoleucine by at least two transfers in this medium. The size of the inoculum was 2%. Cells were harvested by centrifugation at 10,000 × *g* for 30 min at 4°C. Cells were lysed under anaerobic conditions by suspending the cell pellets in 25 mM anaerobic PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (pH 6.8) containing 1 mM dithiothreitol and 1 mM cysteine (1 ml of buffer to 1 g [wet weight] of cells), and then the extract was centrifuged at 30,000 × *g* for 30 min at 4°C as described previously (19). The supernatant was collected and stored under H_2 gas at −20°C.

In vitro acetyl-CoA synthesis. Autotrophically synthesized acetyl-CoA in the cell extract was trapped in lactate as described previously (19). Assays were conducted in 2.6-ml serum vials with butyl rubber stoppers and aluminum seals (West Co., Inc., Phoenixville, Pa.). Extracts were preincubated under H_2 gas at 37°C for 1 h to remove endogenous C_1 substrates. A typical assay contained 25 U of lactate dehydrogenase, 20 nmol of bromoethanesulfonate, 2.5 μmol of

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NADH, and 0.2 ml of extract. The gas phase was H_2-CO_2 (80:20; vol/vol). CO gas (80 kPa) and 1 μmol of CH_2O were included in the assay when indicated. When $^{14}CH_2O$ was included, the specific activity was 7.0×10^6 dpm/μmol. The lactate that formed in the assay was measured spectrophotometrically (11).

Radiolabeling experiments. *M. voltae* was grown in a 160-ml serum bottle containing 10 ml of medium with low concentrations of amino acids and acetate. The bottle was pressurized to 240 kPa with H_2-CO_2 (80:20), and $NaH^{14}CO_3$ was added. The culture was not repressurized during growth. Cells were harvested during the early stationary phase, and the protein was extracted (24). The specific activity of $^{14}CO_2$ in the gas phase of the medium was measured as follows. After the cells were harvested, the remaining CO_2 in the bottle was absorbed in a 2 N NaOH solution by flushing the culture bottle with CO_2-free N_2 gas. The weight of the NaOH solution was carefully predetermined. The amount of CO_2 trapped in the NaOH solution was then calculated from the increase in weight. The amount of radiolabel trapped was determined by liquid scintillation counting.

The specific activity of acetate in the medium was determined after liquid chromatography on a column (Aminex HPX-87H; Bio-Rad Laboratories, Richmond, Calif.) in 0.026 N H_2SO_4–0.5% acetonitrite with a high-performance liquid chromatography system (model 4060; Varian Instruments) and a spectrophotometric detector (LC-75; The Perkin-Elmer Corp., Norwalk, Conn.). The quantity of radiolabel was determined by liquid scintillation counting.

The protein was hydrolyzed to amino acids with 6 N HCl at 108°C for 30 h in vacuo. Radiolabeled amino acids were identified after two-dimensional thin-layer chromatography and radioautography (20). Radiolabeled alanine was converted to lactate by lactate dehydrogenase and glutamate-pyruvate transaminase. The lactate that formed from alanine was further purified by chromatography on Dowex 50W × 8 (H^+ form) and Dowex 2 × 8 (Cl^- form), as described by Fuchs et al. (10).

The specific activities of the medium and cellular amino acids were determined after purification of the dansyl derivatives by liquid chromatography (26). In brief, the lyophilized protein hydrolysate from a 5-ml culture was dissolved in 30 μl of H_2O, and 5 μl was diluted into 195 μl of 0.1 M $NaHCO_3$ (pH 10.5). When the medium was analyzed, 25 μl was diluted in 175 μl of 0.1 M $NaHCO_3$. The solution was then preheated to 60°C prior to the addition of 100 μl of dansyl chloride solution (5 mg/ml) in high-performance liquid chromatography-grade acetonitrile. After the sample was heated for 10 min at 60°C, it was centrifuged for 5 min at 10,000 × *g* at room temperature. Between 10 and 20 μl of the dansylated amino acids was injected onto the liquid chromatograph. Fractions containing the amino acids of interest were collected, and the fractions from four runs were pooled. After analysis by liquid chromatography of 20 μl, to confirm sample purity and to determine the amino acid concentration, 1.0 ml of the pooled fractions was diluted with 2.0 ml of distilled H_2O and 7.0 ml of liquid scintillation cocktail prior to liquid scintillation counting (26).

Purification and degradation of lactate. Lactate was purified either by chromatography on Celite and Dowex 50W (H^+ form) (1) or by the procedure of Fuchs et al. (10). The radiochemical purity of lactate was confirmed by thin-layer chromatography on silica gel with chloroform–1-butanol (80:20) and fluorography (2). The C-1 degradation of lactate was performed by dichromate oxidation (1). The C-2 degradation of lactate (or the C-1 degradation of acetate) was performed by the Schmidt method, and the total degradation of lactate to three CO_2 molecules was performed by the Van Slyke-Folch oxidation procedure (17). The $^{14}CO_2$ that was released from each degradation step was trapped in a 2 N NaOH solution. The millimoles of CO_2 trapped were determined from the increase in weight of the NaOH solution. The radioactivity of $^{14}CO_2$ trapped was determined by liquid scintillation counting of 0.4 ml of the NaOH solution with 20 ml of CO_2 trapping cocktail.

Enzymatic assays. CO dehydrogenase was assayed as described previously (19). Isopropyl malate synthase activity was determined fluorometrically by the procedure of Wiegel and Schlegel (28), except that Tris hydrochloride buffer was substituted with 250 mM PIPES buffer (pH 7.0). Since isopropyl malate was not commercially available, the umbelliferone derivative of malate was the standard for isopropyl malate synthase activity (4). The absorption and emission spectra of umbelliferone and 4-isopropylumbelliferone are very similar, and no more than a 2% deviation of the isopropyl malate synthase activity should occur when the umbelliferone standard is used (4). The protein content of the extract was determined by the procedure of Lowry et al. (16), after treatment in 0.1 M NaOH at 90°C for 30 min.

Materials. All chemicals were of reagent grade or better. H_2 and H_2-CO_2 (80:20; vol/vol) were obtained from Selox Co. (Gainesville, Ga.). Carbon monoxide was purchased from Fisher Scientific Co. (Springfield, N.J.). Lactate dehydrogenase, phosphotransacetylase, NAD^+, NADH, acetyl-CoA, acetylphosphate, 2-ketoisovalerate, coenzyme A, resorcinol, and PIPES were obtained from Sigma Chemical Co. (St. Louis, Mo.). Bromoethanesulfonate was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis). $NaH^{14}CO_3$, $^{14}CH_2O$, [U-^{14}C]leucine, [U-^{14}C]isoleucine, and [U-^{14}C]acetate were obtained from ICN Pharmaceuticals, Inc. (Irvine, Calif.). CO_2 trapping cocktail contained 0.5 liter of toluene, 0.4 liter of methanol, 0.1 liter of ethanolamine, and 2 g of 2,5-bis[5′-*tert*-butylbenzoxazolyo-(2′)]thiophene.

RESULTS AND DISCUSSION

Enzymatic and ^{13}C nuclear magnetic resonance studies have demonstrated that *M. voltae* synthesizes alanine from pyruvate and valine from pyruvate and an active aldehyde via acetolactate synthase (5, 32). Pyruvate is synthesized in turn from acetyl-CoA and CO_2 via pyruvate synthase (10, 18, 19). Not surprisingly, alanine and valine were radiolabeled following growth with [U-^{14}C]acetate or $^{14}CO_2$ (Fig. 1). However, their specific activities were not consistent with their simple formation from exogenous acetate and CO_2 (Table 1). For instance, following growth with $^{14}CO_2$, the specific activity of alanine was 30% higher than expected if it was derived from one CO_2 molecule. Following growth with [U-^{14}C]acetate, the specific activity of alanine was 25% lower than expected if it was synthesized from one acetate molecule. Likewise, valine should contain only one carbon molecule from CO_2, yet its specific activity was twice that of the medium CO_2. In addition, valine should contain two molecules of acetate, yet its specific activity was only 1.3 times the specific activity of the medium acetate.

A possible explanation for these discrepancies was that *M. voltae* was capable of autotrophic acetyl-CoA biosynthesis, even though acetate was required for growth. To test this hypothesis, alanine from cells grown with $^{14}CO_2$ was converted to lactate and degraded (Table 2). Although 64% of the total label was found in the C-1 carbon, significant

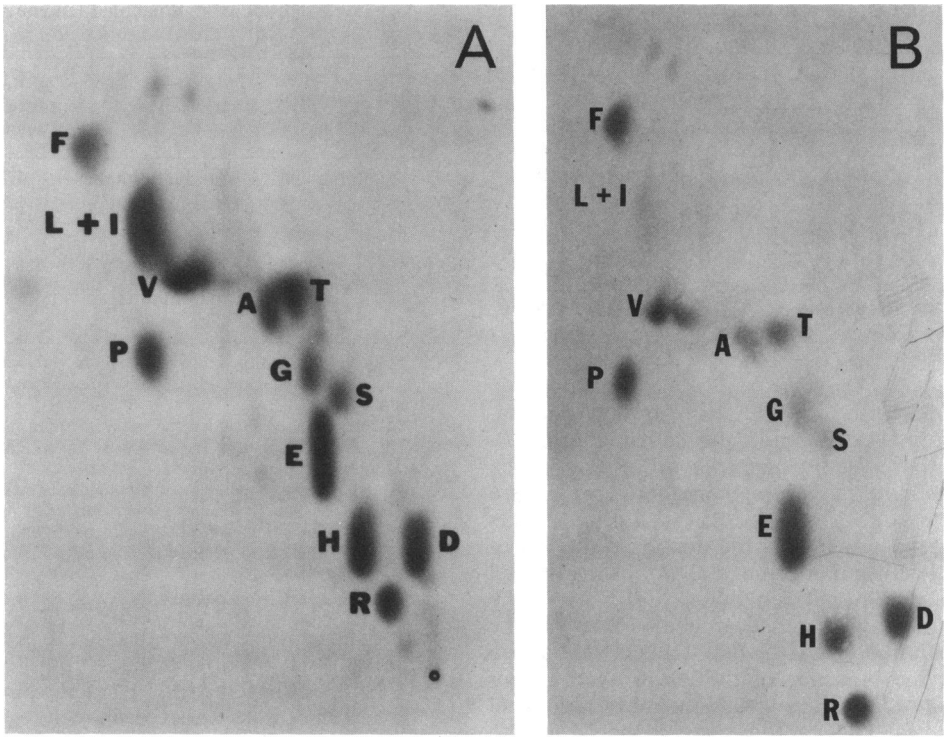

FIG. 1. Radiolabeled amino acids from *M. voltae* following growth in defined medium with $NaH^{14}CO_3$ (A) or [U-^{14}C]acetate (B). Cells were harvested at the early stationary phase. After extraction and hydrolysis of the cellular protein, radiolabeled amino acids were separated by two-dimensional thin-layer chromatography and radioautography. The solvent systems were as follows: 1, methanol-chloroform NH_4OH (80:80:30); 2, phenol-H_2O-KCN (120:40:2 mg). In panel A, the origin is marked (○). The origin is not shown in panel B. The standard one-letter abbreviations for the amino acids are used.

amounts of radiolabel were recovered from the C-2 and C-3 positions, which is consistent with the pathway of autotrophic acetyl-CoA synthesis. From the ratio of label in the C-1 and the C-2 plus C-3 positions of alanine, the relative contribution of endogenous acetate synthesis was estimated to be about 30%. This estimate is close to the level of endogenous synthesis (15 to 30%) that is necessary to explain the radiolabeling pattern observed in Table 1.

CO dehydrogenase is a key enzyme of the autotrophic acetyl-CoA biosynthetic pathway (8, 15, 31). If *M. voltae* is capable of autotrophic acetyl-CoA synthesis, it should contain this enzyme. Low levels of the CO dehydrogenase of 13 nmol of methyl viologen reduced min^{-1} mg of $protein^{-1}$ were found in cell extracts of *M. voltae*. These levels were about 300-fold lower than those found in *M. maripaludis* following autotrophic growth (19). Bott et al. (3) previously failed to detect CO dehydrogenase activity in extracts of *M. voltae*. The sensitivity of their assays was 20 nmol of methyl viologen reduced min^{-1} mg of $protein^{-1}$.

The nature of autotrophic CO_2 fixation in *M. voltae* was further tested by a spectrophotometric assay for autotrophic acetyl-CoA synthesis in which lactate dehydrogenase and NADH were added to cell extracts to trap the acetyl-CoA into lactate (19). In this assay system, lactate from autotrophically synthesized acetyl-CoA was detected in cell extracts of *M. voltae* (Table 3). Furthermore, both CO and CH_2O stimulated lactate (acetyl-CoA) synthesis. These components form intermediates in autotrophic acetyl-CoA bio-

TABLE 1. Incorporation of CO_2 and acetate into amino acids of *M. voltae*[a]

Amino acid	Specific radioactivity (dpm/nmol) after growth with radiolabeled:	
	CO_2[b]	Acetate[c]
Alanine	86	341
Valine	133	604
Isoleucine	48	52
Leucine	47	132

[a] Specific radioactivity of amino acids released by acid hydrolysis of the cellular protein. Values are the averages of at least four determinations, except for the specific radioactivity of isoleucine and leucine after growth with radiolabeled CO_2. In this case, only one determination was made. However, the specific radioactivity of the combined amino acids was also determined to be 52 dpm/nmol of isoleucine-leucine (three determinations).

[b] The specific radioactivity of the CO_2 at the end of growth was 65 dpm/nmol.

[c] The specific radioactivities of the [U-^{14}C]acetate at the beginning and end of growth were 474 and 445 dpm/nmol, respectively.

TABLE 2. Degradation of [^{14}C]alanine purified from cellular proteins of *M. voltae* after growth in the presence of $^{14}CO_2$

Carbon atom of alanine	Specific radioactivity (dpm/mmol, 10^3) of CO_2	% of radiolabel
All	1.74	(33)[a]
C-1	3.34	64
C-2	1.23	24
C-3	0.83	16

[a] A total of 100% of the radiolabel in alanine (after conversion to lactate) was equal to 3 times the specific activity of the CO_2 obtained from the total degradation of lactate.

TABLE 3. CH_2O- and CO-stimulated lactate synthesis in extracts of *M. voltae*

Addition	Lactate formed (nmol)
None[a]	0
+ NADH (2.5 μmol)	0
+ LDH[b] (25 U)	0
+ NADH + LDH	209
+ NADH + LDH + CH_2O (1 μmol)	280
+ NADH + LDH + CO (100 kPa)	235
+ NADH + LDH + CO + CH_2O	328

[a] Without additions, the assay included 12 mg of protein, H_2-CO_2 (80:20), and 0.1 mM bromoethanesulfonate. The length of the assay was 40 min.
[b] LDH, Lactate dehydrogenase.

synthesis. CH_2O reacts chemically with tetrahydromethanopterin to form methylenetetrahydromethanopterin (7). Under a H_2 atmosphere, methylenetetrahydromethanopterin is converted to methyltetrahydromethanopterin, which is a methyl donor for the C-2 of acetyl-CoA (14, 19). CO reacts with CO dehydrogenase, which is the donor for the C-1 of acetyl-CoA (19, 22). To confirm that CH_2O was the methyl donor, the assay was performed with $^{14}CH_2O$, and the lactate formed was purified and degraded (Table 4). The specific incorporation of $^{14}CH_2O$ into the C-3 of lactate further confirmed the presence of the autotrophic acetyl-CoA pathway in *M. voltae*. The small amount of radiolabel found in the C-1 and C-2 carbons was probably due to oxidation of some of the $^{14}CH_2O$ to $^{14}CO_2$. This activity has been observed in extracts of *M. maripaludis* (19).

Because *M. voltae* could synthesize acetate even though exogenous acetate was required for growth, it was of interest to determine whether it could also synthesize the amino acids required for growth, isoleucine and leucine. When *M. voltae* was grown in the presence of $^{14}CO_2$ or [U-^{14}C] acetate, a small amount of radiolabel was incorporated into these amino acids (Fig. 1). The specific incorporation of $^{14}CO_2$ and [U-^{14}C]acetate into leucine and isoleucine was also confirmed by liquid chromatography of the dansyl derivatives (data not shown). However, the specific radioactivities of the purified amino acids were low (Table 1).

To better quantify the contribution of endogenous synthesis in *M. voltae*, the dilution of [U-^{14}C]isoleucine or [U-^{14}C]leucine was determined during growth (Table 5). Thus, the specific radioactivities of both isoleucine and leucine obtained from protein hydrolysates of whole cells were reduced by 32 and 40%, respectively, when compared with the initial specific radioactivity in the medium. Importantly, the amount of these amino acids in the medium at the end of growth increased, and their specific radioactivities were also lower than they were initially. Therefore, *M. voltae* synthesized more than enough of these amino acids for growth, but much of it was excreted into the medium.

TABLE 4. Degradation of radiolabeled lactate formed from $^{14}CH_2O$ in extracts of *M. voltae*

Carbon of lactate	Specific radioactivity (dpm/mmol; 10^4) of CO_2	% of radiolabel
All	7.4	(33)[a]
C-1	7.4	33
C-2	1.2	5
C-3	12.5	56

[a] A total of 100% of the radiolabel in lactate was equal to 3 times the specific activity of the CO_2 obtained from the total degradation of lactate.

TABLE 5. Dilution of radiolabeled leucine and isoleucine during growth of *M. voltae*[a]

Radiolabeled amino acid	Sample assayed[b]	Amt (μmol/culture) of amino acid[c]	Specific radioactivity (dpm/nmol) of amino acid
[U-^{14}]leucine	Initial medium	4.20	3,660
	Final medium	4.80	2,610
	Cells	0.34	2,220
[U-^{14}C]isoleucine	Initial medium	3.00	3,050
	Final medium	3.80	2,400
	Cells	0.37	2,080

[a] Cells were grown for 2 days in medium with acetate and [U-^{14}C]leucine and unlabeled isoleucine or [U-^{14}C]isoleucine and unlabeled leucine. The inoculum was 4%.
[b] Samples assayed included the initial medium immediately after inoculation, the final medium after the cells were harvested, and the cellular protein hydrolysate at the end of growth.
[c] Amount of either leucine or isoleucine per 10 ml of culture. The amount of cellular leucine and isoleucine was calculated from the cell yield and the amino acid composition of the whole cells of 358 nmol of leucine mg of cellular dry weight^{-1} and 245 nmol of isoleucine mg of cellular dry weight^{-1}.

To confirm these observations, extracts of *M. voltae* were examined for isopropyl malate synthase, which is the first enzyme that is specific for the leucine biosynthetic pathway. This enzyme was detected in cell extracts of both *M. maripaludis* and *M. voltae*. Activity was linear with both the protein concentration and time (Fig. 2). However, it was not

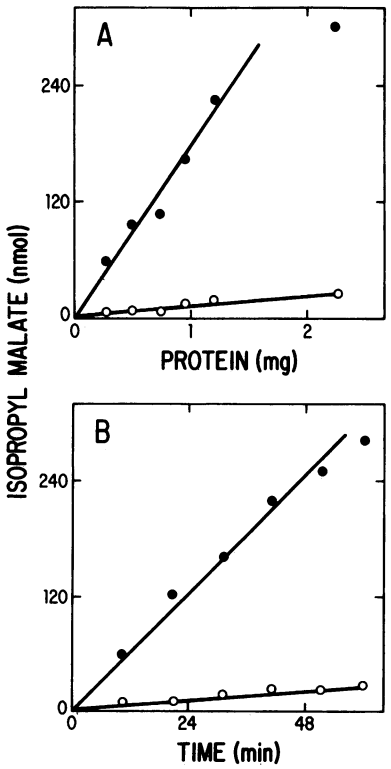

FIG. 2. Isopropyl malate synthase activity in *M. voltae* and *M. maripaludis*. (A) Effect of protein content on enzyme activity. The assay time was 20 min. (B) Time course of isopropyl malate synthase activity. The assay contained 2.5 mg of protein. Symbols: ●, *M. maripaludis*; ○, *M. voltae*.

subject to the feedback inhibition by the end product leucine, as has been shown in other bacteria (29). *M. maripaludis* is capable of autotrophic growth from H_2 and CO_2, and it can synthesize all the amino acids for growth from CO_2. The specific activity of isopropyl malate synthase was expected to be high, and the levels found were 9.0 nmol min^{-1} mg of $protein^{-1}$. In contrast, only low levels of this enzyme activity (0.8 nmol min^{-1} mg of $protein^{-1}$) were found in extracts of *M. voltae*. This level was less than the amount (about 3 nmol min^{-1} mg of $protein^{-1}$) that is necessary to support growth at its normal generation time of 2 h in this medium. However, it was sufficient to make the amounts of leucine synthesized in the radiolabeling experiment, if biosynthesis continued during the linear and early stationary growth phases.

In conclusion, results of radiolabeling and enzyme studies demonstrated that *M. voltae* is capable of synthesizing acetate by the pathway of autotrophic acetyl-CoA biosynthesis. The specific activity of autotrophic acetyl-CoA synthesis in extracts of *M. voltae* is about 20% of that found in extracts of *M. maripaludis* (19). In addition, both leucine and isoleucine, which are required for *M. voltae* to grow in laboratory conditions, can also be synthesized from CO_2 and acetate. It is of interest that although *M. voltae* requires exogenous acetate, leucine, and isoleucine for growth, it also has the capacity to synthesize these organic compounds.

The reason for these requirements is not known. The possibility that the original culture of *M. voltae* underwent mutation is unlikely because related methanococci with the same phenotypes have been isolated from salt marshes (25). Another possibility is that *M. voltae* has adapted to growth in environments where these nutrients are abundant, and it has lost the ability to grow in the absence of high external concentrations. The observation that both isoleucine and leucine are excreted into the medium is consistent with this interpretation. However, the observation that *M. voltae* also contains a high-affinity transport system for isoleucine tends to argue against this hypothesis (12). A third possibility may be that the amino acids and acetate are required for the extracellular synthesis of cell wall components or some other essential component. However, there is little precedent for this type of absolute requirement. Lastly, the requirement may be a consequence of a regulatory imbalance under the rather artificial conditions of batch culture. Thus, in nature cells may seldom be exposed to the high concentrations of H_2 that are favorable for optimal growth in batch culture. Future investigations will be necessary to resolve these issues.

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LITERATURE CITED

1. **Aronoff, S.** 1956. Techniques of radiobiochemistry. The Iowa State College Press, Ames, Iowa.
2. **Bonner, W. M., and J. D. Stedman.** 1978. Efficient fluorography of ^3H and ^{14}C on thin layers. Anal. Biochem. **89**:247–256.
3. **Bott, M. H., B. Eikmanns, and R. K. Thauer.** 1985. Defective formation and/or utilization of carbon monoxide in H_2/CO_2 fermenting methanogens dependent of acetate as carbon source. Arch. Microbiol. **143**:266–269.
4. **Calvo, J. M., J. C. Bartholomew, and B. I. Stieglitz.** 1969. Fluorometric assay of enzymatic reactions involving acetyl coenzyme A in aldol condensations. Anal. Biochem. **28**:164–181.
5. **Ekiel, I., K. F. Jarrell, and G. D. Sprott.** 1985. Amino acid biosynthesis and sodium-dependent transport in *Methanococcus voltae*, as revealed by ^{13}C NMR. Eur. J. Biochem. **149**: 437–444.
6. **Ekiel, I., I. C. P. Smith, and G. D. Sprott.** 1984. Biosynthesis of isoleucine in methanogenic bacteria: a ^{13}C NMR study. Biochemistry **23**:1683–1687.
7. **Escalante-Semerena, J. C., and R. S. Wolfe.** 1985. Tetrahydromethanopterin-dependent methanogenesis from nonphysiological C_1 donors in *Methanobacterium thermoautotrophicum*. J. Bacteriol. **161**:696–701.
8. **Fuchs, G.** 1986. CO_2 fixation in acetogenic bacteria: variations on a theme. FEMS Microbiol. Rev. **39**:181–213.
9. **Fuchs, G., and E. Stupperich.** 1986. Carbon assimilation pathway in archaebacteria. Syst. Appl. Microbiol. **7**:364–369.
10. **Fuchs, G., E. Stupperich, and R. K. Thauer.** 1978. Acetate assimilation and the synthesis of alanine, aspartate and glutamate in *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **117**:61–66.
11. **Gutmann, I., and A. W. Wahlefeld.** 1983. L-(+)-Lactate determination with lactate dehydrogenase and NAD, p. 100. *In* H. A. Bergermeyer (ed.), Methods of enzymatic analysis, 3rd ed. Verlag Chemie, Deerfield Beach, Fla.
12. **Jarrell, K. F., S. E. Bird, and G. D. Sprott.** 1984. Sodium-dependent isoleucine transport in the methanogenic archaebacterium *Methanococcus voltae*. FEBS Lett. **166**:357–361.
13. **Jones, W. J., M. J. B. Paynter, and R. Gupta.** 1983. Characterization of *Methanococcus maripaludis* sp. nov., a new methanogen isolated from salt marsh sediment. Arch. Microbiol. **135**: 91–97.
14. **Länge, S., and G. Fuchs.** 1987. Autotrophic synthesis of activated acetic acid from CO_2 in *Methanobacterium thermoautotrophicum*. Synthesis from tetrahydromethanopterin-bound C_1 units and carbon monoxide. Eur. J. Biochem. **163**:147–154.
15. **Ljungdahl, L. G.** 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. Annu. Rev. Microbiol. **40**:415–450.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
17. **Sakami, W.** 1955. Handbook of isotope tracer methods. Western Reserve University, Cleveland, Ohio.
18. **Shieh, J. S., and W. B. Whitman.** 1987. Pathway of acetate assimilation in autotrophic and heterotrophic methanococci. J. Bacteriol. **169**:5327–5329.
19. **Shieh, J. S., and W. B. Whitman.** 1988. Autotrophic acetyl coenzyme A synthesis in *Methanococcus maripaludis*. J. Bacteriol. **170**:3072–3079.
20. **Stahl, E.** 1969. Thin-layer chromatography, 2nd ed., p. 742. Springer-Verlag, New York.
21. **Stupperich, E., and G. Fuchs.** 1984. Autotrophic synthesis of activated acetic acid from two CO_2 in *Methanobacterium thermoautotrophicum*. II. Evidence for different origins of acetate carbon atoms. Arch. Microbiol. **139**:14–20.
22. **Stupperich, E., K. E. Hammel, G. Fuchs, and R. K. Thauer.** 1983. Carbon monoxide fixation into the carboxyl group of acetyl coenzyme A during autotrophic growth of *Methanobacterium*. FEBS Lett. **152**:21–23.
23. **Whitman, W. B.** 1985. Methanogenic bacteria, p. 3–84. *In* C. R. Woese and R. S. Wolfe (ed.), The bacteria, vol. VIII: archaebacteria. Academic Press, Inc., Orlando, Fla.
24. **Whitman, W. B., E. Ankwanda, and R. S. Wolfe.** 1982. Nutrition and carbon metabolism of *Methanococcus voltae*. J. Bacteriol. **149**:852–863.
25. **Whitman, W. B., J. S. Shieh, S. H. Sohn, D. S. Caras, and U. Premachandran.** 1986. Isolation and characterization of 22 mesophilic methanococci. Syst. Appl. Microbiol. **7**:235–240.

26. **Whitman, W. B., S. H. Sohn, S. U. Kuk, R. Y. Xing.** 1987. Role of amino acids and vitamins in nutrition of mesophilic *Methanococcus* spp. Appl. Environ. Microbiol. **53**:2373–2378.
27. **Widdel, F.** 1986. Growth of methanogenic bacteria in pure culture with 2-propanol and other alcohols as hydrogen donors. Appl. Environ. Microbiol. **51**:1056–1062.
28. **Wiegel, J., and H. G. Schlegel.** 1977. 2-Isopropyl malate synthase from *Alcaligenes eutrophus* H 16. I. Purification and general properties. Arch. Microbiol. **112**:239–246.
29. **Wiegel, J., and H. G. Schlegel.** 1977. 2-Isopropyl malate synthase from *Alcaligenes eutrophus* H 16. II. Substrate specificity and kinetics. Arch. Microbiol. **112**:247–254.
30. **Wolfe, R. S.** 1985. Unusual coenzymes of methanogenesis. Trends Biochem. Sci. **10**:396–399.
31. **Wood, H. G., S. W. Ragsdale, and E. Pezacka.** 1986. The acetyl-CoA pathway: a newly discovered pathway of autotrophic growth. Trends Biochem. Sci. **11**:14–18.
32. **Xing, R. Y., and W. B. Whitman.** 1987. Sulfometuron methyl-sensitive and -resistant acetolactate synthase of the archaebacteria *Methanococcus* spp. J. Bacteriol. **169**:4486–4492.