Pathway of acetate assimilation in autotrophic and heterotrophic methanococci.

J S Shieh and W B Whitman
J. Bacteriol. **1987, 169(11):5327.**

JOURNAL OF BACTERIOLOGY, Nov. 1987, p. 5327–5329
0021-9193/87/115327-03$02.00/0

Vol. 169, No. 11

Pathway of Acetate Assimilation in Autotrophic and Heterotrophic Methanococci

JERSONG SHIEH AND WILLIAM B. WHITMAN*
Department of Microbiology, University of Georgia, Athens, Georgia 30602

Received 15 June 1987/Accepted 18 August 1987

The autotroph *Methanococcus maripaludis* contained high levels of acetate-coenzyme A ligase, pyruvate synthase, pyruvate,water dikinase, pyruvate carboxylase, and the enzymes of the incomplete reductive tricarboxylic acid cycle. Phosphoenolpyruvate carboxykinase, citrate synthase, and isocitrate dehydrogenase were not detected. In contrast, the heterotroph *Methanococcus* sp. strain A3 contained acetate kinase, and acetate coenzyme A ligase was virtually absent.

The methanogenic bacteria are a diverse group of anaerobic archaebacteria, which catabolize a limited number of one-carbon compounds and acetate to methane. Acetate is also a required carbon source for many methanogens, and in the autotrophic species of *Methanobacterium* and *Methanosarcina*, acetyl coenzyme A (acetyl-CoA) is the initial product of CO_2 fixation (8, 9, 24). Therefore, acetate is central to the carbon metabolism of autotrophic as well as heterotrophic species. The methanococci are one of the three major phylogenetic groups of the methanogens (1, 17). By the criterion of 16S rRNA structure, they are about as related to the other methanogens as the gram-negative eubacteria are related to the gram-positive eubacteria (17). The pattern of incorporation of stable isotopes into the amino acids of *Methanococcus voltae* is consistent with the presence of an incomplete reverse tricarboxylic acid cycle, similar to that found in *Methanobacterium thermoautotrophicum* and *Methanospirillum hungatei* (4, 5, 9). In this report, we demonstrate the presence of the enzymes of the incomplete reductive tricarboxylic acid cycle in the methanococci. Moreover, during autotrophic growth, *Methanococcus maripaludis* possessed high levels of acetate-CoA ligase. In contrast, the heterotroph *Methanococcus* sp. strain A3 contained acetate kinase.

Growth of *Methanococcus maripaludis* and *Methanococcus* sp. strain A3 was as described previously (11, 25). Cell-free extracts were prepared under strictly anaerobic conditions after cell pellets were thawed in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.). Cells lysed upon the addition of 25 mM K-PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer, pH 6.8, containing 1 mM dithiothreitol and 1 mM cysteine, which was saturated with H_2 gas. Pancreatic DNase (1 mg/20 g of cells [wet weight]) was added. The cell extract became homogeneous after 30 min of incubation in the chamber at room temperature and was then centrifuged at 30,000 × *g* for 30 min at 4°C in a polypropylene centrifugation bottle that had been equilibrated in an anaerobic chamber for at least 1 day. The supernatant was collected in an anaerobic chamber, dialyzed against 50 volumes of the same buffer used for lysis, and stored under H_2 gas at −20°C. Protein in cell extracts was determined by the procedure of Lowry et al. (15). Bovine serum albumin was the standard. All assays were performed at room temperature. Except for the assay for fumarate hydratase, spectrophotometric assays were performed under strictly anaerobic conditions.

Acetate-CoA ligase and succinate-CoA ligase were measured by the procedure of Oberlies et al. (16). Acetate kinase was measured both in the direction of acetate formation and in the direction of acetyl phosphate formation by the procedures of Smith and Lequerica (19). Pyruvate synthase was measured by trapping the pyruvate formed from acetyl-CoA and CO_2 as lactate with lactate dehydrogenase (J.-S. Shieh and W. B. Whitman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, I72, p. 184). Pyruvate synthase and 2-oxoglutarate synthase were also measured by CoA- and keto acid-dependent methyl viologen reduction (26). Pyruvate,water dikinase was measured spectrophotometrically by coupling ATP-dependent pyruvate consumption to the reduction of NAD by lactate dehydrogenase (7). Activity was compared in undialyzed extracts containing 2.5 mM P_i and dialyzed, Dowex-treated extracts, which contained less than 1.4 μM P_i. In addition, the ATP used in the assay was found to be essentially free of P_i, and it could contribute no more than 0.01 μM P_i to the enzyme assay. Pyruvate kinase was measured by the procedure of Fujii and Miwa (10). Phosphoenolpyruvate carboxylase was measured as phosphoenolpyruvate-dependent oxaloacetate formation (12). Pyruvate carboxylase was measured in the same manner except that pyruvate and ATP were substituted for phosphoenolpyruvate. Phosphoenolpyruvate carboxykinase (PP_i) was determined as phosphoenolpyruvate-, P_i-, Mg^{2+}-, and bicarbonate-dependent oxaloacetate formation (3). Phosphoenolpyruvate carboxykinase (ATP) was measured as phosphoenolpyruvate-, ATP-, Mg^{2+}-, and bicarbonate-dependent oxaloacetate formation (12). Citrate (*si*)-synthase was determined as acetyl-CoA-dependent oxaloacetate consumption (3). Malate dehydrogenase was measured as oxaloacetate-dependent NADH oxidation (26). Succinate dehydrogenase (fumarate reductase) was determined as fumarate-dependent reduced benzyl viologen oxidation (26). Isocitrate dehydrogenase was measured as isocitrate-dependent reduction of NADP or NAD (23). Fumarate hydratase was measured as the malate-dependent formation of fumarate, which was detected spectrophotometrically at 240 nm (20).

The levels of the enzymes of the reductive tricarboxylic acid pathway were very similar in both methanococci (Table 1). Cell extracts catalyzed the conversion of acetyl-CoA and CO_2 to pyruvate, albeit at a slow rate. Because the pyruvate

* Corresponding author.

TABLE 1. Enzymes of acetate assimilation in the methanococci

Enzyme (EC no.)	Sp act (nmol min^{-1} mg of $protein^{-1}$)[a]	
	Methanococcus maripaludis	*Methanococcus* sp. strain A3
Acetate-CoA ligase (6.2.1.1)	425.0	5.3
Acetate kinase (2.7.2.1)		
Acetyl phosphate formation	<0.1	41.0
Acetate formation	4.6	757.0
Pyruvate synthase (1.2.7.1)		
Pyruvate formation	1.3	0.7
Methyl viologen reduction	66.0	43.0
Pyruvate,water dikinase (2.7.9.2)	13.5	9.5
Phosphoenolpyruvate carboxylase (4.1.1.31)	<0.1	<0.1
Phosphoenolpyruvate carboxykinase (PP_i) (4.1.1.38)	<0.1	<0.1
Phosphoenolpyruvate carboxykinase (ATP) (4.1.1.49)	<0.1	<0.1
Pyruvate carboxylase (6.4.1.1)	30.0	11.0
Malate dehydrogenase (1.1.1.37)	38.0	3.5
Fumarate hydratase (4.2.1.2)	12.0	ND
Succinate dehydrogenase (1.3.99.1)	44.0	25.0
Succinate-CoA ligase (6.2.1.5)	1,060.0	850.0
2-Oxoglutarate synthase (1.2.7.3)	66.0	43.0
Citrate (*si*)-synthase (4.1.3.7)	<0.1	<0.1
Isocitrate dehydrogenase (1.1.1.42)	<0.1	<0.1

[a] Specific activities are the averages of duplicate assays of at least two extracts. *Methanococcus maripaludis* was grown in mineral medium without acetate. *Methanococcus* sp. strain A3 was grown in medium containing the required carbon sources acetate, isoleucine, and leucine. ND, Not done.

synthase is extremely oxygen labile and otherwise unstable, the in vitro activity may underestimate the levels in living cells (26). The activity of the reverse reaction, the CoA-dependent reduction of methyl viologen by pyruvate, was much higher, and it was comparable with the activity found in *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri* (23, 26).

Pyruvate was converted to phosphoenolpyruvate by a pyruvate,water dikinase activity, which was present in both extracts (Table 1). Because 5 mM ADP inhibited the pyruvate,water dikinase by 60% (data not shown), ADP may be an effector as described previously for *Methanobacterium thermoautotrophicum* (7). Because no P_i-dependent activity was found in dephosphorylated extracts, pyruvate,P_i dikinase (E.C. 2.7.9.1) was absent. Similarly, pyruvate was not formed in either extract from phosphoenolpyruvate plus ADP. Thus, pyruvate kinase (EC 2.7.1.40) was also not present in levels greater than 0.1 nmol min^{-1} mg of $protein^{-1}$.

Oxaloacetate formation was not dependent on phosphoenolpyruvate, unlike that in the *Methanobacterium thermoautotrophicum*. Instead, pyruvate carboxylase activity was found in both methanococci. This activity was dependent on pyruvate, ATP, and bicarbonate. The apparent K_m for pyruvate was 0.3 mM. In dialyzed extracts, activity was not inhibited by 16 U of the biotin-binding protein avidin. Presumably, if biotin is a coenzyme of this enzyme, it must be tightly bound. Phosphoenolpyruvate did not substitute for pyruvate in this assay, and the presence or absence of ATP and PP_i had no effect (data not shown). Thus, phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase (ATP), and phosphoenolpyruvate carboxykinase (PP_i) were apparently absent. As a further control, when commercially available phosphoenolpyruvate carboxylase was added to extracts, full activity was detected (data not shown). Therefore, extracts did not contain inhibitors or competing enzymes which obscured these activities.

Other enzymes found in the methanococci were malate dehydrogenase, succinate dehydrogenase (fumarate reductase), succinate-CoA ligase, and 2-oxoglutarate synthase (Table 1). The apparent succinate-CoA ligase activity was not dependent on adenylate kinase and was not affected by the adenylate kinase inhibitor diadenosine pentaphosphate. Therefore, ADP rather than AMP was formed in the reaction, which distinguishes it from acetate-CoA ligase. Citrate synthase and isocitrate dehydrogenase were not detected in either extract. Commercially available enzymes (obtained from Sigma Chemical Co., St. Louis, Mo.) were readily detected in extracts, which served as positive controls for these assays. Following growth with acetate, extracts of *Methanococcus maripaludis* contained the same levels of pyruvate carboxylase and malate dehydrogenase as autotrophically grown cells did. Likewise, phosphoenolpyruvate carboxykinase (PP_i), phosphoenolpyruvate carboxykinase (ATP), phosphoenolpyruvate carboxylase, citrate synthase, and isocitrate dehydrogenase were undetectable in acetate-grown cells.

The initial enzyme of acetate assimilation differs in various methanogens. *Methanosarcina barkeri* has an acetate kinase, which forms acetyl phosphate and ADP from ATP and acetate (14, 19). *Methanobacterium thermoautotrophicum* and *Methanothrix soehngenii* contain acetate-CoA ligase, which forms acetyl-CoA and AMP from acetate, CoA, and ATP (13, 16, 18). Extracts of *Methanococcus maripaludis* grown autotrophically contained high levels of acetate-CoA ligase activity (Table 1). Because the adenylate kinase inhibitor diadenosine pentaphosphate at a concentration of 1 mM produced 97% inhibition of the coupled assay, AMP and not ADP was a product. This experiment confirmed the identity of the ligase. The apparent K_ms of the ligase for acetate and CoA were 90 and 300 μM, respectively. *Methanococcus maripaludis* can obtain up to 60% of its cellular carbon from exogenous acetate (data not shown). In extracts of acetate-grown cells, the level of acetate-CoA ligase was reduced to 82 nmol min^{-1} mg^{-1} or about one-fifth of the autotrophic level. Likewise, the specific activity of acetate-CoA ligase in extracts of acetate-grown *Methanobacterium thermoautotrophicum* is less than that found in extracts of autotrophically grown cells (16). *Methanococcus* sp. strain A3, a heterotrophic isolate which requires acetate for growth (25), contained only very low levels of acetate-CoA ligase (Table 1). However, this activity was also inhibited by 1 mM diadenosine pentaphosphate (data not shown). In addition, strain A3 contained high levels of acetate kinase, an enzyme that was virtually absent in *Methanococcus maripaludis* (Table 1). The apparent K_m for acetate of the kinase was 0.25 mM (data not shown). Therefore, the initial enzymes of acetate assimilation in these bacteria differ. Presumably, the facultatively autotrophic bacterium used an enzyme with a higher affinity for acetate because it was adapted for growth in environments where the concentration of acetate was low. This conclusion is supported by the observation that the specific activity of the acetate-CoA ligase decreased in cells grown with abundant acetate.

The carbon metabolism of only a few methanogens has been examined in detail. For *Methanobacterium thermoautotrophicum* and *Methanospirillum hungatei*, the incomplete reductive tricarboxylic acid cycle has been described (5, 9).

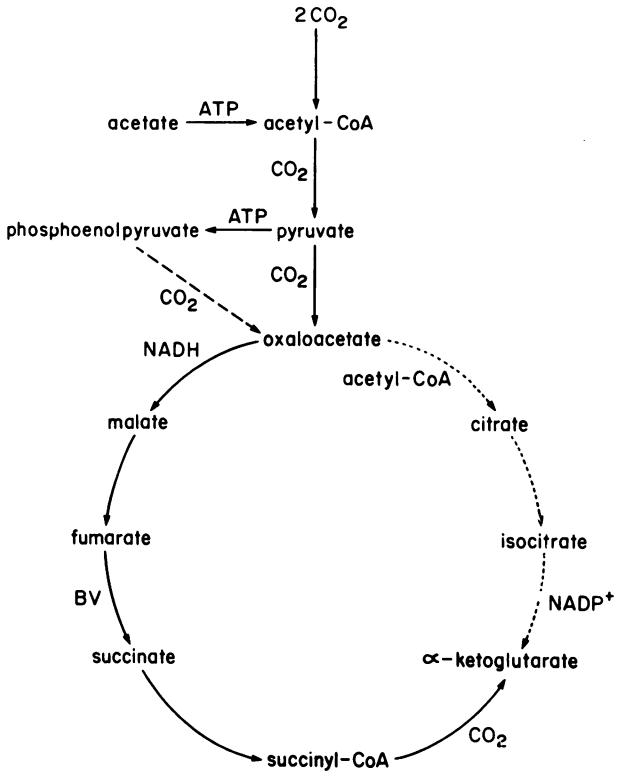

FIG. 1. Pathways of carbon assimilation in methanogenic bacteria. Enzymes demonstrated in *Methanococcus maripaludis* are shown by the solid lines. *Methanobacterium thermoautotrophicum* contains these enzymes, except that phosphoenolpyruvate carboxylase is substituted for pyruvate carboxylase (9, 12). *Methanosarcina barkeri* contains the oxidative branch of the tricarboxylic acid cycle shown by the broken lines (19, 23). In addition, *Methanosarcina barkeri* and *Methanococcus* sp. strain A3 contain acetate kinase instead of acetate-CoA ligase (14, 19). Therefore, acetyl phosphate is an intermediate in the formation of acetyl-CoA. BV, Benzyl viologen.

For *Methanosarcina barkeri* and *Methanothrix concilii*, the incomplete oxidative tricarboxylic acid cycle is found (Fig. 1; 2, 6, 21–23). Failure to demonstrate citrate synthase and isocitrate dehydrogenase in *Methanococcus maripaludis* and strain A3 excludes the oxidative pathway. Although phosphoenolpyruvate carboxylase could not be detected, the pathway of carbon assimilation in the methanococci resembles that in *Methanobacterium thermoautotrophicum*, in which α-ketogluturate is derived from an incomplete reductive tricarboxylic acid cycle.

This work was supported by National Science Foundation grant PCM-8351355 and the Georgia Power Co.

LITERATURE CITED

1. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
2. **Daniels, L., and J. G. Zeikus.** 1978. One-carbon metabolism in methanogenic bacteria: analysis of short-term fixation products of $^{14}CO_2$ and $^{14}CH_3OH$ incorporated into whole cells. J. Bacteriol. **136**:75–84.
3. **Eden, G., and G. Fuchs.** 1983. Autotrophic CO_2 fixation in *Acetobacterium woodii*. II. Demonstration of enzymes involved. Arch. Microbiol. **135**:68–73.
4. **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.
5. **Ekiel, I., I. C. P. Smith, and G. D. Sprott.** 1983. Biosynthetic pathways in *Methanospirillum hungatei* as determined by ^{13}C nuclear magnetic resonance. J. Bacteriol. **156**:316–326.
6. **Ekiel, I., G. D. Sprott, and G. B. Patel.** 1985. Acetate and CO_2 assimilation by *Methanothrix concilii*. J. Bacteriol. **162**:905–908.
7. **Eyzaguirre, J., K. Jansen, and G. Fuchs.** 1982. Phosphoenolpyruvate synthetase in *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **132**:67–74.
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 pathways in archaebacteria. Syst. Appl. Microbiol. **7**:364–369.
10. **Fujii, H., and S. Miwa.** 1983. Pyruvate kinase, p. 496–501. *In* H. A. Bergymeyer (ed.), Methods of enzymatic analysis, 3rd ed., vol. 3. Verlag Chemie International, Inc., Deerfield Beach, Fla.
11. **Jones, W. J., Paynter, M. J. B., and R. Gupta.** 1983. Characterization of *Methanococcus maripaludis* sp. nov., a new methanogen isolated from salt marsh sediment. Arch. Microbiol. **135**:91–97.
12. **Kenealy, W. R., and J. G. Zeikus.** 1982. Characterization and function of phosphoenolpyruvate carboxylase in *Methanobacterium thermoautotrophicum*. FEMS Microbiol. Lett. **14**:7–10.
13. **Kohler, H. P. E., and A. J. B. Zehnder.** 1984. Carbon monoxide dehydrogenase and acetate thiokinase in *Methanothrix soehngenii*. FEMS Microbiol. Lett. **21**:287–292.
14. **Laufer, K., B. Eikmanns, U. Frimmer, and R. K. Thauer.** 1987. Methanogenesis from acetate by *Methanosarcina barkeri*: catalysis of acetate formation from methyl iodide, CO_2, and H_2 by the enzyme system involved. Z. Naturforsch. Sect. C **42**:360–372.
15. **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.
16. **Oberlies, G., G. Fuchs, and R. K. Thauer.** 1980. Acetate thiokinase and the assimilation of acetate in *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **128**:248–252.
17. **Pace, N. R., G. J. Olsen, and C. R. Woese.** 1986. Ribosomal RNA phylogeny and the primary lines of evolutionary descent. Cell **45**:325–326.
18. **Pellerin, P., B. Gruson, G. Prensier, G. Albagnac, and P. Debeire.** 1987. Glycogen in *Methanothrix*. Arch. Microbiol. **146**: 377–381.
19. **Smith, M. R., and J. L. Lequerica.** 1985. *Methanosarcina* mutant unable to produce methane or assimilate carbon from acetate. J. Bacteriol. **164**:618–625.
20. **Stitt, M.** 1983. Fumarase, p. 359–362. *In* H. A. Bergmeyer (ed.), Methods of enzymatic analysis, 3rd ed., vol. 4. Verlag Chemie International, Inc., Deerfield Beach, Fla.
21. **Weimer, P. J., and J. G. Zeikus.** 1978. One carbon metabolism in methanogenic bacteria: cellular characterization and growth of *Methanosarcina barkeri*. Arch. Microbiol. **119**:49–57.
22. **Weimer, P. J., and J. G. Zeikus.** 1978. Acetate metabolism in *Methanosarcina barkeri*. Arch. Microbiol. **119**:175–182.
23. **Weimer, P. J., and J. G. Zeikus.** 1979. Acetate assimilation pathway of *Methanosarcina barkeri*. J. Bacteriol. **137**:332–339.
24. **Whitman, W. B.** 1985. Methanogenic bacteria, p. 3–84. *In* C. R. Woese and R. S. Wolfe (ed.), The bacteria, vol. 8. Academic Press, Inc., New York.
25. **Whitman, W. B., J. Shieh, D. S. Caras, and U. Premachandran.** 1986. Isolation and characterization of 22 mesophilic methanococci. Syst. Appl. Microbiol. **7**:235–240.
26. **Zeikus, J. G., G. Fuchs, W. Kenealy, and R. K. Thauer.** 1977. Oxidoreductase involved in cell carbon synthesis of *Methanobacterium thermoautotrophicum*. J. Bacteriol. **132**:604–613.