

Protein Content and Enzyme Activities in Methanol- and Acetate-Grown *Methanosarcina thermophila*

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Received 4 August 1989/Accepted 6 December 1989

The cell extract protein content of acetate- and methanol-grown *Methanosarcina thermophila* TM-1 was examined by two-dimensional polyacrylamide gel electrophoresis. More than 100 mutually exclusive spots were present in acetate- and methanol-grown cells. Spots corresponding to acetate kinase, phosphotransacetylase, and the five subunits of the carbon monoxide dehydrogenase complex were identified in acetate-grown cells. Activities of formylmethanofuran dehydrogenase, formylmethanofuran:tetrahydromethanopterin formyltransferase, 5,10-methenyltetrahydromethanopterin cyclohydrolase, methylene tetrahydromethanopterin:coenzyme F₄₂₀ oxidoreductase, formate dehydrogenase, and carbonic anhydrase were examined in acetate- and methanol-grown *Methanosarcina thermophila*. Levels of formyltransferase in either acetate- or methanol-grown *Methanosarcina thermophila* were approximately half the levels detected in H₂-CO₂-grown *Methanobacterium thermoautotrophicum*. All other enzyme activities were significantly lower in acetate- and methanol-grown *Methanosarcina thermophila*.

Of the known methanogens, *Methanosarcina* species are the most catabolically diverse and are therefore amenable to the study of catabolite influence on protein synthesis. *Methanosarcina thermophila* TM-1 is capable of using acetate, methanol, or methylated amines as growth substrates (28). Except for the reductive demethylation of methyl coenzyme M, the pathways for utilization of acetate or methanol by the methanogenic archaeobacteria (see Fig. 3) (23, 25) are distinct from the pathway for reduction of carbon dioxide (20). When acetate is used, acetate kinase and phosphotransacetylase catalyze the activation of acetate to acetyl coenzyme A prior to the proposed cleavage of the carbon-carbon bond by the carbon monoxide dehydrogenase (CODH) complex (23). The levels of these enzymes are severalfold greater in acetate-grown cells than they are in methanol-grown cells (1, 15, 23). Electrons for the demethylation of methyl coenzyme M derive from the oxidation of methanol (see reaction 5 in Fig. 3) or the carbonyl group of acetate (see reaction 2 in Fig. 3). The presence of formylmethanofuran dehydrogenase activity in extracts of methanol-grown *Methanosarcina barkeri* is consistent with the proposal that methanol is oxidized by a reversal of the carbon dioxide reduction pathway (5). Growth of *Methanosarcina thermophila* TM-1 is supported by acetate or methanol, but the organism reduces carbon dioxide to methane only after an extended adaptation period, and growth is poor (28). Here we present results of two-dimensional (2-D) gel electrophoretic analyses of whole-cell proteins which estimate the extent of regulation by the growth substrate and report the activities of the enzymes that catalyze one-carbon redox reactions in extracts of acetate- and methanol-grown cells.

MATERIALS AND METHODS

Organism and 2-D electrophoresis. *Methanosarcina thermophila* (29) was grown either on 100 mM acetate or 100 mM methanol to the mid-log phase, and extracts were prepared by using a French pressure cell as described previously (22). Protein was determined by either the bicinchonic acid method (standard assay) (Pierce Chemical Co., Rockford, Ill.) or by a protein assay (Bio-Rad Laboratories, Richmond, Calif.), according to the instructions of the manufacturer, by using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard. Samples for electrophoresis were prepared by the method of Potts (19). The 2-D electrophoresis was performed as described by O'Farrell (17), except that 3-[(3-cholamidopropyl)dimethylammonia]-1-propanesulfonate was substituted for Nonidet P-40. The cathode solution was 20 mM NaOH, and the anode solution was 20 mM H₃PO₄. Tube gels (inner diameter, 1.5 mm; length, 130 mm) were focused at 20°C for 16 h at 800 V followed by 2 h at 1,500 V. For denaturing (sodium dodecyl sulfate) electrophoresis in the second dimension, 12% polyacrylamide gels were used as described by Laemmli (13) (30 mA per gel, 20°C). No equilibration was done between the first and second dimensions, to minimize diffusion of proteins from the tube gels. Gels were stained with Coomassie blue R-250 for 14 h or were stained with silver (27) to detect low-abundance proteins. Purified acetate kinase was a gift from D. J. Aceti (1), CODH was a gift from K. C. Terlesky (23), and phosphotransacetylase was a gift from L. L. Lundie (15).

Enzyme activities. Formylmethanofuran dehydrogenase, formylmethanofuran:tetrahydromethanopterin formyltransferase, 5,10-methenyltetrahydromethanopterin cyclohydrolase, methylene tetrahydromethanopterin:coenzyme F₄₂₀ oxidoreductase, and formate dehydrogenase activities were assayed as described before by using coenzymes that were isolated from *Methanobacterium thermoautotrophicum* (5, 7, 8, 10, 21). Carbonic anhydrase was assayed as described before (12).

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Immunological analysis. Antiserum specific to the formylmethanofuran:tetrahydromethanopterin formyltransferase of *Methanobacterium thermoautotrophicum* was produced in New Zealand White rabbits. Western blot (immunoblot) analysis was performed as described before (3). Anti-formyltransferase antiserum was diluted 1:10,000 in phosphate-buffered saline (10 mM potassium phosphate [pH 7.0], 10 mM NaCl, 0.1% [vol/vol] Triton X-100 [PBST]) containing 0.1% casein and 0.1% gelatin, washed with PBST, incubated with ¹²⁵I-labeled goat anti-rabbit immunoglobulin G conjugate (0.6 μ Ci; Dupont, NEN Research Products, Boston, Mass.), washed with PBST, and autoradiographed on diagnostic film (SB 5; Eastman Kodak Co., Rochester, N.Y.).

RESULTS AND DISCUSSION

2-D electrophoresis. Approximately 454 spots were resolved in silver-stained gels of proteins from acetate-grown cells and 406 spots were resolved from methanol-grown cells (Fig. 1A and B). The intensities and patterns of the spots were reproducible by either staining procedure. Different proteins, at the same concentration, may silver stain with different intensities (18); however, comparison of spots of the same protein was quantitative. Spots with apparent equal intensities in both gels were used as standards when protein patterns were compared. Acidic proteins predominated in cells that were grown on either substrate. This 2-D protein pattern is in contrast to that of *Escherichia coli* NC3, in which neither acidic nor basic proteins predominate, and also that of *Methanococcus thermolithotrophicus*, in which basic proteins predominate (4, 11). At least 141 spots were present in methanol-grown cells but not in acetate-grown cells, and at least 139 spots were present in acetate-grown cells but not in methanol-grown cells; examples are shown in Fig. 1C and D. The results may not reflect the actual number of protomers, since the pattern of some spots suggested charge train heterogeneity (Fig. 1A through D). The basis for this apparent heterogeneity in *Methanosarcina thermophila* is not known. It is possible to induce charge heterogeneity of proteins by carbamylation with urea (9). Heterogeneity may also be induced by oxygen radicals (6); however, 2-D electrophoresis analysis of *Methanosarcina thermophila* proteins prepared under anaerobic, nonreducing conditions showed a pattern similar to that of proteins prepared under aerobic conditions (data not shown).

The 2-D electrophoresis of pure acetate kinase from *Methanosarcina thermophila* yielded two spots (data not shown) which corresponded to the two polypeptides from acetate-grown cells indicated in Fig. 1A. It was previously reported (1) that acetate kinase is composed of two subunits with the same N-terminal amino acid sequence and molecular weight; however, the results reported here suggest that the subunits have different charge properties. It is interesting that acetate kinase from *E. coli* is covalently modified by phosphorylation during catalytic turnover (2). The 2-D electrophoresis of pure, monomeric phosphotransacetylase yielded a single spot (data not shown) which corresponded to the polypeptide from acetate-grown cells indicated in Fig. 1A.

Spots corresponding to acetate kinase or phosphotransacetylase were either absent or present at a lower intensity in gels of methanol-grown cells (Fig. 1B). These results are consistent with the previously reported amounts of enzyme activities and materials that cross-react with anti-acetate kinase or anti-phosphotransacetylase antiserum (1, 15). The presence of unidentified spots in acetate-grown but not in

methanol-grown cells implies their involvement in acetate metabolism. For example, high levels of carbonic anhydrase activity are present in acetate-grown but not in methanol-grown *Methanosarcina thermophila* (Table 1). It is hypothesized that this enzyme may function in acetate transport in *Methanosarcina barkeri* (12).

Native and denaturing one-dimensional electrophoresis has shown that the intact CODH complex is present in acetate-grown but not in methanol-grown cells, and at least the largest (α) and smallest (ϵ) of the five subunits are present in much lower concentrations in methanol-grown cells (23). The 2-D electrophoresis of the pure CODH complex resolved the five subunits (data not shown) that corresponded in location to the polypeptides from acetate-grown cells, as indicated in Fig. 1C. Comparison of panels C and D of Fig. 1 confirmed earlier results (23), which suggested that the α subunit of CODH from acetate-grown cells is present in higher concentrations. The spot corresponding to the ϵ subunit was only barely visible in the Coomassie blue R-250-stained 2-D gels of acetate- and methanol-grown cells (Fig. 1C and D), which precluded any quantitative comparison. No conclusions could be drawn regarding the identification of the large spots from methanol-grown cells that appeared at positions corresponding to the β , γ , and δ subunits of CODH (Fig. 1D). Either these subunits are present at a higher concentration in methanol-grown cells or additional proteins are synthesized that migrate to similar positions as the β , γ , and δ subunits of CODH. Further studies are necessary to distinguish between these possibilities.

Formylmethanofuran:tetrahydromethanopterin formyltransferase catalyzes an early step in the pathway of carbon dioxide reduction to methane (8). Although acetate-grown *Methanosarcina thermophila* cells are unable to reduce carbon dioxide to methane (28), four polypeptides with the same molecular weight were present in acetate-grown *Methanosarcina thermophila* cells that cross-reacted with anti-formyltransferase antiserum from *Methanobacterium thermoautotrophicum* (Fig. 2). Spots that corresponded in location to the cross-reacting material were visible in 2-D gels of both acetate- and methanol-grown cells. High levels of formyltransferase activity were present in both acetate- and methanol-grown cells (Table 1). The pattern in Fig. 2 implies charge train heterogeneity; possible microheterogeneity of the formyltransferase from *Methanobacterium thermoautotrophicum* has been noted previously (8).

Enzyme activities. Low levels of formylmethanofuran dehydrogenase activity were detected in acetate-grown cells of *Methanosarcina thermophila*, but methanol-grown cells contained higher levels of activity than did H₂-CO₂-grown *Methanobacterium thermoautotrophicum* (Table 1). Formyltransferase activities in methanol-grown cells of *Methanosarcina thermophila* were approximately half of those detected in H₂-CO₂-grown *Methanobacterium thermoautotrophicum*; acetate-grown *Methanosarcina thermophila* cells contained slightly lower activities than did methanol-grown cells. Methanol- and acetate-grown cells of *Methanosarcina thermophila* contained low levels of cyclohydrolyase and oxidoreductase activities when compared with those of H₂-CO₂-grown *Methanobacterium thermoautotrophicum* cells. Functionally similar cofactors from *Methanosarcina* and *Methanobacterium* species have minor structural differences (24); thus, activities in extracts of *Methanosarcina thermophila* could be underestimated since the cofactors used in the assays were isolated from *Methanobacterium thermoautotrophicum*.

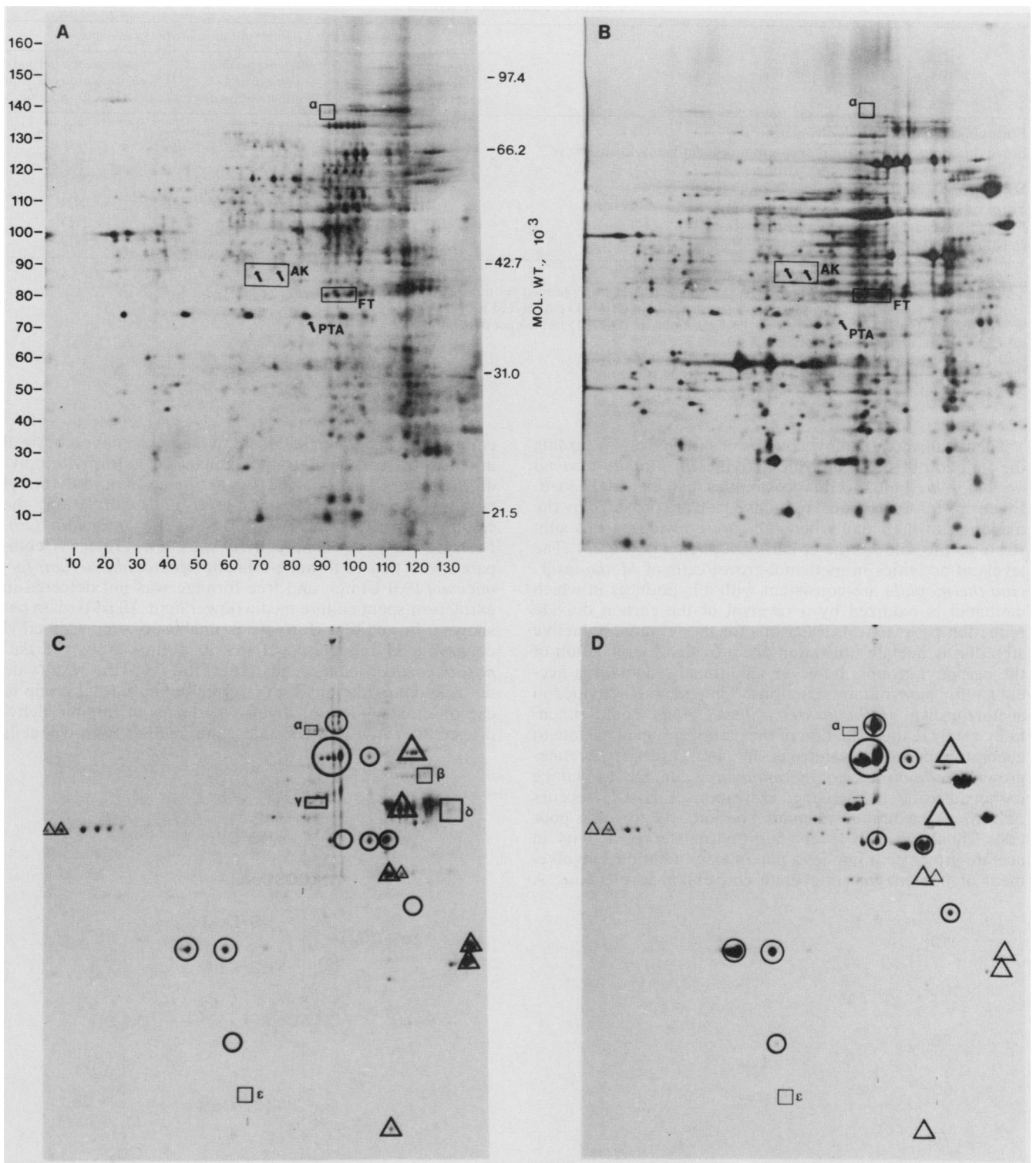


FIG. 1. A 2-D electrophoretic analysis of *Methanosarcina thermophila* proteins. The polypeptide maps of cell extract protein (20 µg) from cells grown on either 100 mM acetate or 100 mM methanol are shown in panels A and B, respectively. The identified polypeptides include the α subunit of CODH, acetate kinase (AK), phosphotransacetylase (PTA), and the putative formyltransferase region (FT). Gels in panels A and B were silver stained. (C and D) Gels were loaded with 75 µg of cell extract protein of acetate- and methanol-grown cells, respectively. Gels were stained with Coomassie blue R-250. The identified polypeptides include α , β , γ , δ , and ϵ subunits of CODH. Symbols: Δ , examples of proteins that were present in higher levels in acetate-grown cells than in methanol-grown cells; \circ , examples of proteins that were present in higher levels in methanol-grown cells than in acetate-grown cells. Axes represent relative positions of spots. The increasing isoelectric point is from right to left in each panel (pH range, ~ 4.3 to 7.5). Molecular weight markers include rabbit muscle phosphorylase *b* (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (42,700), bovine carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500).

TABLE 1. Enzyme activities

Enzyme	Enzyme activity (U/mg) in the following organisms:		
	<i>Methanosarcina thermophila</i> (acetate-grown)	<i>Methanosarcina thermophila</i> (methanol-grown)	<i>Methanobacterium thermoautotrophicum</i> (H ₂ /CO ₂ -grown)
Formylmethanofuran dehydrogenase ^a	0.08	1.5	1.0
Formylmethanofuran:tetrahydromethanopterin formyltransferase ^b	0.7	1.0	2.1
5,10-Methenyl-H ₄ MPT cyclohydrolase ^c	0.03	0.09	1.2
Methylene-H ₄ MPT:coenzyme F ₄₂₀ oxidoreductase ^d	0.02	0.05	3.1
Formate dehydrogenase (13 mM formate) ^e	0.4	0.6	ND ^f
Formate dehydrogenase (40 mM formate)	0.2	0.7	ND
Carbonic anhydrase ^g	0.7	NA ^h	ND

^a One unit = 1 μmol of methyl viologen reduced per min.

^b One unit = 1 μmol of 5-formyl-tetrahydromethanopterin (H₄MPT) formed per min.

^c One unit = 1 μmol of methenyl-tetrahydromethanopterin (H₄MPT) hydrolyzed per min.

^d One unit = 1 nmol of methenyl-tetrahydromethanopterin (H₄MPT) formed per min.

^e One unit = 1 μmol of methyl viologen reduced per min.

^f ND, Not determined.

^g One unit = time required for a decrease in 2 pH units.

^h NA, No activity detected (level of sensitivity, 0.3 U).

The results do not allow conclusions to be made regarding the function of these enzyme activities in *Methanosarcina thermophila*, but several possibilities can be envisioned. Electrons for the terminal reductive step are provided by the oxidation of methanol when cells are growing on this substrate as the sole source of carbon and energy (Fig. 3). The levels of activities in methanol-grown cells of *Methanosarcina thermophila* are consistent with a hypothesis in which methanol is oxidized by a reversal of the carbon dioxide reduction pathway (5). Electrons for the terminal reductive step during acetate utilization are provided by oxidation of the carbonyl groups; however, additional reductant is necessary for biosynthetic reactions. The enzyme activities in acetate-grown *Methanosarcina thermophila* could potentially catalyze the oxidation of the methyl group of acetate to generate accessory electrons for biosynthesis. Acetate-grown *Methanosarcina thermophila* is unable to reduce carbon dioxide to methane; utilization of H₂-CO₂ occurs only after an extended adaptation period, and growth is poor (28). Thus, the high level of formyltransferase activity in acetate-grown cells implies a potential for additional involvement of this enzyme in acetate conversion to methane. A

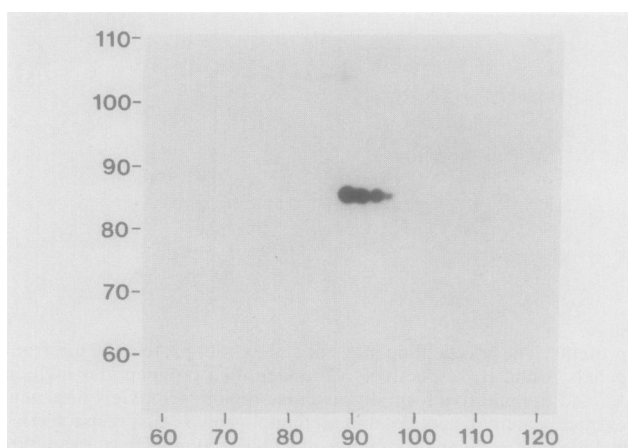


FIG. 2. Western blot (immunoblot) analysis of putative formyltransferase in extracts of acetate-grown *Methanosarcina thermophila*. The 2-D gels were loaded with 75 μg of cell extract protein, and the blots were probed with anti-formyltransferase antiserum. Coordinates on the x and y axes correspond to coordinates in Fig. 1.

pathway for acetate metabolism to methane is hypothesized in which the carbonyl group is converted to formyltetrahydromethanopterin followed by the release of the formyl group coupled to a substrate-level phosphorylation; the resulting formate is finally oxidized to carbon dioxide (26). Formate dehydrogenase activity was low (Table 1) compared with that of formate-utilizing *Methanobacterium formicicum* (9.0 U/mg), and free formate was not detected in extracts or spent culture media (lower limit, 10 μM) (data not shown). In addition, formate is unable to support methyl coenzyme M reductase activity in acetate-grown *Methanosarcina thermophila* cells (16). However, the results do not rule out oxidation of an enzyme-bound formyl group to carbon dioxide. The relatively low levels of formate dehydrogenase activity in acetate- and methanol-grown cells

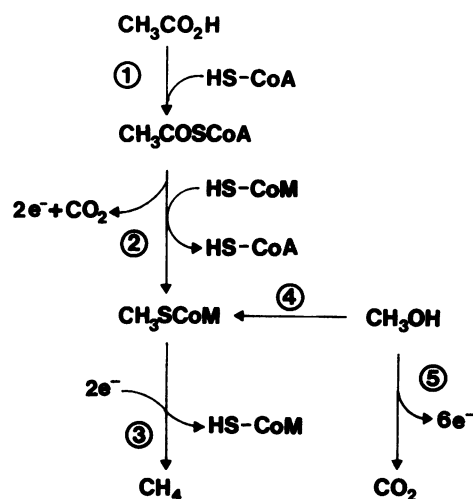


FIG. 3. Proposed pathways for the conversion of acetate or methanol to methane. Pathways: 1, activation of acetate to acetyl coenzyme A (CoA) by acetate kinase and phosphotransacetylase; 2, cleavage of the carbon-carbon bond of acetyl coenzyme A catalyzed by the CODH complex and release of electrons (e⁻) for the reductive demethylation of methyl coenzyme M (CoM) to methane; 3, reductive demethylation of methyl coenzyme M to methane; 4, methyl group transfer from methanol to coenzyme M; 5, oxidation of methanol to CO₂ providing electrons for the reductive demethylation of methyl coenzyme M to methane.

could potentially function to provide formyl groups for cell carbon synthesis, as recently proposed for *Methanobacterium thermoautotrophicum* (22a). Other possibilities for the high levels of formyltransferase include cycling of a formyl group in the hypothetical mechanism proposed for substrate-level phosphorylation in methanogens (14).

ACKNOWLEDGMENTS

This study was supported in part by grant 5086-260-1255 from the Gas Research Institute and grant DE-FG05-87ER 13730 A000 from the U.S. Department of Energy (to J.G.F.) and by Public Health Service grant AI12277 from the National Institutes of Health (to R. S. Wolfe).

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