APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1981, p. 388-391
0099-2240/81/020388-04$02.00/0 Vol. 41, No. 2

Levels of Coenzyme F_{420}, Coenzyme M, Hydrogenase, and Methylcoenzyme M Methylreductase in Acetate-Grown *Methanosarcina*

LARRY BARESI† AND R. S. WOLFE*
Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Methanosarcina barkeri **strain 227 maintained on an acetate medium for 2 years was found to possess hydrogenase, methylcoenzyme M methylreductase, coenzyme F_{420}, and coenzyme M. The levels of these constituents in acetate-grown cells were similar to those found in cells of the same strain grown on methanol or hydogen and carbon dioxide.**

Knowledge of methanogenesis from acetate has lagged far behind that of methanogenesis from hydrogen and carbon dioxide, where 2-(methylthio)ethanesulfonic acid (CH_3-S-CoM) has been shown to be a substrate for methylreductase (6–8, 12) and where coenzyme F_{420} (4, 5, 13) and hydrogenase (5, 6, 13, 15) have been shown to play roles in electron transport. With few exceptions, such as *Methanosarcina* strain TM-1 (17) and the "large rod" (16), all methanogens oxidize hydrogen and reduce CO_2 to methane (1). Only members of the genus *Methanosarcina* have been reported to carry out methanogenesis also from methanol and acetate. Recently CH_3-S-CoM has been shown to be formed during methanogenesis from methanol (10). *Methanosarcina barkeri* strain 227 is capable of growth on H_2-CO_2 (80:20), methanol, or acetate (11). By use of this strain it is possible to grow cells separately on each of these substrates and to compare the levels of various components. Since little is known about the enzymology of methane formation from acetate, we report here on levels of 2-mercaptoethanesulfonic acid, CH_3-S-CoM methylreductase, hydrogenase, and coenzyme F_{420} in acetate-grown cells of *M. barkeri* strain 227. A comparison is made with levels of these compounds found in cells of this strain grown on H_2-CO_2 or methanol.

MATERIALS AND METHODS

Organisms and culture conditions. *M. barkeri* strain 227 was obtained from R. A. Mah (University of California at Los Angeles, Los Angeles) and cultivated in the following basal medium (values in grams per liter): NH_4Cl, 0.33; $MgCl_2 \cdot 6H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.0025; $NiCl_2 \cdot 6H_2O$, 0.00047; resazurin, 0.001; L-cysteine hydrochloride$\cdot H_2O$, 0.5; yeast extract (Difco Laboratories, Detroit, Mich.), 2; Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 2. For growth of cells on acetate, the medium was supplemented with (grams per liter): Na_2HPO_4, 2.2; $NaH_2PO_4 \cdot H_2O$, 3.4; $Na_2S \cdot 9H_2O$, 0.1; sodium acetate, 8.2. When H_2-CO_2 (80:20) was the substrate, the basal medium supplement consisted of (grams per liter): Na_2HPO_4, 0.53; $NaH_2PO_4 \cdot H_2O$, 0.86; $Na_2S \cdot 9H_2O$, 0.1; $NaHCO_3$, 5. When methanol was the substrate, the basal medium was supplemented with (grams per liter): Na_2HPO_4, 2.2; $NaH_2PO_4 \cdot H_2O$, 3.4; $Na_2S \cdot 9H_2O$, 0.1; $NaHCO_3$, 0.1; methanol, 20. The gas atmosphere for both the acetate-grown and the methanol-grown cells was nitrogen. All gases used in the procedures described in this communication were scrubbed free of oxygen by passage over heated copper filings at 350°C. Anaerobic procedures described by Balch and coworkers were used (1, 2). Sterile sodium sulfide was added aseptically to the sterile medium after autoclaving and before inoculation.

Cells were routinely cultured in 50 ml of medium in a 125-ml serum vial (Wheaton Scientific Div., Wheaton Industries, Millville, N.J.) or in 400 ml of medium in a 1-liter bottle (no. 219760, Wheaton Scientific) modified to accommodate syringe injection as described by Balch and co-workers (1, 2). Serum vials were initially inoculated from anaerobic plates (1). Aseptic transfer from the plate to the vial was carried out in an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.). With a sterile blunt needle the colony was picked and then injected through the sterile stopper into the appropriate medium; the culture was incubated at 37°C. To use this culture as an inoculum, the atmosphere was pressurized with nitrogen or, alternatively, the pressure of methane produced within the vial by the organism was used to force the culture into a bottle that contained sterile medium. This was conveniently done (Fig. 1) by use of a double-male Luer lock adapter (Becton, Dickinson & Co., Rutherford, N.J.) to each end of which was attached an 18-gauge hypodermic needle; the probe was autoclaved with the needle shields in place. The sterile medium in the vessel designated to receive the inoculum was aseptically placed under a slight positive pressure of nitrogen (2), and one end of the sterile probe was pushed through the sterile rubber stopper, releasing the limited internal pressure and clearing the adapter of oxygen. As the pressure release neared

† Present address: Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA 91103.

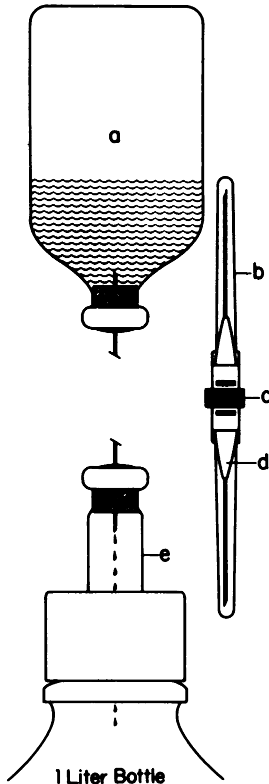

FIG. 1. *Inoculating probe developed for anaerobic transfer of inocula too large to be conveniently transferred by syringe. The procedure is described in the text. (a) Culture in a 150-ml serum vial; (b) plastic shield; (c) double-male Luer lock adapter; (d) 18-gauge hypodermic needle; (e) 1-liter bottle fitted with aluminum sealed tube. (From reference 1.)*

completion, the opposite end of the probe was aseptically pushed through the flamed rubber stopper of the inoculating vessel; the pressure in the inoculating vessel forced the inoculum into the receiving vessel. After inoculation the needle in the receiving vessel was removed first. Cells also were cultured on acetate or methanol in 20-liter carboys. (This technique is the most efficient and reliable method that we have found for anaerobically transferring large inocula of 50 to 200 ml of culture.)

To harvest cells the culture medium was anaerobically siphoned from the culture vessel, leaving a layer of cells on the bottom of the vessel. The cell slurry was anaerobically transferred to 50-ml centrifuge tubes that were anaerobically sealed before centrifugation. The cell pellet in each tube was washed once in about 50 ml of anaerobic medium of the same composition as the growth medium, but without substrate, i.e., acetate, methanol, or hydrogen. Harvested cells were used immediately for extract preparation.

Preparation of cell extract. Cells were disrupted by passage through a French pressure cell at 20,000 lb/in^2. The pressure cell was loaded in an anaerobic chamber. The effluent from the apparatus was collected anaerobically in a closed stainless steel centrifuge tube under a gentle stream of nitrogen that was passed into the tube through a rubber stopper and vented by a second hypodermic needle used as an exit port. The closed centrifuge tube of lysed cells was returned to the anaerobic chamber, where it was sealed with a stainless steel cap; the tube was removed from the chamber and centrifuged at $40,000 \times g$ for 30 min at 4°C. The tube was then returned to the anaerobic chamber, where the supernatant solution (hereafter referred to as cell extract) was decanted in 2-ml amounts, each portion being stored in a 5-ml serum bottle capped with a black rubber stopper. Each bottle was then gassed with nitrogen and stored at −20°C until used.

Assays. The CH_3-S-CoM methylreductase assay was performed by procedures described previously (6, 7, 12). The reaction mixture was modified to contain the following additions: 10 μl of a solution of 0.1 M adenosine 5′-triphosphate and 0.5 M $MgCl_2$ and 20 μl of a solution of 31 mM TES buffer [*N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (pH 6.0) and 12.5 mM 2-mercaptoethanol. The reaction was initiated at 37°C by addition of 10 μl of 0.05 M CH_3-S-CoM. Hydrogenase was assayed by procedures described previously (5) by following reduction of flavin mononucleotide. The coenzyme F_{420} content of cell extract was estimated after the extract had been concentrated by rotor evaporation. In certain experiments the extract was boiled for 15 min and then centrifuged at $40,000 \times g$ before being concentrated. The initial rate of nicotinamide adenine dinucleotide phosphate reduction in the coenzyme F_{420}-nicotinamide adenine dinucleotide phosphate-linked hydrogenase system in extracts of *Methanobacterium thermoautotrophicum* as described by Eirich et al. (5) was used to quantitate coenzyme F_{420}. In certain preparations coenzyme F_{420} was quantitated spectrophotometrically, using $E^{401}_{1M} = 25 \times 10^3$ M^{-1} cm^{-1} (7). The amounts of coenzyme M in heat-treated extracts of cells were determined as described by Balch and Wolfe (3). Methane was determined with a Packard gas chromatograph model 428 equipped with a hydrogen flame detector and a glass column (180 cm by 4 mm) packed with Super Q (Alltech Associates, Inc., Arlington Heights, Ill.).

Chemical. CH_3-S-CoM was synthesized by W. E. Ellefson, following the procedure described by Romesser and Balch (9). Flavin mononucleotide, adenosine 5′-triphosphatase, nicotinamide adenine dinucleotide phosphate, TES, and 2-mercaptoethanol were obtained from Sigma Chemical Co., St. Louis, Mo. Gases were obtained commercially and were scrubbed free of oxygen as described above.

RESULTS

For cells grown on H_2-CO_2 the cell yield after 5 to 12 days of incubation averaged 4 g (wet weight)/liter of growth medium; for cells grown on methanol for the same time the yield was 2.5 g, and for acetate-grown cells the yield was 1 g after 21 to 28 days of incubation.

Table 1 shows the specific activities of hydrogenase and CH_3-S-CoM methylreductase as well as the levels of coenzyme F_{420} and coenzyme M that were found in cells of *M. barkeri* strain 227.

TABLE 1. *Quantitation of enzyme activities and cofactor concentrations for cells grown on different methanogenic substrates*[a]

Substrate for cell growth	Prepn no.	CH_3-S-CoM methylreductase (nmol of CH_4 formed/min per mg of protein)	Hydrogenase (μmol of FMN reduced/min per mg of protein)	Coenzyme F_{420} (ng/mg of protein)	Coenzyme M (nmol/mg of protein)
H_2-CO_2	1	12.5 ± 0.03[b]	0.49 ± 0.03[b]	7.3 ± 0.00[b,c]	80 ± 19[d]
	2	12.0			
Acetate	3	18.4	0.31 ± 0.03[b]	7.6 ± 0.00[b]	90 ± 20[d]
	4	7.7 ± 1.0[b]			
Methanol	5	8.6 ± 2.0[b]	0.51	7.0[e]	72 ± 0.5[d]

[a] Standard deviations are indicated.
[b] Duplicate determinations.
[c] Enzymic assay.
[d] Quadruplicate determinations.
[e] Spectrophotometric assay.

Separate batches of the organism were grown on H_2-CO_2 (80:20), acetate, or methanol; cells from each substrate had been maintained and subcultured exclusively on that substrate for over 1 year. Of the four components assayed, there was rather good agreement among the levels found in cells from the three substrates. Efforts were made to prepare extract from cells that shared a common history, such as age of culture, conditions of storage, and condition of breakage, as well as to keep variation in protein concentration to a minimum. However, due to the long growth period (4 weeks or more) on acetate, it was not possible to provide rigorously similar preparations. The widest variation was found in the CH_3-S-CoM methylreductase assay, where values ranged from about 8 to 18 nmol of methane formed per min per mg of protein.

Figure 2A shows results of additional studies on the CH_3-S-CoM methylreductase and on the ability of extracts from acetate-grown or hydrogen-grown cells to form methane from H_2-CO_2. When extract from acetate-grown cells was tested in the assay under an H_2-CO_2 atmosphere with CH_3-S-CoM present, methane was produced at a rate of 18.8 nmol/min per mg of protein, whereas in the absence of added CH_3-S-CoM, the rate was 0.53 nmol/min per mg of protein. Similar results were obtained with extract from hydrogen-grown cells, where the rate of methane formation was 10.6 nmol/min per mg of protein in the presence of CH_3-S-CoM and 0.5 nmol/min per mg of protein in the absence of added CH_3-S-CoM. Each extract possessed an active CH_3-S-CoM methylreductase, but methane formation was negligible in an H_2-CO_2 atmosphere in the absence of added CH_3-S-CoM.

Figure 2B shows results obtained when the CH_3-S-CoM methylreductase reaction was carried out under a nitrogen atmosphere with and without added CH_3-S-CoM. For hydrogen-

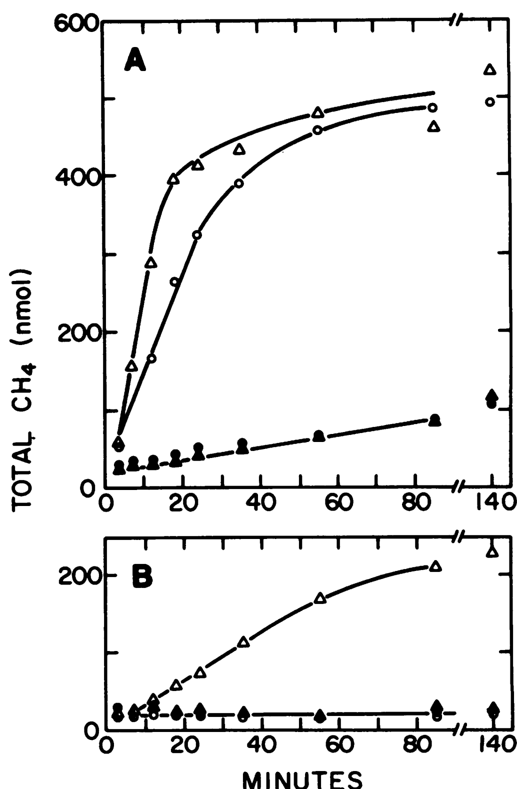

FIG. 2. *Formation of methane from CH_3-S-CoM by extracts of hydrogen-grown or acetate-grown cells. Reaction mixtures for the CH_3-S-CoM methylreductase were constituted as described in the text. (A) H_2-CO_2 (80:20) atmosphere in each reaction vial; (B) Nitrogen atmosphere in each vial. Symbols: triangles, vials containing extract from acetate-grown cells (1.2 mg of protein with added CH_3-S-CoM (△) and without CH_3-S-CoM (▲); circles, reaction mixtures containing extract (1.2 mg of protein) from hydrogen-grown cells with added CH_3-S-CoM (○) and without CH_3-S-CoM (●).*

grown cells, no methane was produced in the absence of hydrogen or hydrogen and carbon dioxide. Yet for acetate-grown cells, the cell extract had the capacity to reduce about 40% of the CH_3-S-CoM to methane. The exact nature of this reduction is unknown, but preincubation of the mixture under a nitrogen atmosphere for 10 min at 37°C before the addition of CH_3-S-CoM decreased the amount of methane produced (data not shown).

DISCUSSION

There exists a dearth of evidence concerning components of the methanogenic system in acetate-grown methanogens. By use of acetate-adapted cells of *M. barkeri* strain 227, we now have made a first step toward an understanding of the process; cells of this strain that were subcultured only on acetate as substrate over a 2-year period were found to possess hydrogenase and CH_3-S-CoM methylreductase activities as well as coenzyme F_{420} and coenzyme M. When levels of these components in acetate-grown cells were compared with levels found in cells grown on H_2-CO_2 or methanol, we found considerable similarities. Results of the present studies extend the observation of Weimer and Zeikus (14) and Shapiro and Wolfe (10) that cells of *M. barkeri* cultivated on methanol possess hydrogenase. Hydrogenase appeas to be a constitutive activity in strain 227; on the basis of these results it would be expected that cells grown on various methylamines also would exhibit hydrogenase activity. Different hydrogenases have been found in *M. thermoautotrophicum*, and it may be that the hydrogenase produced by cells of *M. barkeri* growing on hydrogen and carbon dioxide is different than that produced by cells growing on methanol or acetate.

The level of coenzyme F_{420} (about 7 ng/mg of protein) detected in cells grown on each of the three substrates is about 50% of that reported by Eirich et al. (5) for *M. barkeri* strain MS. This variation may be due to strain differences; however, the important finding is that acetate-grown cells of strain 227 possess levels of coenzyme F_{420} equal to those produced on other substrates. The levels of coenzyme M detected in strain 227 under the three growth conditions were similar. In contrast, Balch and Wolfe (3) reported a 2.5-fold excess of coenzyme M in cells of strain MS grown on methanol compared with cells grown on hydrogen and carbon dioxide.

Results of these studies show the presence of a functional CH_3-S-CoM methylreductase system in strain 227 that is independent of growth substrate. Recently, Smith and Mah (11) reported a diauxic growth response for strain 227 when cells were grown on a mixture of methanol and acetate, and results reported by Shapiro and Wolfe (10) suggested that the methanol-2-mercaptoethanesulfonic acid methyltransferase was constitutive, being found in both cells grown on hydrogen-carbon dioxide and cells grown on methanol.

ACKNOWLEDGMENT

This work was supported by National Science Foundation grant PCM 78-25141.

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. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32:**781–791.
3. **Balch, W. E., and R. S. Wolfe.** 1979. Specificity and biological distribution of coenzyme M (2-mercaptoethanesulfonic acid). J. Bacteriol. **137:**256–263.
4. **Eirich, L. D., G. D. Vogels, and R. S. Wolfe.** 1978. The structure of coenzyme F_{420}, a novel electron carrier isolated from *Methanobacterium* strain M.o.H. Biochemistry **17:**4583–4593.
5. **Eirich, L. D., G. D. Vogels, and R. S. Wolfe.** 1979. Distribution of coenzyme F_{420} and properties of its hydrolytic fragments. J. Bacteriol. **140:**20–27.
6. **Gunsalus, R. P., and R. S. Wolfe.** 1978. ATP activation and properties of the methyl coenzyme M reductase system in *Methanobacterium thermoautotrophicum*. J. Bacteriol. **135:**851–857.
7. **Gunsalus, R. P., and R. S. Wolfe.** 1980. Methyl coenzyme M reductase from *Methanobacterium thermoautotrophicum*. J. Biol. Chem. **255:**1891–1895.
8. **McBride, B. C., and R. S. Wolfe.** 1971. A new coenzyme of methyl-transfer, coenzyme M. Biochemistry **10:**2317–2324.
9. **Romesser, J. A., and W. E. Balch.** 1980. Coenzyme M: preparation and assay. Methods Enzymol. **67:**545–552.
10. **Shapiro, S., and R. S. Wolfe.** 1980. Methyl-coenzyme M, an intermediate in methanogenic dissimilation of C_1 compounds by *Methanosarcina barkeri*. J. Bacteriol. **141:**728–734.
11. **Smith, M. R., and R. A. Mah.** 1978. Growth and methanogenesis by *Methanosarcina* strain 227 on acetate and methanol. Appl. Environ. Microbiol. **36:**870–879.
12. **Taylor, C. D., and R. S. Wolfe.** 1974. Structure and methylation of coenzyme M. J. Biol. Chem. **249:**4879–4885.
13. **Tzeng, S. F., R. S. Wolfe, and M. P. Bryant.** 1975. Factor 420-dependent pyridine nucleotide-linked hydrogenase system of *Methanobacterium ruminantium*. J. Bacteriol. **121:**184–191.
14. **Weimer, P. J., and J. G. Zeikus.** 1978. Acetate metabolism in *Methanosarcina barkeri*. Arch. Microbiol. **119:**175–182.
15. **Wolfe, R. S., and I. J. Higgins.** 1979. Microbial biochemistry of methane a study in contrasts, p. 267–353. *In* J. R. Quayle (ed.), International review of biochemistry, vol. 21. Microbial biochemistry. University Park Press, Baltimore.
16. **Zehnder, A. J., B. A. Huser, T. D. Brock, and K. Wuhrmann.** 1980. Characterization of an acetate-decarboxylating, non-hydrogen-oxidizing methane bacterium. Arch. Microbiol. **124:**1–11.
17. **Zinder, S. H., and R. A. Mah.** 1979. Isolation and characterization of a thermophilic strain of *Methanosarcina* unable to use H_2–CO_2 for methanogenesis. Appl. Environ. Microbiol. **38:**996–1008.