Levels of Coenzyme F$_{420}$, Coenzyme M, Hydrogenase, and Methylcoenzyme M Methylreductase in Acetate-Grown Methanosarcina

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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 hydrogen 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$_4$Cl, 0.33; MgCl$_2$-6H$_2$O, 0.1; FeCl$_3$-6H$_2$O, 0.0025; NaCl, 0.00047; resazurin, 0.001; L-cysteine hydrochloride-H$_2$O, 0.5; yeast extract (Difco Laboratories, Detroit, Mich.), 2; Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 2.

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For growth of cells on acetate, the medium was supplemented with (grams per liter): Na$_2$HPO$_4$, 2.2; Na$_2$HPO$_4$-1.2H$_2$O, 3.4; Na$_2$S-9H$_2$O, 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$_2$HPO$_4$, 0.53; Na$_2$HPO$_4$·H$_2$O, 0.86; Na$_2$S-9H$_2$O, 0.1; NaHCO$_3$, 5. When methanol was the substrate, the basal medium was supplemented with (grams per liter): Na$_2$HPO$_4$, 2.2; NaH$_2$PO$_4$·H$_2$O, 3.4; Na$_2$S-9H$_2$O, 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 co-workers 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
fuge 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 × 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 CH3-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 MgCl2 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-mercaptopethanol. The reaction was initiated at 37°C by addition of 10 μl of 0.05 M CH3-S-CoM. Hydrogenase was assayed by procedures described previously (5) by following reduction of flavin mononucleotide. The coenzyme F420 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 × g before being concentrated. The initial rate of nicotinamide adenine dinucleotide phosphate reduction in the coenzyme F420-nicotinamide adenine dinucleotide phosphate-linked hydrogenase system in extracts of Methanobacterium thermoautotrophicum as described by Eirich et al. (5) was used to quantitate coenzyme F420. In certain preparations coenzyme F420 was quantitated spectrophotometrically, using E1%1cm = 25 × 103 M−1 cm−1 (7). The amounts of coenzyme M in heat-treated extracts of cells were determined as described before 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 (Altech Associates, Inc., Arlington Heights, Ill.).

Chemical. CH3-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-mercaptopethanol 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 H2-CO2, 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 CH3-S-CoM methylreductase as well as the levels of coenzyme F420 and coenzyme M that were found in cells of M. barkeri strain 227.
Separate batches of the organism were grown on H\textsubscript{2}-CO\textsubscript{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\textsubscript{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\textsubscript{3}-S-CoM methylreductase and on the ability of extracts from acetate-grown or hydrogen-grown cells to form methane from H\textsubscript{2}-CO\textsubscript{2}. When extract from acetate-grown cells was tested in the assay under an H\textsubscript{2}-CO\textsubscript{2} atmosphere with CH\textsubscript{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\textsubscript{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\textsubscript{3}-S-CoM and 0.5 nmol/min per mg of protein in the absence of added CH\textsubscript{3}-S-CoM. Each extract possessed an active CH\textsubscript{3}-S-CoM methylreductase, but methane formation was negligible in an H\textsubscript{2}-CO\textsubscript{2} atmosphere in the absence of added CH\textsubscript{3}-S-CoM.

Figure 2B shows results obtained when the CH\textsubscript{3}-S-CoM methylreductase reaction was carried out under a nitrogen atmosphere with and without added CH\textsubscript{3}-S-CoM. For hydrogen-
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 CH₂S-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 CH₂S-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 CH₂S-CoM methylreductase activities as well as coenzyme F₄₂₀ and coenzyme M. When levels of these components in acetate-grown cells were compared with levels found in cells grown on H₂-CO₂ 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 appears to be a constitutive activity in strain 227; on the basis of these results it would be expected that cells grown on various methyamines 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 F₄₂₀ (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 F₄₂₀ 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 CH₂S-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.

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