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Nutritional and Biochemical Characterization of *Methanospirillum hungatii*

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To ascertain its physiological similarity to other methanogenic bacteria, *Methanospirillum hungatii*, the type species of the genus, was characterized nutritionally and biochemically. Good growth occurred in a medium consisting of mineral salts, cysteine sulfide reducing buffer, and an H_2-CO_2 (80:20) atmosphere. Addition of amino acids and B vitamins stimulated growth. Cell-free extracts contained methylcobalamin-coenzyme M methyltransferase, methylreductase, and formate hydrogenlyase. Cells contained coenzyme M and coenzyme F_{420}. Coenzyme F_{420} was required for formate hydrogenlyase activity. Coenzyme F_{420} purified from *M. hungatii* had identical properties to that purified from species of *Methanobacterium*. The physiological basis of the family *Methanobacteriaceae* is strengthened by these findings.

Methanospirillum hungatii, a recently described methanogenic species (7, 8, 14), has been shown by electron micrographic studies to have unusual morphological characteristics that clearly set it apart from all other known organisms (8, 22). The substructure of the rigid cell envelope is unique in its regular pattern and lateral arrangement of the "structural elements" (22). In addition, the organism possesses a curious structure termed a "cell spacer" that is formed between cells. The function of this structure is unknown. At the end of each chain of cells is the "end component," which may be a modified "cell spacer." Long chains of cells produce light and dark striations throughout a colony, forming one of the most interesting and characteristic colonies among the bacteria (8).

Methanogenic bacteria share physiological and biochemical characteristics such as the ability to anaerobically oxidize hydrogen and reduce carbon dioxide to methane (2). Two unique coenzymes, coenzyme M and coenzyme F_{420}, have been shown to be involved in this process (17-19). We were interested in learning whether *M. hungatii*, which has such unusual morphological characteristics, was similar in its biochemical characteristics to other methanogenic bacteria. We present here results which clearly show that, although this organism is morphologically distinct, it is physiologically and biochemically a typical methanogen.

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MATERIALS AND METHODS

Media. Mineral salts medium contained the following constituents at the indicated final percent composition by weight: KH_2PO_4, 0.021; K_2HPO_4, 0.021; $(NH_4)_2SO_4$, 0.021; NaCl, 0.042; $MgSO_4 \cdot 7H_2O$, 0.0084; $CaCl_2 \cdot 2H_2O$, 0.0056; resazurin, 0.0001; Na_2CO_3, 0.4; cysteine-hydrochloride, 0.025; $Na_2S \cdot 9H_2O$, 0.25. Before sterilization, the medium was adjusted to pH 7.2 with HCl. The final pH after sterilization and equilibration with a gas mixture of H_2-CO_2 (80:20) was 7.0. Media used for nutritional studies were prepared by supplementing mineral salts medium with yeast extract (Difco), Casamino Acids (Difco), or vitamin B solution (20).

Cultural procedures. The type strain (ATCC 27890) of *M. hungatii*, the type species of the genus *Methanospirillum*, was used in this study; the isolation and maintenance of this organism have been described previously (8).

Nutritional studies were performed in 500-ml anaerobic shake flasks as described by Bryant et al. (3), with a glass sampling tube (40 by 15 mm) fitted into the side of the flask; a rubber stopper was inserted in the open end of the tube. The Hungate technique (9), as modified by Bryant and Robinson (4), was used. The flow rate of H_2-CO_2 (80:20) was 50 ml/min, and each culture was incubated at 45°C with shaking. Each flask contained 200 ml of medium and was inoculated with a 5-ml test tube culture previously grown in mineral salts medium. Growth was followed by periodically removing a 3-ml sample and measuring the absorbance at 660 nm in a 1-cm light path.

M. hungatii was mass cultured as described by Bryant et al. (3) in mineral salts medium supplemented with 0.4% each of yeast extract (Difco) and Trypticase (BBL). Fermentors were sparged with H_2-CO_2 (80:20) at a flow rate of 300 ml/min. The growth temperature was 41°C, and the pH was

maintained between 7.0 and 7.4. Inocula (200 ml) were grown in shake flasks (3).

Preparation of extracts. Crude cell-free extracts were prepared by passing a suspension of 20 g (wet weight) of packed cells in 20 ml of 0.05 M TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (pH 7.2) through a French pressure cell at 15,000 lb/in^2. The partially oxidized cell lysate was immediately gassed with H_2 by the Hungate technique, which produced a rapid reduction of coenzyme F_{420} (6). The cell lysate was centrifuged at 46,000 × *g* for 30 min in a Sorvall refrigerated centrifuge at 4°C. Anaerobic conditions were maintained by using capped 50-ml stainless-steel centrifuge tubes flushed with argon. The clear dark-brown supernatant solution was stored under a hydrogen atmosphere at −20°C.

Diethylaminoethyl (DEAE)-cellulose-treated extract was prepared in the following manner. Crude extract (10 ml) was applied to a DEAE-cellulose column (1.2 by 7 cm) previously equilibrated with 0.05 M TES buffer (pH 7.1). The extract was eluted in the cold with the same buffer at a flow rate of 3 ml/h. The extract was collected in a volume of 7 ml, gassed immediately with H_2, and stored at 4°C. Protein was estimated by the method of Lowry et al. (10). Crystalline bovine serum albumin was used as a protein standard.

Partial purification of coenzyme F_{420}. Methods used for the purification of coenzyme F_{420} were similar to those used previously (18). The amount of coenzyme isolated from *M. hungatii* was estimated by using the reported extinction coefficient (6). Spectra were obtained with a Cary model 14 spectrophotometer.

Enzymatic assays. Methane biosynthesis in crude cell extract was measured as described by Wolin et al. (20), except that the side arm was sealed with a small serum stopper and vented with a 21-gauge needle during final gassing of the flask with H_2.

Flasks were placed in a 40°C water bath and shaken until endogenous methane production ceased. The reaction was initiated by tipping in the substrate from the side arm or replacing the H_2 gas phase with H_2-CO_2 (80:20). The final pH of the reaction mixture was 7.0. Each 1.2-ml reaction mixture contained: TES buffer, pH 7.2, 50 μmol; adenosine 5′-triphosphate (ATP), 15 μmol; $MgCl_2$, 15 μmol; crude cell-free extract, 45 mg of protein; and the test substrate. Test substrates were: sodium formate, 100 μmol; sodium acetate, 100 μmol; sodium benzoate, 100 μmol; methylcobalamin, 7.2 μmol, or coenzyme M as 2,2′-dithiodiethanesulfonic acid [(S-CoM)$_2$], 45 nmol. Methane was measured with a Packard 7800 series gas chromatograph fitted with a silica gel column and a flame ionization detector. Helium was used as the carrier gas and the column was operated at 50°C. Methane was quantitated by measuring peak heights.

Formate hydrogenlyase assays with DEAE-cellulose-treated extract were carried out in small-scale reaction tubes as described by Taylor and Wolfe (16). All constituents except enzyme were added to the tube, which then was sealed with a serum stopper. The tube was flushed with nitrogen at a flow rate of 10 to 13 ml/min for 15 min. The gassing needles were removed, and the reaction was initiated by injecting enzyme. Each reaction mixture (0.59 ml) contained: TES buffer, pH 7.2, 50 μmol; $MgCl_2$, 3.5 μmol; sodium formate where indicated, 5 μmol; coenzyme F_{420} where indicated, 1.6 μg; dithiothreitol, 1.5 μmol; DEAE-cellulose-treated cell-free extract, 21 mg of protein. The reaction temperature was 40°C. Hydrogen evolution was measured with a Packard 7800 series gas chromatograph fitted with a silica gel column and an electron capture detector. Helium was used as the carrier gas and the column was operated at 0°C in an ice bath.

Methods for the assay of methylcobalamin-coenzyme M methyltransferase in crude cell-free extracts of *M. hungatii* were as described by Taylor and Wolfe (16). The samples were counted at an efficiency of 86 ± 1%, and the specific activity of [*methyl*-^{14}C]methylcobalamin ($^{14}CH_3$-B_{12}) was 2.33 × 10^6 dpm/μmol. Components used in the reaction mixtures (0.029 ml) were: TES buffer, pH 7.2, 50 μmol; ATP, 5 μmol; $MgCl_2$, 1.8 μmol; crude cell-free extract, 5.9 mg of protein; $^{14}CH_3$-B_{12}, 700 nmol; (S-CoM)$_2$, 90 nmol; tripolyphosphate, 7.8 μmol; nicotinamide adenine dinucleotide phosphate (NADPH), 150 nmol. The reaction temperature was 40°C.

Methylreductase in crude cell-free extracts of *M. hungatii* was assayed in small-scale reaction tubes as described previously (17). The reaction mixture (0.3 ml) contained: TES buffer, pH 7.2, 50 μmol; $MgCl_2$, 1.8 μmol; ATP, 2.5 μmol; crude cell-free extract, 4.6 mg of protein; 2-(methylthio)ethanesulfonic acid (CH_3-S-CoM), 1.32 μmol. The reaction temperature was 40°C.

The simplified assay with crude cell extracts as described by Taylor and Wolfe (16) was used to demonstrate the presence of coenzyme M in *M. hungatii*. Crude cell-free extract from *Methanobacterium* MOH was used as a source of methyltransferase. The reaction was allowed to proceed for 30 min.

Chemicals. Synthesis of methylcobalamin was performed as previously described (16); [*methyl*-^{14}C]methyliodide was purchased from Amersham/Searle, and cyanocobalamin was from Sigma Chemical Co. Sephadex QAE-A-25 and Sephadex G-10 were purchased from Pharmacia Fine Chemicals, Inc.; (S-CoM)$_2$, CH_3-S-CoM, and 2-mercaptoethanesulfonic acid (HS-CoM) were kindly provided by Craig Taylor.

RESULTS

Nutritional characteristics. Significant growth was obtained in the mineral salts medium with an H_2-CO_2 (80:20) atmosphere (Fig. 1). When this medium was supplemented with 0.4% Casamino Acids (Difco), growth was stimulated. The addition of 0.4% yeast extract (Difco) with Casamino Acids further stimulated growth. The vitamin B solution (1%, vol/vol) in combination with Casamino Acids partially replaced the stimulatory effect of yeast extract. Similar results were obtained if Trypticase

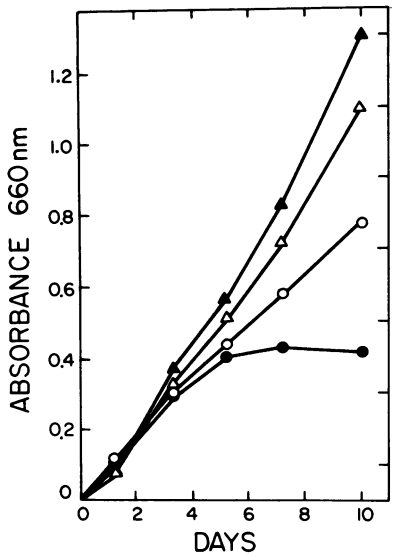

FIG. 1. *Stimulation of growth by B vitamins, yeast extract, and Casamino Acids. Growth in mineral salts medium with:* (●) *no additions;* (○) *0.4% (wt/vol) Casamino Acids added;* (△) *0.4% (wt/vol) Casamino Acids and 1.0% (vol/vol) B vitamin solution added;* (▲) *0.4% (wt/vol) each of Casamino Acids and yeast extract (Difco) added.*

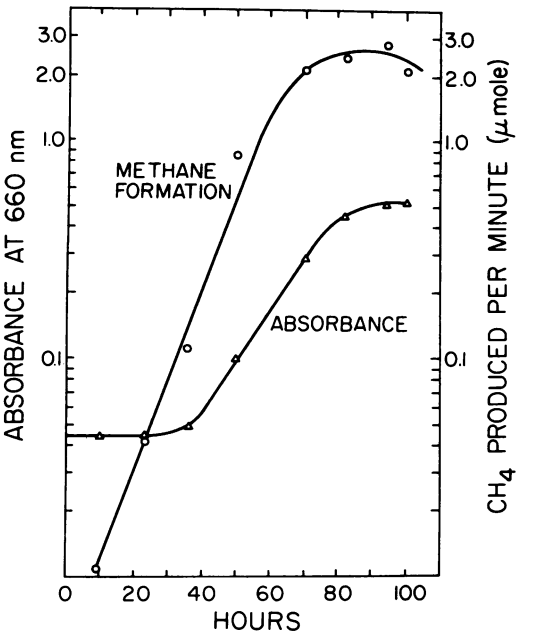

FIG. 2. *Methane formation and growth of Methanospirillum hungatii JF in a 12-liter fermentor.*

(BBL) was used instead of Casamino Acids. In a separate experiment, growth was not stimulated when 1 mg each of CH_3-S-CoM and HS-CoM or 0.14% (wt/vol) sodium acetate was added to a culture in 200 ml of mineral salts medium.

Mass culture. Cell yields of 25 g (wet weight) per 12-liter fermentor were consistently obtained. Growth followed methane production during the exponential and stationary phases (Fig. 2); the mean doubling time was 17 h.

Methane formation in cell-free extracts. Figure 3 illustrates the ability of various substrates to serve as precursors of methane formation in cell-free extracts. When only H_2 was included in the gas phase, a small endogenous level of CH_4 formation was observed. When acetate or formate was added, no significant methane was produced above the endogenous level. Benzoate appeared to inhibit endogenous methane formation. Methane formation from H_2-CO_2 (80:20) subsided after 30 min, and then increased sharply when 15 μmol of ATP was introduced at 75 min. Roberton and Wolfe (13) observed a similar stimulation of methane production by addition of ATP to extracts of *Methanobacterium* MOH, and they have concluded that ATP is required only in catalytic quantities. Compared with TES buffer (pH 7.2), tris(hydroxymethyl)aminomethane buffer (pH

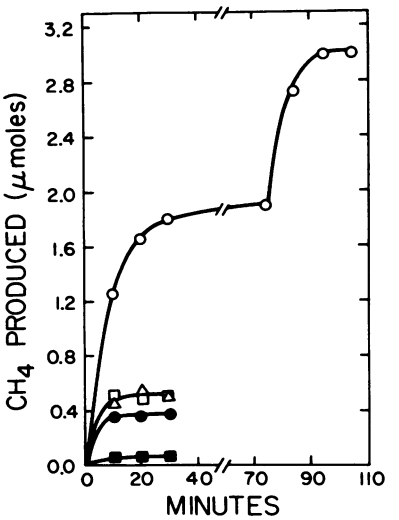

FIG. 3. *Methane formation from various substrates in crude cell-free extracts. Each 1.2-ml reaction mixture contained: TES buffer, pH 7.2, 50 μmol; ATP, 15 μmol; $MgCl_2$, 15 μmol; crude cell-free extract, 45 mg of protein; and the test substrate. Gas phase: H_2. Test substrates were:* (○) *H_2 gas phase replaced with H_2-CO_2 (80:20);* (△) *sodium acetate, 100 μmol;* (●) *sodium formate, 100 μmol;* (■) *sodium benzoate, 100 μmol;* (□) *no added substrate. After 75 min, 15 μmol of ATP was added to the reaction mixture, which contained H_2-CO_2 (80:20) in the gas phase.*

7.2) inhibited methane formation from H_2-CO_2 by 10%. Methylcobalamin could also serve as a substrate for methane formation in crude cell-free extracts, as illustrated in Fig. 4. The addition of $(S\text{-}CoM)_2$, 45 nmol, greatly stimulated methane formation from CH_3-B_{12}.

Enzyme activities. Formate hydrogenlyase, methyltransferase, and methylreductase were present in cell-free extracts of *M. hungatii* (Table 1). It was shown previously that coenzyme F_{420} is required for formate hydrogenlyase activity and for the pyridine nucleotide-linked hydrogenase system in *M. ruminantium* (19). Evidence presented in Fig. 5 shows that coenzyme F_{420} purified from *M. hungatii* is required for formate hydrogenlyase activity by extracts of *M. hungatii* from which the anionic material (including coenzyme F_{420}) was removed by passage through a DEAE-cellulose column. Dithiothreitol was required for activity but did not serve as an electron donor for H_2 formation, since H_2 was not formed when formate was absent.

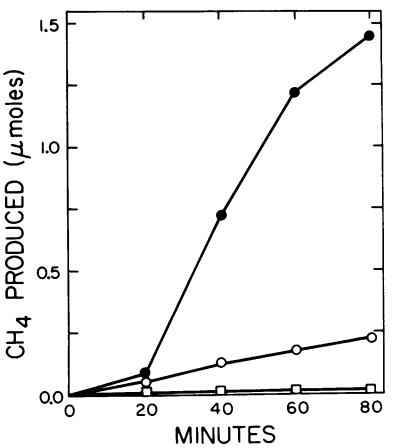

FIG. 4. *Methane formation from methylcobalamin in crude cell-free extracts. Each 1.2-ml reaction mixture contained: TES buffer, pH 7.2, 50 μmol; ATP, 15 μmol; $MgCl_2$, 15 μmol; and crude cell-free extract, 45 mg of protein. Symbols:* (●) *methylcobalamin, 7.2 μmol, and coenzyme M, 45 nmol;* (○) *methylcobalmin, 7.2 μmol;* (□) *coenzyme M, 45 μmol.*

TABLE 1. *Enzyme activities in crude cell-free extracts of Methanospirillum hungatii JF*

Enzyme	Sp act[a]
Formate hydrogenlyase	1.300
Methylcobalamin-coenzyme M methyltransferase	0.014
Methylreductase	0.080

[a] Specific activity is given as micromoles of product formed per hour per milligram of crude cell-free extract protein.

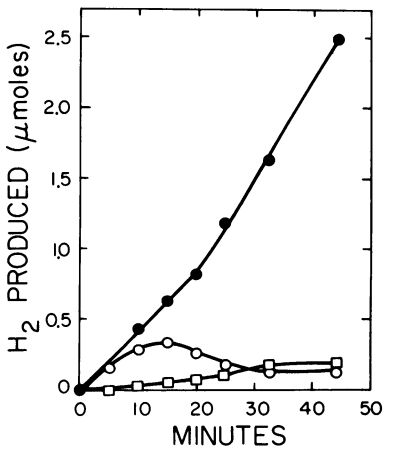

FIG. 5. *Requirement of coenzyme F_{420} for formate hydrogenlyase activity in DEAE-cellulose-treated extracts. Each reaction mixture (0.59 ml) contained: TES buffer, pH 7.2, 50 μmol; $MgCl_2$, 3.5 μmol; dithiothreitol, 1.5 μmol; DEAE-cellulose-treated cell-free extract, 21 mg of protein. Symbols:* (●) *coenzyme F_{420}, 1.6 μg, and sodium formate, 5 μmol;* (○) *coenzyme F_{420}, 1.6 μg;* (□) *sodium formate, 5 μmol.*

Presence of coenzyme M and coenzyme F_{420}. Coenzyme M was not detected when whole-cell hot-water extracts of *M. hungatii* were assayed by the transmethylation assay with crude cell-free extract from *Methanobacterium* MOH. The methylation of coenzyme M (purified from *Methanobacterium* MOH) was inhibited 50% when a hot-water extract from *M. hungatii* was added to the reaction mixture. Coenzyme M was partially separated from the inhibitor by passage of the hot-water extract through a Sephadex QAE-A25 column, employing the same conditions used during the purification of coenzyme F_{420}. Coenzyme M activity eluted with the coenzyme F_{420} peak at 2 M ammonium acetate; this also occurs in extracts of *Methanobacterium* MOH (17).

Coenzyme F_{420} purified from *M. hungatii* was very similar to coenzyme F_{420} isolated from *Methanobacterium* MOH (6) and *Methanobacterium ruminantium* (18). The ultraviolet-visible spectra of coenzyme F_{420} appear in Fig. 6. Each cuvette contained 32.3 μg of purified coenzyme F_{420} from *M. hungatii* in 1 ml of 0.1 M NaOH or 0.1 M HCl. The absorption maximum (420 nm) of coenzyme F_{420} (which was dissolved in 0.1 M NaOH) shifted to a shorter wavelength when the coenzyme was dissolved in 0.1 M HCl. Absorbancy at each absorption maximum increased when the pH was changed from acid to base.

DISCUSSION

Results of our studies lend considerable sup-

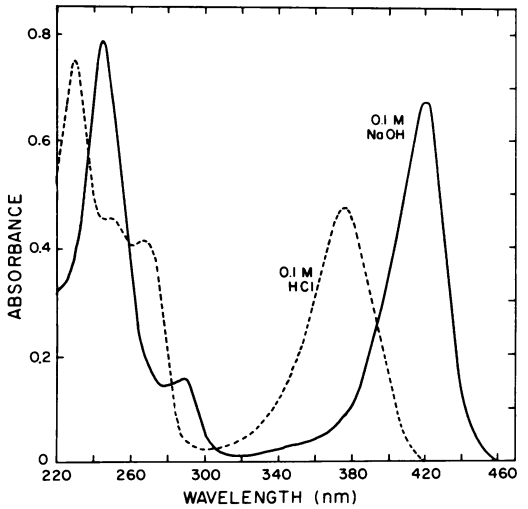

FIG. 6. *Ultraviolet-visible spectra of coenzyme F_{420} purified from M. hungatii JF. The cuvettes contained 32.2 μg of purified coenzyme F_{420} in 1 ml of 0.1 M NaOH or 0.1 M HCl.*

port to Barker's proposal that the methanogenic bacteria be placed in a single family that is based on the unique physiology of methanogenesis (1, 2). In view of the unusual morphological properties of *M. hungatii* (7, 8, 14, 22), we examined this organism to ascertain its physiological and enzymatic similarities to other methanogenic bacteria. *M. hungatii* contains both coenzyme M, which participates in methylcobalamin-coenzyme M methyltransferase and methylreductase reactions, and coenzyme F_{420}, which is required in the formate hydrogenlyase system. Nutritionally, the organism is a typical methanogen, growing in a medium consisting of mineral salts, cysteine sulfide, and H_2-CO_2 (80:20) (or formate). Addition of B vitamins and Casamino Acids stimulates growth.

Barker's proposal that all methanogenic bacteria be placed in the same family was based on knowledge of the nutrition, physiology, and ecological niche of the methanogenic bacteria as viewed in 1956. Twenty years later, it is obvious that these criteria can be extended to subcellular systems. Now, evidence is at hand that greatly strengthens his proposal: (i) results of analysis of eight species of methanogenic bacteria, which are available to us, indicate that they all possess coenzyme M; this coenzyme has not been detected in a wide variety of other organisms and tissues that have been examined (W. E. Balch, unpublished data). (ii) All known methanogenic bacteria possess coenzyme F_{420}, and, so far, we have not detected this coenzyme in other organisms (unpublished data). (iii) Current evidence indicates that methanogenic bacteria do not possess peptidoglycan in their cell walls (Otto Kandler, personal communication). (iv) All known methanogenic bacteria possess hydrogenase and require strong reducing environments; molecular hydrogen is a common substrate. (v) Results obtained in the laboratory of Carl Woese indicate that, when the oligonucleotide patterns of degraded 16S ribosomal ribonucleic acid from each of 50 different diverse bacteria are compared, the patterns of the methanogenic bacteria cluster as a distinct group, representing the most ancient evolutionary divergence so far detectable in the procaryotes (W. E. Blach, L. J. Magrum, G. E. Fox, R. S. Wolfe, and C. R. Woese, J. Mol. Evol., in press). (vi) Cytochromes have not yet been detected in methanogenic bacteria. Thus, from a number of directions, evidence is accumulating that methanogenic bacteria share a common set of biochemical characteristics that clearly distinguish them as one of nature's unique physiological families. *M. hungatii* shares these characteristics and physiologically is a typical methanogen.

ACKNOWLEDGMENTS

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