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Formate-Dependent H_2 Production by the Mesophilic Methanogen *Methanococcus maripaludis*▽

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***Methanococcus maripaludis*, an H_2- and formate-utilizing methanogen, produced H_2 at high rates from formate. The rates and kinetics of H_2 production depended upon the growth conditions, and H_2 availability during growth was a major factor. Specific activities of resting cells grown with formate or H_2 were 0.4 to 1.4 $U \cdot mg^{-1}$ (dry weight). H_2 production in formate-grown cells followed Michaelis-Menten kinetics, and the concentration of formate required for half-maximal activity (K_f) was 3.6 mM. In contrast, in H_2-grown cells this process followed sigmoidal kinetics, and the K_f was 9 mM. A key enzyme for formate-dependent H_2 production was formate dehydrogenase, Fdh. H_2 production and growth were severely reduced in a mutant containing a deletion of the gene encoding the Fdh1 isozyme, indicating that it was the primary Fdh. In contrast, a mutant containing a deletion of the gene encoding the Fdh2 isozyme possessed near-wild-type activities, indicating that this isozyme did not play a major role. H_2 production by a mutant containing a deletion of the coenzyme F_{420}-reducing hydrogenase Fru was also severely reduced, suggesting that the major pathway of H_2 production comprised Fdh1 and Fru. Because a Δ*fru*-Δ*frc* mutant retained 10% of the wild-type activity, an additional pathway is present. Mutants possessing deletions of the gene encoding the F_{420}-dependent methylene-H_4MTP dehydrogenase (Mtd) or the H_2-forming methylene-H_4MTP dehydrogenase (Hmd) also possessed reduced activity, which suggested that this second pathway was comprised of Fdh1-Mtd-Hmd. In contrast to H_2 production, the cellular rates of methanogenesis were unaffected in these mutants, which suggested that the observed H_2 production was not a direct intermediate of methanogenesis. In conclusion, high rates of formate-dependent H_2 production demonstrated the potential of *M. maripaludis* for the microbial production of H_2 from formate.**

Many hydrogenotrophic methanogens use H_2 or formate for the reduction of CO_2 to obtain energy for growth. *Methanococcus maripaludis*, the model microorganism in this study, is a hydrogenotrophic, formate-utilizing, mesophilic methanogen. It is common in salt marsh sediments, from which it was isolated (12). An extraordinarily active H_2 consumer, *M. maripaludis* is exceptionally well equipped with enzymes responsible for H_2 metabolism. *M. maripaludis* contains genes for seven different hydrogenases, whose expression depends upon the growth conditions (18). It possesses two membrane-bound, energy-converting [Ni-Fe] hydrogenases, designated Eha and Ehb, that are involved in the reduction of low-potential ferredoxins for anabolism (16, 26). There are also four cytoplasmic [Ni-Fe] hydrogenases, including two coenzyme F_{420}-reducing (Fru and Frc) and two coenzyme F_{420}-nonreducing (Vhu and Vhc) hydrogenases. One hydrogenase of each type (Fru and Vhu) contains a selenocysteinyl residue. The other hydrogenases (Frc and Vhc) contain cysteinyl residues at homologous positions (18). The selenocysteine-containing isozymes are abundant during cultivation in medium containing selenium, and the cysteine-containing isozymes (Frc and Vhc) are produced only upon selenium limitation (27). Lastly, the cells contain a cytoplasmic [Fe-S] cluster-free hydrogenase, the H_2-forming methylenetetrahydromethanopterin (methylene-H_4MPT) dehydrogenase (Hmd), which in other species has been shown to play an important role at high levels of H_2 or under nickel limitation (1, 2, 29).

When formate is the substrate, it is oxidized for the reduction of CO_2 to methane. The key enzyme for formate utilization is formate dehydrogenase, Fdh. The genome of *M. maripaludis* harbors two sets of genes encoding Fdh, *fdhA1B1* and *fdhA2B2* (33). Both Fdhs contain selenocysteinyl residues. While *fdhA1B1* are found in an apparent operon with genes for a putative formate transporter and carbonic anhydrase, *fdhA2B2* are not linked with other genes in formate utilization. In methanococci as well as in methanobacteria, the deazaflavin coenzyme F_{420} is the electron acceptor of the Fdhs (5, 20).

Formate-hydrogen lyase activity (reaction 1) is common in methanococci and other methanogens (5, 6, 11, 20, 34). Reaction 1: $HCO_2^- + H_2O \rightarrow HCO_3^- + H_2$ (+1.3 kJ/reaction).

Two pathways are likely to contribute to this activity in whole cells of *M. maripaludis*. In the first pathway, reduced coenzyme F_{420} ($F_{420}H_2$) generated by Fdh is oxidized by the reversible F_{420}-dependent hydrogenase Fru (Fig. 1). In the second pathway, $F_{420}H_2$ is oxidized by the F_{420}-dependent methylene-H_4MPT dehydrogenase (Mtd) and Hmd (1, 2, 29) (Fig. 1). While previous studies demonstrated H_2 production from formate for some methanogens (7), the rates were very low and the pathways were not determined.

Because of the low energy yield of CH_4 production, these microorganisms possess extremely high specific activities for enzymes involved in methanogenesis, including the hydroge-

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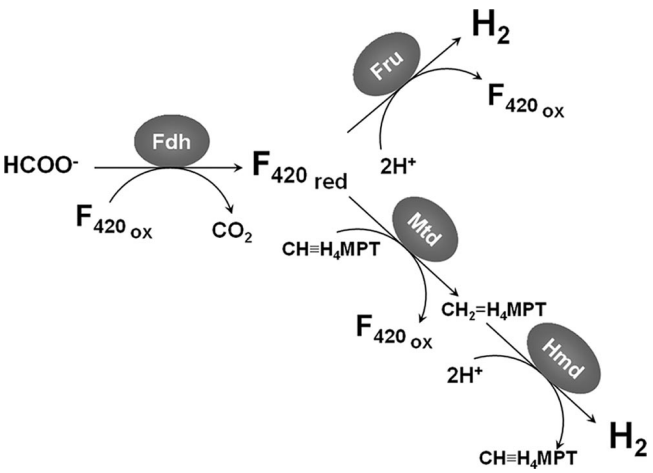

FIG. 1. Potential pathways of H_2 production in the hydrogenotrophic methanogens. The first pathway includes Fdh and Fru. In this pathway, Fdh reduces F_{420}. In selenium-grown cells, $F_{420}H_2$ is oxidized by the [Ni-Fe] hydrogenase Fru. In the second pathway, $F_{420}H_2$ is oxidized by Mtd to reduce methenyl-H_4MPT to methylene-H_4MPT, which is then reoxidized by Hmd, a Ni-free hydrogenase, to produce H_2. Abbreviations: Fdh, formate dehydrogenase; Fru, F_{420}-reducing hydrogenase; $F_{420\ ox}$, oxidized coenzyme F_{420}; $F_{420\ red}$, reduced coenzyme F_{420}; Mtd, F_{420}-dependent methylene-H_4MPT dehydrogenase; Hmd, H_2-forming methylene-H_4MPT dehydrogenase.

nases used for H_2 consumption. If H_2 utilization were a reversible process, high rates of H_2 production would be possible. To test this hypothesis, the rates of H_2 production from formate were tested in *M. maripaludis*, a representative of a diversified and important group of methanogens.

MATERIALS AND METHODS

Strains and growth conditions. *M. maripaludis* wild-type strain S2 and the mutant strains are listed in Table 1. For growth experiments, the strains were grown in 28-ml Balch tubes (4) filled with 5 ml of either McNA (McN minimal medium [32] supplemented with 10 mM acetate) or McCV medium (McNA medium supplemented with 0.2% yeast extract and Casamino Acids as well as vitamin solution [32]). The tubes were pressurized to 276 kPa with H_2-CO_2 gas (80:20 [vol/vol]) and grown at 37°C. When sodium formate was used (100 mM), 100 mM Tris-HCl solution, pH 7, was added to reduce the increase in pH. The tubes were also pressurized to 138 kPa with N_2-CO_2 (80:20 [vol/vol]).

For obtaining cell mass, *M. maripaludis* cultures were grown in 1-liter Wheaton bottles (3) with 100 ml of McNA or McCV medium and 138 kPa of H_2-CO_2 (80:20 [vol/vol]) at 37°C. When sodium formate was the substrate (0.4 M), the bottles were flushed with N_2-CO_2 (80:20 [vol/vol]) and pressurized to 35 kPa.

Cells were collected by centrifugation in sealed plastic centrifuge bottles that had been equilibrated in an anaerobic glove box (Coy Laboratories, Ann Arbor, MI) for at least 24 h to remove O_2 adsorbed to the plastic. The cells were centrifuged at 6,000 × *g* for 20 min at 4°C using a Beckman model J2-21 centrifuge (Beckman Coulter, Inc., Fullerton, CA) fitted with a Beckman JA-14 rotor. The cells were resuspended in 1/100 of the initial volume of an anaerobic buffer containing 50 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), 400 mM NaCl, 20 mM KCl, 20 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM dithiothreitol, pH 6.9.

H_2 measurements. The H_2 measurements were performed at 37°C in a custom-made anaerobic, water-jacketed cuvette (2.8 ml) fitted with a rubber stopper and gassed with O_2-free N_2 (31). The concentration of the dissolved H_2 in the anaerobic buffer was measured using a modified amperometric O_2 Clark-type electrode (15, 31) (Yellow Springs Instrument, Yellow Springs, OH) connected to a picoammeter PA2000 (Unisense, Aarhus, Denmark). The connections for the electrode (6.3-mm TRS connector) and picoammeter (BNC connector) were incompatible, and so the appropriate connection was manufactured. Standard curves were prepared with H_2-saturated distilled water. Cell suspensions of 0.1 mg (dry weight) were added via microsyringes to 1 ml of the same buffer used to suspend the cells. The assay was started by adding sodium formate. One unit was defined as 1 μmol of H_2 produced per minute. The cell dry weight was calculated from the slope of a standard curve relating absorbance at 600 nm to dry weight. From this curve, a suspension with an A_{600} of 1 corresponded to 0.34 mg (dry weight) · ml^{-1}.

CH_4 detection. Resting cells (0.1 mg [dry weight]) suspensions in 0.5 ml of buffer were transferred to 3.5-ml vials under an atmosphere of N_2. The assay was initiated by adding formate to a final concentration of 20 mM or flushing the vials with H_2-CO_2 (80:20 [vol/vol]) for 1 min. The samples were incubated at 37°C for 10 min. CH_4 was detected with an SRI 8610-C gas chromatograph (SRI Instruments, Torrance, CA) fitted with on-column injection, a Porpak Q teflon column at 90°C, and a flame ionization detector operating at 150°C. The carrier gas was N_2. One unit was defined as 1 μmol of CH_4 produced per minute.

Preparation of cell extracts. All procedures were performed anaerobically. Cells were collected by centrifugation as described above and loaded into a chilled French pressure cell in the anaerobic glove box. Cell extracts were prepared by passing 10 ml of cell suspension (about 10 mg [dry weight] per ml) through the French pressure cell operated at 65 MPa equipped with a 22-gauge needle. Cell extracts were collected in the sealed tubes or serum bottles previously equilibrated in the anaerobic glove box. Subsequently, the extracts were centrifuged at 10,000 × *g* for 20 min at 4°C. Protein concentrations were determined using the Bradford protein kit (Bio-Rad, Hercules, CA).

Enzymatic assays. The Fdh and F_{420}-reducing hydrogenase activities were measured spectrophotometrically under anaerobic conditions. H_2-dependent F_{420} reduction was assayed in 1 ml of anaerobic buffer containing 100 mM PIPES, pH 6.9, 20 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM dithiothreitol. The final concentration of F_{420} was 10 μM. The 1.6-ml glass cuvettes were sealed with rubber stoppers and flushed with O_2-free H_2 for 5 min before each assay. Changes in absorbance at a λ of 420 nm were measured using a Beckman DU-640B spectrophotometer. An extinction coefficient (ε_{420}) of 40 mM · cm^{-1} was used for calculations. One unit was defined as 1 μmol of coenzyme F_{420} reduced per minute. F_{420} was purified from the *M. maripaludis* cell paste according to a modification of the method of Eirich et al. (9).

Fdh activity was assayed in 1 ml of the same buffer described above plus 2 mM methyl viologen (MV). The cuvettes were flushed with O_2-free N_2 for 5 min before each assay. The MV was reduced with a few microliters of 200 mM dithionite until the assay buffer turned slightly blue. The cell extract was added, and the reaction was initiated by adding formate to a final concentration of 10 mM. Changes in absorbance at a λ of 605 nm were recorded, and an extinction coefficient (ε_{605}) of 13.9 mM · cm^{-1} was used for calculations. One unit was defined as 2 μmol of MV reduced per minute.

RESULTS AND DISCUSSION

Initial rates of H_2 production. Previous reports of formate-dependent H_2 production measured formation of headspace H_2 during growth (7, 19, 28). However, preliminary measurements of the rate of production of dissolved H_2 with an H_2 probe and using resting cells far exceeded these reports. Therefore, the earlier measurements underestimated the production rate, possibly because H_2 uptake was occurring simultaneously or gas transfer to the headspace was rate limiting.

To examine these high rates in more detail, further experiments were performed to standardize the reaction conditions. Whole cells were grown either with H_2 or formate, washed in

TABLE 1. Strains used in this study

Strain	Genotype	Reference
S2	Wild type	32
MM707	Δ*fdhA1*	33
MM708	Δ*fdhA2*	33
MM709	Δ*fdhA1*-Δ*fdhA2*	33
MM1145	Δ*fruA*	19
MM1183	Δ*frcA*	19
MM1184	Δ*fruA*-Δ*frcA*	19
MM1097	Δ*hmd*	19
MM1020	Δ*mtd*	19

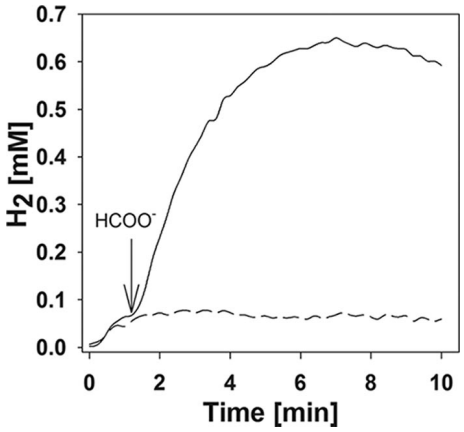

FIG. 2. Initial rates of H_2 production from formate (solid line) by resting cells of *M. maripaludis*. Cells were grown with formate, washed in buffer, and resuspended in the reaction cuvette. Assays were initiated by adding 20 mM sodium formate (arrow). Results from incubation of the cell suspension in the absence of formate is shown by the broken line.

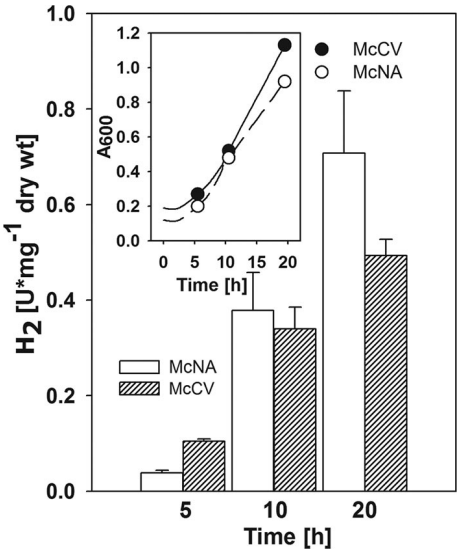

FIG. 3. Changes in formate-dependent H_2 production by resting cells following growth under H_2 limitation. The wild-type S2 was grown in minimal or McNA (○) and rich or McCV (●) medium in 1-liter Wheaton bottles with 100 ml of broth. At the beginning of the experiment, the bottles were pressurized with 138 kPa of H_2-CO_2 (80:20 [vol/vol]). Cell samples (20 ml) were collected at different absorbances, as indicated by the points on the inset growth curves. After each sampling, the bottles were repressurized with N_2-CO_2 (80:20 [vol/vol]). This resulted in H_2 limitation but maintained the concentration of CO_2. Error bars represent 1 standard deviation from the four measurements.

buffer, and resuspended in a reaction cuvette. In both cases, formate-dependent H_2 production proceeded linearly for the first minute (Fig. 2). In the subsequent 2 to 5 minutes, the apparent rate of H_2 generation declined until a plateau was reached. With 20 mM formate, the maximal H_2 concentrations ranged between 0.4 and 0.8 mM, depending upon the experiment. These concentrations approached 3 mM, the concentration expected at equilibrium. The observed rate would be lower than the true production rate if H_2 utilization were occurring simultaneously. However, H_2 utilization for methanogenesis was not likely to be a factor, because the initial concentration of CO_2 was very low. In addition, the inhibition of methanogenesis by bromoethanesulfonate (1 mM) had little effect on the initial rates or maximum values of H_2 production (data not shown). Therefore, the initial observed rate was not affected by simultaneous H_2 consumption for methanogenesis.

Both H_2- and formate-grown cells produced H_2 from formate at comparable rates. Although the rates depended greatly upon the experiment (see below), the rates varied from about 0.4 to 1.4 $U \cdot mg^{-1}$ (dry weight) regardless of how the cells were grown (data not shown). These results suggested that the levels of Fdh and hydrogenase were high in both cell types. Since these enzymes are essential components of the methanogenesis system, the rates of methanogenesis were also compared. In one experiment, the rates of methanogenesis by H_2-grown cells were 0.37 ± 0.02 $U \cdot mg^{-1}$ (dry weight) and 0.32 ± 0.04 $U \cdot mg^{-1}$ (dry weight) with H_2 or formate, respectively (means ± standard deviations of triplicate measurements with two independently grown cultures). Similarly, the rates of methanogenesis by formate-grown cells were 0.25 ± 0.02 $U \cdot mg^{-1}$ (dry weight) and 0.43 ± 0.04 $U \cdot mg^{-1}$ (dry weight) with H_2 or formate, respectively. Since four molecules of H_2 or formate are consumed per molecule of CH_4 formed, the capacity of H_2 or formate consumption is comparable to the rate of formate-dependent H_2 production regardless of the growth substrate.

Influence of growth conditions on cellular rates of H_2 production. The rate of formate-dependent H_2 production varied greatly between experiments. To determine if some of this variability resulted from the growth phase, H_2-grown cultures were monitored for formate-dependent H_2 production (Fig. 3). In batch cultures of methanococci, exponential growth is only observed at low cell densities (25). Above an absorbance of ~0.4, growth becomes linear as the rate of H_2 transfer to the aqueous phase becomes rate limiting. Finally, the linear phase usually ends at absorbances of ~0.8, as cells enter early stationary phase. When cultures were allowed to nearly exhaust H_2 late in growth, the specific activity of H_2 production increased 6- to 10-fold (Fig. 3). In addition to growth phase, the medium composition also affected these rates. At the end of growth, cells grown in the minimal medium possessed about 30% higher specific activities than cells grown in the rich medium (Fig. 3). Methanococci require higher levels of H_2 for anabolism during growth on minimal medium (26). Taken together, these results were consistent with a role for H_2 limitation in the regulation of H_2 production.

To examine directly the role of H_2 partial pressure on the specific activity of H_2 production, cultures were grown in bottles with very large headspaces in order to minimize gas pressure fluctuations due to H_2 consumption. Parallel cultures were grown with H_2 partial pressures of 220 and 80 kPa to absorbances of 0.45 to 0.50. The specific activities of H_2 production were 0.12 ± 0.03 and 0.41 ± 0.04 $U \cdot mg^{-1}$ (dry weight), respectively. Thus, the levels of H_2 during growth had a direct effect on the expression of the enzymes involved in H_2 production. These results are consistent with microarray observations, where the levels of mRNA for both the formate

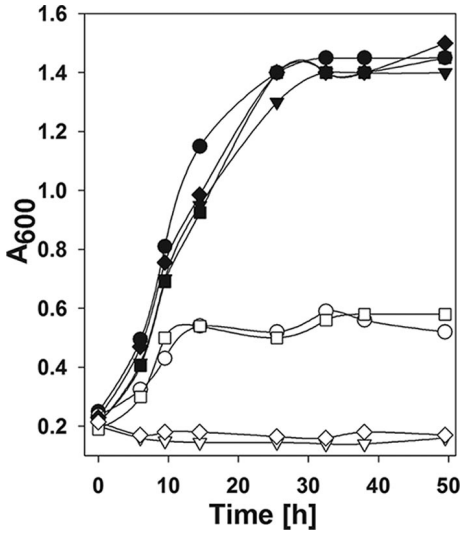

FIG. 4. Growth of the wild-type and *fdh* mutant strains with H_2 or formate. Solid symbols, growth with H_2 (276 kPa; H_2-CO_2 80:20 [vol/vol]); open symbols, growth with formate (100 mM). Circles, S2; inverted triangles, Δ*fdhA1*; squares, Δ*fdhA2*; diamonds, Δ*fdhA1-A2*. Each point represents the mean value of two replicates. Similar curves were obtained in a replicate experiment.

dehydrogenase (*fdh1*) and F_{420}-reducing hydrogenase (*fru*) genes are significantly higher during H_2 limitation (17).

Role of formate dehydrogenase. To ascertain the importance of the two Fdh isozymes in H_2 production, the mutant strains Δ*fdhA1*, Δ*fdhA2*, and Δ*fdhA1*-Δ*fdhA2* were tested for their ability to grow with formate, MV-dependent Fdh activity, and for H_2 production. The double mutant Δ*fdhA1*-Δ*fdhA2*, which was constructed by marker exchange of internal portions of both *fdhA1* and *fdhA2*, was unable to utilize formate for either growth, which was followed for 140 h, or H_2 production (Fig. 4, Table 2, and data not shown). In addition, in extracts of H_2-grown cells, the MV-dependent Fdh activity was <0.02 $U \cdot mg^{-1}$ (dry weight). Thus, H_2 production required Fdh. MV-dependent Fdh activities in cell extracts of the Δ*fdhA2* mutant and the wild-type S2 strains were 1.0 ± 0.25 and 3.6 ± 1.2 $U \cdot mg^{-1}$ (dry weight), respectively. In contrast, growth on formate of strain Δ*fdhA2* was comparable to that of the wild type, and H_2 production was only slightly reduced. Therefore, the rate of formate oxidation did not appear to limit growth and H_2 production in this mutant. Notably, the Δ*fdhA1* mutant failed to grow with formate without an extended incubation of 70 h and produced H_2 poorly (Fig. 4, Table 2, and data not shown). In addition, the MV-dependent Fdh activity was <0.02 $U \cdot mg^{-1}$ (dry weight). Subsequent transfers of formate-grown cultures of the Δ*fdhA1* mutant to the fresh medium decreased the lag phase, and the third subculture grew with formate after a lag of about 48 h. Presumably, mutations at other sites on the genome were responsible for the adaptation of the Δ*fdhA1* mutant to growth on formate. For instance, increased expression of Fdh2 could account for this phenotype. Thus, Fdh2 played only a small role in H_2 production, and Fdh1 appeared to be the major isozyme in formate utilization under these conditions. In previous studies, the Δ*fdhA1* mutant grew with formate at similar rates as the Δ*fdhA2* strain (33). Presumably, this difference reflects the differences in the medium composition and the experimental design.

TABLE 2. Activities of formate-dependent H_2 production by *fdh* mutant strains[a]

Genotype	Sp act ($U \cdot mg^{-1}$ [dry wt])
Wild type	0.32 ± 0.05
Δ*fdhA1*	0.04 ± 0.01
Δ*fdhA2*	0.29 ± 0.02
Δ*fdhA1*-Δ*fdhA2*	<0.02

[a] Cultures were grown with H_2 at 136 kPa of H_2-CO_2, (80:20 [vol/vol]) to an absorbance of about 0.5. The values are means ± standard deviations of triplicate measurements with two independently grown cultures.

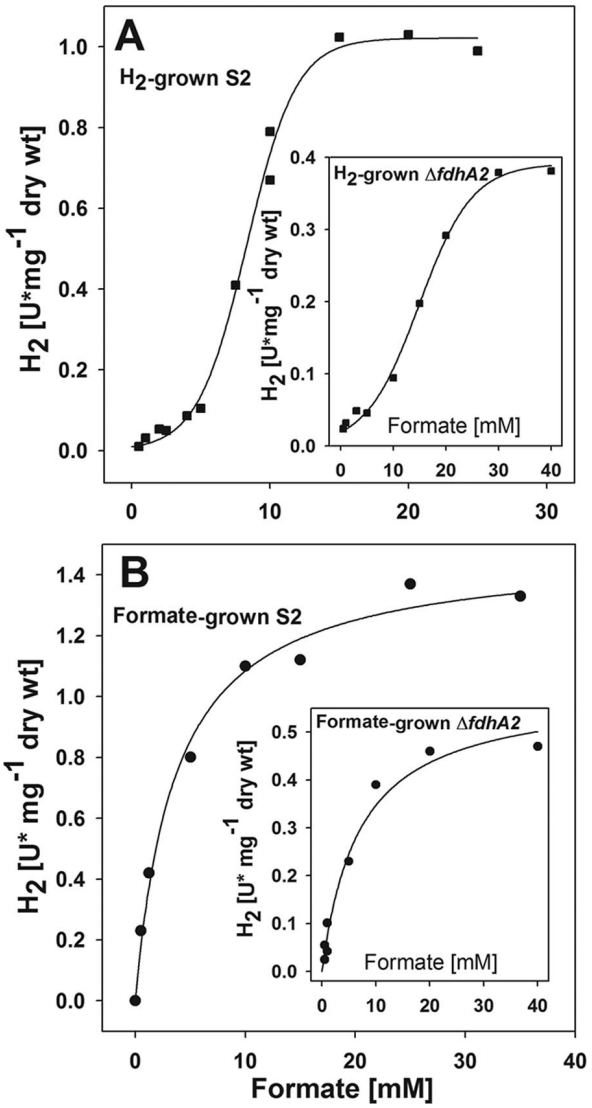

FIG. 5. Kinetics of formate-dependent H_2 production by resting cells of the wild-type strain S2 previously grown either with H_2 (A) or formate (B). The kinetics of the Δ*fdhA2* mutant are shown in the insets. The S2 and Δ*fdhA2* mutant strains were grown to absorbances of about 0.8 and 0.6, respectively.

Kinetics of formate-dependent H_2 production by whole cells. The kinetics depended on the growth substrates of the cells.

TABLE 3. Activities of formate-dependent H_2 and CH_4 production for hydrogenase and methylene-H_4MPT dehydrogenase mutant strains[a]

Genotype	Sp act (U · mg^{-1} [dry wt])	
	H_2	CH_4
Wild type	0.56 ± 0.10	0.36 ± 0.03
Δ*fruA*-Δ*frcA*	0.06 ± 0.02	0.20 ± 0.04
Δ*hmd*	0.40 ± 0.08	0.12 ± 0.02
Δ*mtd*	0.35 ± 0.05	0.13 ± 0.02

[a] Cells were grown with formate to an absorbance of about 0.6, except for Δ*mtd*, for which the absorbance was about 0.4. H_2 and CH_4 production levels were measured using cells from the same culture. The values are means ± 1 standard deviation of triplicate measurements from each of two independently grown cultures.

For incubations of formate-grown cells, H_2 production followed Michaelis-Menten kinetics. The K_f, or concentration of formate required for half-maximal activity, was 3.6 ± 0.5 mM (mean ± 1 standard deviation), and the V_f, or the maximal specific activity, was 1.5 ± 0.1 U · mg^{-1} (dry weight) (Fig. 5). For H_2-grown cells, H_2 production followed sigmoidal kinetics, and the kinetic values were 9 ± 1 mM and 1.1 ± 0.2 U · mg^{-1} (dry weight), respectively. The sigmoidal kinetics for the H_2-grown cells were not due to the presence of two isozymes in the wild-type cells, because the Δ*fdhA2* mutant possessed similar kinetics (Fig. 5). Instead, the biphasic kinetics suggested that the formate transporter FdhC plays a significant role in the kinetics of H_2 production. Expression of *fdhC* is reduced in H_2-grown cells (33). Thus, at low formate concentrations, low levels of the transporter may limit the rate of formate uptake and H_2 production. High formate concentrations would then compensate for low levels of the transporter. In contrast, the levels of the transporter may be sufficient in the formate-grown cells so that uptake is no longer rate limiting at millimolar formate concentrations.

Pathways of H_2 production. Given the requirement for Fdh, two pathways of H_2 production were likely (Fig. 1). In selenium-grown cells, the major pathway was expected to include the oxidation of Fdh-generated $F_{420}H_2$ by the F_{420}-dependent [Ni-Fe] hydrogenase Fru. The selenium-independent isozyme Frc was not expected to play a role, because it would not be expressed under these conditions (27). In extracts of cells grown with both selenium and formate, the specific activities of MV-dependent Fdh and Fru were 18 and 2.1 U · mg^{-1} of the total protein. Assuming that 60% of the cell was protein, the corresponding cellular specific activities were 10.8 and 1.3 U · mg^{-1} (dry weight), values that were consistent with a role in whole-cell H_2 production. To examine this point further, the H_2 production activities of a series of in-frame deletion mutants in the F_{420}-dependent hydrogenases, Δ*fruA*, Δ*frcA*, and Δ*fruA*-Δ*frcA*, were examined. The activity of the Δ*fruA*-Δ*frcA* mutant was severely reduced (Table 3). The Δ*fruA* mutant possessed nearly identical activity as the double mutant, suggesting the FruA played an important role (data not shown). In contrast, the activity of the Δ*frcA* mutant was identical to the wild type (data not shown), as expected if this enzyme is not produced in the presence of selenium. These observations clearly suggested that F_{420} is an intermediate in the process and provided evidence for an F_{420}-dependent formate-hydrogen lyase system in *M. maripaludis.* Because the Δ*fruA*-Δ*frcA* mutant retained about 10% of the wild-type H_2 production activity (Table 3), an additional pathway must be present. Deletions of either *mtd* or *hmd* reduced the activity by about 30 to 40%, which indicated that the second system utilized the H_4MPT-dependent pathway (Fig. 1).

Is H_2 an intermediate of methanogenesis with formate? The rates of H_2 generation were comparable to the rates of methanogenesis, which suggested that H_2 was produced in sufficient amounts to be an intermediate during methanogenesis with formate. During hydrogenotrophic growth, H_2 is proposed to be the electron donor for the Eha-dependent reduction of CO_2 to formylmethanofuran as well as the heterodisulfide reductase, the first and last steps of methanogenesis, respectively (for a review see reference 10). During growth with formate, F_{420} is initially reduced. If methanogenesis proceeds in a fashion similar to hydrogenotrophic growth, H_2 could be generated from $F_{420}H_2$ to produce the reductant for the first and last steps of methanogenesis. To test this hypothesis, rates of formate-dependent H_2 and CH_4 production were measured in cells derived from the same cultures in order to reduce the variability caused by growth and handling (Table 3). In these

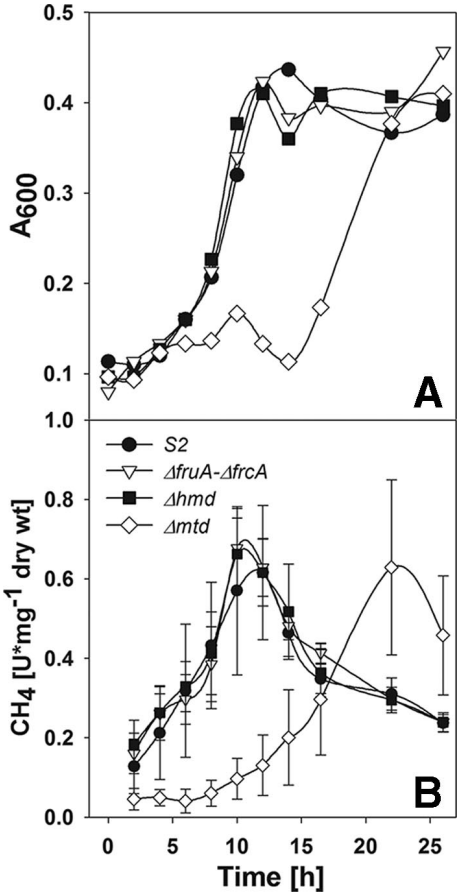

FIG. 6. Growth (A) and specific activity of CH_4 production (B) by the wild-type S2 (●), Δ*fruA*-Δ*frcA* (▽), Δ*hmd*, (■), and Δ*mtd* (◇) mutant strains grown with formate (100 mM). Each point represents the mean value of three replicates, and error bars in panel B represent 1 standard deviation. Similar curves were obtained in a replicate experiment.

experiments, there was little correlation between the rates of H_2 and CH_4 production in the wild type and Δ*fruA*-Δ*frcA*, Δ*hmd*, and Δ*mtd* mutants. In fact, for the Δ*fruA*-Δ*frcA* mutant, the rate of methanogenesis exceeded the rate of H_2 production, which seemed to preclude the possibility that H_2 could be an obligate intermediate of methanogenesis.

Methanogenesis is very O_2 sensitive, and it is possible that the activity may have been damaged during preparation of the cell suspensions used in this experiment. Therefore, the rates of methanogenesis were also determined during growth of the wild type and mutants without preparation of resting cells. For the wild type, the specific activity for methanogenesis was low except during the exponential and linear growth phases (Fig. 6). For the Δ*fruA*-Δ*frcA* and Δ*hmd* mutants, growth and methanogenesis were nearly the same as the wild type. Even though the growth and CH_4 production were delayed for the Δ*mtd* mutant (Fig. 6), the maximum rate of methanogenesis during the exponential growth phase was nearly the same as that of the wild type. For this mutant, H_2 must first accumulate in the medium to allow for activity of the low-affinity Hmd before growth commences (19). In conclusion, while the rate of H_2 production measured with a H_2 probe is too low to be an obligatory intermediate for methanogenesis from formate, it is still formally possible that a H_2 cycle could still exist within the cell. In this case, the cellular H_2 levels would not equilibrate with the bulk H_2 dissolved in the medium. However, this possibility seems unlikely, given that inactivation of each of the major pathways of H_2 production, either in the Δ*fruA*-Δ*frcA* or the Δ*hmd* and Δ*mtd* mutants, had little effect on the cellular rate of methanogenesis from formate.

Recently, a novel hypothesis for energy conservation by the hydrogenotrophic methanogens was proposed (30). This model predicts that the exergonic H_2-dependent reduction of the heterodisulfide is not membrane associated and does not generate a proton or sodium motive force. Instead, this exergonic reaction is directly coupled to the endergonic reduction of the low-potential ferredoxin required for CO_2 reduction by flavin-mediated electron bifurcation. According to this model, one low-potential electron from H_2 oxidation partially reduces the ferredoxin, while a high-potential electron partially reduces the heterodisulfide. An additional cycle is required to fully reduce both the ferredoxin and heterodisulfide (30). This model avoids the necessity of an energy-conserving hydrogenase, such as Eha, to reduce the low-potential ferredoxin. While the observation that H_2 is not an obligate intermediate during growth with formate supports this model, it remains unclear how $F_{420}H_2$ generated from formate donates electrons for the flavin-mediated electron bifurcation. If H_2 is not an intermediate, an enzymatic complex with the function of $F_{420}H_2$:flavin oxidoreductase would be required. At present, a candidate for this enzyme has not been identified, either from biochemical or genome analyses.

Possible applications. The growing energy demand, environmental concerns, and limited resources of fossil fuels draw attention to H_2, which is a clean and efficient energy source. Microbial H_2 production has involved a range of approaches (8, 13, 14, 24). Methanogens are very active H_2 consumers; if these systems could be used for H_2 production, high rates would be possible. To test this concept, H_2 production from formate by resting cells of methanococci was evaluated. Rates of formate-dependent H_2 production have been previously examined in a few bacteria. The methylotrophs *Methylomonas albus* and *Methylosinus trichosporium* produced H_2 at rates of 1.6 and 0.4 mU · mg^{-1} (dry weight), respectively, after 5 hours of incubation under anaerobic conditions (21). Similar low rates were observed for *Shewanella oneidensis* MR-1 (23) and *Alcaligenes eutrophus* (22). The highest rates of 1.7 to 4.2 U · mg^{-1} (dry weight) were obtained with genetically engineered *Escherichia coli* strains (35). The rates obtained from wild-type methanococci, up to 1.4 U · mg^{-1} (dry weight), were comparable. However, because of the equilibrium constant, high concentrations of H_2 cannot be produced from formate regardless of the catalyst employed. Therefore, biotechnological applications would require an efficient means of harvesting H_2 at low levels. Because $F_{420}H_2$ is a key intermediate in methanococcal H_2 production, one might speculate that the substrate range could be increased by genetically engineering methanococci to couple the reduction of F_{420} to the oxidation of substrates other than formate.

While our studies were focused on *M. maripaludis*, other methanogens also possess many of the activities required for formate-dependent H_2 production and could be candidates for biotechnological applications. Thus, use of thermophilic or freshwater species might extend this application to a much broader range of conditions. The use of formate-utilizing methanogens appears to be the optimal solution. In this approach the growth and H_2 production are decoupled. In such bioreactors, formate first is used to obtain the cell mass. Then, by the continuous supply of this substrate, the cell mass could be an efficient catalyst for H_2 production. For wild-type cells, the rates of formate-dependent H_2 production are among the highest reported for prokaryotes. Further optimization of the growth and reaction conditions as well as genetic engineering could potentially increase these rates yet again. These observations open the possibilities for the use of methanococci in bioreactors for the generation of H_2 from the relatively inexpensive chemical, which can be derived from biomass (35).

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REFERENCES

1. **Afting, C., A. Hochheimer, and R. K. Thauer.** 1998. Function of H_2-forming methylenetetrahydromethanopterin dehydrogenase from *Methanobacterium thermoautotrophicum* in coenzyme F_{420} reduction with H_2. Arch. Microbiol. **169:**206–210.
2. **Afting, C., E. Kremmer, C. Brucker, A. Hochheimer, and R. K. Thauer.** 2000. Regulation of the synthesis of H_2-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) and of HmdII and HmdIII in *Methanothermobacter marburgensis*. Arch. Microbiol. **174:**225–232.
3. **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.
4. **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.

5. **Baron, S. F., and J. G. Ferry.** 1989. Reconstitution and properties of a coenzyme F_{420}-mediated formate hydrogenlyase system in *Methanobacterium formicicum*. J. Bacteriol. **171:**3854–3859.
6. **Belay, N., R. Sparling, and L. Daniels.** 1986. Relationship of formate to growth and methanogenesis by *Methanococcus thermolithotrophicus*. Appl. Environ. Microbiol. **52:**1080–1085.
7. **Bleicher, K., and J. Winter.** 1994. Formate production and utilization by methanogens and by sewage sludge consortia: interference with the concept of interspecies formate transfer. Appl. Microbiol. Biotechnol. **40:**910–915.
8. **Dutta, D., D. De, S. Chaudhuri, and S. K. Bhattacharya.** 2005. Hydrogen production by cyanobacteria. Microb. Cell. Fact. **4:**36.
9. **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.
10. **Ferry, J. G., and K. A. Kastead.** 2007. Methanogenesis, p. 288–314. *In* R. Cavicchioli (ed.), *Archaea*: molecular and cellular biology. ASM Press, Washington, DC.
11. **Ferry, J. G., and R. S. Wolfe.** 1977. Nutritional and biochemical characterization of *Methanospirillum hungatii*. Appl. Environ. Microbiol. **34:**371–376.
12. **Franklin, M. J., W. J. Wiebe, and W. B. Whitman.** 1988. Populations of methanogenic bacteria in a Georgia salt marsh. Appl. Environ. Microbiol. **54:**1151–1157.
13. **Hallenbeck, P. C.** 2005. Fundamentals of the fermentative production of hydrogen. Water Sci. Technol. **52:**21–29.
14. **Hallenbeck, P. C., and J. R. Benemann.** 2002. Biological hydrogen production; fundamentals and limiting processes. Int. J. Hydrogen Energy **27:**1185–1193.
15. **Hanus, F. J., K. R. Carter, and H. J. Evans.** 1980. Techniques for measurement of hydrogen evolution by nodules. Methods Enzymol. **69:**731–737.
16. **Hedderich, R.** 2004. Energy-converting [NiFe] hydrogenases from archaea and extremophiles: ancestors of complex I. J. Bioenerg. Biomembr. **36:**65–75.
17. **Hendrickson, E. L., A. K. Haydock, B. C. Moore, W. B. Whitman, and J. A. Leigh.** 2007. Functionally distinct genes regulated by hydrogen limitation and growth rate in methanogenic Archaea. Proc. Natl. Acad. Sci. USA **104:**8930–8934.
18. **Hendrickson, E. L., R. Kaul, Y. Zhou, D. Bovee, P. Chapman, J. Chung, E. Conway de Macario, J. A. Dodsworth, W. Gillett, D. E. Graham, M. Hackett, A. K. Haydock, A. Kang, M. L. Land, R. Levy, T. J. Lie, T. A. Major, B. C. Moore, I. Porat, A. Palmeiri, G. Rouse, C. Saenphimmachak, D. Soll, S. Van Dien, T. Wang, W. B. Whitman, Q. Xia, Y. Zhang, F. W. Larimer, M. V. Olson, and J. A. Leigh.** 2004. Complete genome sequence of the genetically tractable hydrogenotrophic methanogen *Methanococcus maripaludis*. J. Bacteriol. **186:**6956–6969.
19. **Hendrickson, E. L., and J. A. Leigh.** 2008. Roles of coenzyme F_{420}-reducing hydrogenases and hydrogen- and F_{420}-dependent methylenetetrahydromethanopterin dehydrogenases in reduction of F_{420} and production of hydrogen during methanogenesis. J. Bacteriol. **190:**4818–4821.
20. **Jones, J. B., and T. C. Stadtman.** 1980. Reconstitution of a formate-$NADP^+$ oxidoreductase from formate dehydrogenase and a 5-deazaflavin-linked $NADP^+$ reductase isolated from *Methanococcus vannielii*. J. Biol. Chem. **255:**1049–1053.
21. **Kawamura, S., J. G. O′Neil, and J. F. Wilkinson.** 1983. Hydrogen production by methylotrophs under anaerobic conditions. J. Ferment. Technol. **61:**151–156.
22. **Klibanov, A. M., B. N. Alberti, and S. E. Zale.** 1982. Enzymatic synthesis of formic acid from hydrogen and carbon dioxide and production of hydrogen from formic acid. Biotechnol. Bioeng. **24:**25–36.
23. **Meshulam-Simon, G., S. Behrens, A. D. Choo, and A. M. Spormann.** 2007. Hydrogen metabolism in *Shewanella oneidensis* MR-1. Appl. Environ. Microbiol. **73:**1153–1165.
24. **Nandi, R., and S. Sengupta.** 1998. Microbial production of hydrogen: an overview. Crit. Rev. Microbiol. **24:**61–84.
25. **Pennings, J. L., P. Vermeij, L. M. de Poorter, J. T. Keltjens, and G. D. Vogels.** 2000. Adaptation of methane formation and enzyme contents during growth of *Methanobacterium thermoautotrophicum* (strain ΔH) in a fed-batch fermentor. Antonie van Leeuwenhoek **77:**281–291.
26. **Porat, I., W. Kim, E. L. Hendrickson, Q. Xia, Y. Zhang, T. Wang, F. Taub, B. C. Moore, I. J. Anderson, M. Hackett, J. A. Leigh, and W. B. Whitman.** 2006. Disruption of the operon encoding Ehb hydrogenase limits anabolic CO_2 assimilation in the archaeon *Methanococcus maripaludis*. J. Bacteriol. **188:**1373–1380.
27. **Rother, M., I. Mathes, F. Lottspeich, and A. Bock.** 2003. Inactivation of the *selB* gene in *Methanococcus maripaludis*: effect on synthesis of selenoproteins and their sulfur-containing homologs. J. Bacteriol. **185:**107–114.
28. **Schauer, N. L., and J. G. Ferry.** 1980. Metabolism of formate in *Methanobacterium formicicum*. J. Bacteriol. **142:**800–807.
29. **Thauer, R. K.** 1998. Biochemistry of methanogenesis: a tribute to Marjory Stephenson. Microbiology **144:**2377–2406.
30. **Thauer, R. K., A. K. Kaster, H. Seedorf, W. Buckel, and R. Hedderich.** 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. Nat. Rev. Microbiol. **6:**579–591.
31. **Wang, R. T.** 1980. Amperometric hydrogen electrode. Methods Enzymol. **69:**409–413.
32. **Whitman, W. B., J. Shieh, S. Sohn, D. S. Caras, and U. Premachandran.** 1986. Isolation and characterization of 22 mesophilic methanococci. Syst. Appl. Microbiol. **7:**235–240.
33. **Wood, G. E., A. K. Haydock, and J. A. Leigh.** 2003. Function and regulation of the formate dehydrogenase genes of the methanogenic archaeon *Methanococcus maripaludis*. J. Bacteriol. **185:**2548–2554.
34. **Yamazaki, S.** 1982. A selenium-containing hydrogenase from *Methanococcus vannielii*. Identification of the selenium moiety as a selenocysteine residue. J. Biol. Chem. **257:**7926–7929.
35. **Yoshida, A., T. Nishimura, H. Kawaguchi, M. Inui, and H. Yukawa.** 2005. Enhanced hydrogen production from formic acid by formate hydrogen lyase-overexpressing *Escherichia coli* strains. Appl. Environ. Microbiol. **71:**6762–6768.