

# Formate-Dependent H<sub>2</sub> Production by the Mesophilic Methanogen *Methanococcus maripaludis*<sup>▽</sup>

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*Methanococcus maripaludis*, an H<sub>2</sub>- and formate-utilizing methanogen, produced H<sub>2</sub> at high rates from formate. The rates and kinetics of H<sub>2</sub> production depended upon the growth conditions, and H<sub>2</sub> availability during growth was a major factor. Specific activities of resting cells grown with formate or H<sub>2</sub> were 0.4 to 1.4 U · mg<sup>-1</sup> (dry weight). H<sub>2</sub> 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<sub>2</sub>-grown cells this process followed sigmoidal kinetics, and the  $K_f$  was 9 mM. A key enzyme for formate-dependent H<sub>2</sub> production was formate dehydrogenase, Fdh. H<sub>2</sub> 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<sub>2</sub> production by a mutant containing a deletion of the coenzyme F<sub>420</sub>-reducing hydrogenase Fru was also severely reduced, suggesting that the major pathway of H<sub>2</sub> production comprised Fdh1 and Fru. Because a  $\Delta fru\text{-}\Delta frc$  mutant retained 10% of the wild-type activity, an additional pathway is present. Mutants possessing deletions of the gene encoding the F<sub>420</sub>-dependent methylene-H<sub>4</sub>MTP dehydrogenase (Mtd) or the H<sub>2</sub>-forming methylene-H<sub>4</sub>MTP dehydrogenase (Hmd) also possessed reduced activity, which suggested that this second pathway was comprised of Fdh1-Mtd-Hmd. In contrast to H<sub>2</sub> production, the cellular rates of methanogenesis were unaffected in these mutants, which suggested that the observed H<sub>2</sub> production was not a direct intermediate of methanogenesis. In conclusion, high rates of formate-dependent H<sub>2</sub> production demonstrated the potential of *M. maripaludis* for the microbial production of H<sub>2</sub> from formate.

Many hydrogenotrophic methanogens use H<sub>2</sub> or formate for the reduction of CO<sub>2</sub> 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<sub>2</sub> consumer, *M. maripaludis* is exceptionally well equipped with enzymes responsible for H<sub>2</sub> 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<sub>420</sub>-reducing (Fru and Frc) and two coenzyme F<sub>420</sub>-nonreducing (Vhu and Vhc) hydrogenases. One hydrogenase of each type (Fru and Vhu) contains a selenocysteine residue. The other hydrogenases (Frc and Vhc) contain cysteine 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<sub>2</sub>-forming methylenetetrahydromethanopterin (methylene-H<sub>4</sub>MPT) dehydro-

genase (Hmd), which in other species has been shown to play an important role at high levels of H<sub>2</sub> or under nickel limitation (1, 2, 29).

When formate is the substrate, it is oxidized for the reduction of CO<sub>2</sub> 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 selenocysteine 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<sub>420</sub> 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:  $\text{HCO}_2^- + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{H}_2$  (+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<sub>420</sub> (F<sub>420</sub>H<sub>2</sub>) generated by Fdh is oxidized by the reversible F<sub>420</sub>-dependent hydrogenase Fru (Fig. 1). In the second pathway, F<sub>420</sub>H<sub>2</sub> is oxidized by the F<sub>420</sub>-dependent methylene-H<sub>4</sub>MPT dehydrogenase (Mtd) and Hmd (1, 2, 29) (Fig. 1). While previous studies demonstrated H<sub>2</sub> 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<sub>4</sub> production, these microorganisms possess extremely high specific activities for enzymes involved in methanogenesis, including the hydroge-

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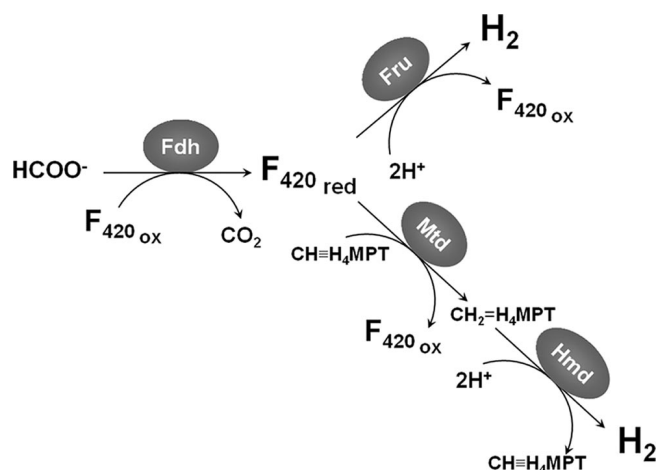


FIG. 1. Potential pathways of H<sub>2</sub> production in the hydrogenotrophic methanogens. The first pathway includes Fdh and Fru. In this pathway, Fdh reduces F<sub>420</sub>. In selenium-grown cells, F<sub>420</sub>H<sub>2</sub> is oxidized by the [Ni-Fe] hydrogenase Fru. In the second pathway, F<sub>420</sub>H<sub>2</sub> is oxidized by Mtd to reduce methenyl-H<sub>4</sub>MPT to methylene-H<sub>4</sub>MPT, which is then reoxidized by Hmd, a Ni-free hydrogenase, to produce H<sub>2</sub>. Abbreviations: Fdh, formate dehydrogenase; Fru, F<sub>420</sub>-reducing hydrogenase; F<sub>420</sub>ox, oxidized coenzyme F<sub>420</sub>; F<sub>420</sub>red, reduced coenzyme F<sub>420</sub>; Mtd, F<sub>420</sub>-dependent methylene-H<sub>4</sub>MPT dehydrogenase; Hmd, H<sub>2</sub>-forming methylene-H<sub>4</sub>MPT dehydrogenase.

nases used for H<sub>2</sub> consumption. If H<sub>2</sub> utilization were a reversible process, high rates of H<sub>2</sub> production would be possible. To test this hypothesis, the rates of H<sub>2</sub> 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<sub>2</sub>-CO<sub>2</sub> 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<sub>2</sub>-CO<sub>2</sub> (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<sub>2</sub>-CO<sub>2</sub> (80:20 [vol/vol]) at 37°C. When sodium formate was the substrate (0.4 M), the bottles were flushed with N<sub>2</sub>-CO<sub>2</sub> (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<sub>2</sub> 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<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 5 mM dithiothreitol, pH 6.9.

**H<sub>2</sub> measurements.** The H<sub>2</sub> 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<sub>2</sub>-free N<sub>2</sub> (31). The concentration of the dissolved H<sub>2</sub> in the anaerobic buffer was measured using a modified amperometric O<sub>2</sub> 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<sub>2</sub>-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<sub>2</sub> 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<sub>600</sub> of 1 corresponded to 0.34 mg (dry weight) · ml<sup>-1</sup>.

**CH<sub>4</sub> 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<sub>2</sub>. The assay was initiated by adding formate to a final concentration of 20 mM or flushing the vials with H<sub>2</sub>-CO<sub>2</sub> (80:20 [vol/vol]) for 1 min. The samples were incubated at 37°C for 10 min. CH<sub>4</sub> was detected with an SRI 8610-C gas chromatograph (SRI Instruments, Torrance, CA) fitted with on-column injection, a Porapak Q teflon column at 90°C, and a flame ionization detector operating at 150°C. The carrier gas was N<sub>2</sub>. One unit was defined as 1 μmol of CH<sub>4</sub> 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<sub>420</sub>-reducing hydrogenase activities were measured spectrophotometrically under anaerobic conditions. H<sub>2</sub>-dependent F<sub>420</sub> 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<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 5 mM dithiothreitol. The final concentration of F<sub>420</sub> was 10 μM. The 1.6-ml glass cuvettes were sealed with rubber stoppers and flushed with O<sub>2</sub>-free H<sub>2</sub> 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<sub>420</sub>) of 40 mM · cm<sup>-1</sup> was used for calculations. One unit was defined as 1 μmol of coenzyme F<sub>420</sub> reduced per minute. F<sub>420</sub> 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<sub>2</sub>-free N<sub>2</sub> 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<sub>605</sub>) of 13.9 mM · cm<sup>-1</sup> was used for calculations. One unit was defined as 2 μmol of MV reduced per minute.

## RESULTS AND DISCUSSION

**Initial rates of H<sub>2</sub> production.** Previous reports of formate-dependent H<sub>2</sub> production measured formation of headspace H<sub>2</sub> during growth (7, 19, 28). However, preliminary measurements of the rate of production of dissolved H<sub>2</sub> with an H<sub>2</sub> probe and using resting cells far exceeded these reports. Therefore, the earlier measurements underestimated the production rate, possibly because H<sub>2</sub> 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<sub>2</sub> or formate, washed in

TABLE 1. Strains used in this study

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

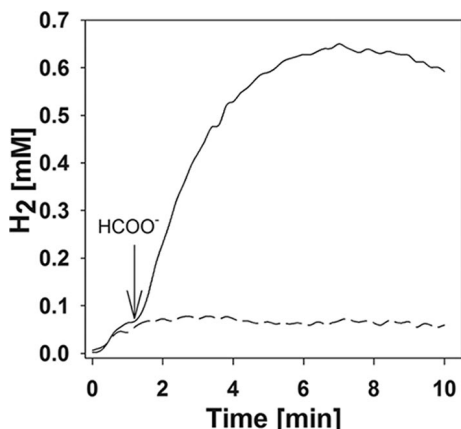


FIG. 2. Initial rates of  $H_2$  production from formate (solid line) by resting cells of *M. maripaludis*. Cells were grown with formate, washed in buffer, and resuspended in the reaction cuvette. Assays were initiated by adding 20 mM sodium formate (arrow). Results from incubation of the cell suspension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

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  $0.37 \pm 0.02 U \cdot mg^{-1}$  (dry weight) and  $0.32 \pm 0.04 U \cdot mg^{-1}$  (dry weight) with  $H_2$  or formate, respectively (means  $\pm$  standard deviations of triplicate measurements with two independently grown cultures). Similarly, the rates of methanogenesis by formate-grown cells were  $0.25 \pm 0.02 U \cdot mg^{-1}$  (dry weight) and  $0.43 \pm 0.04 U \cdot mg^{-1}$  (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

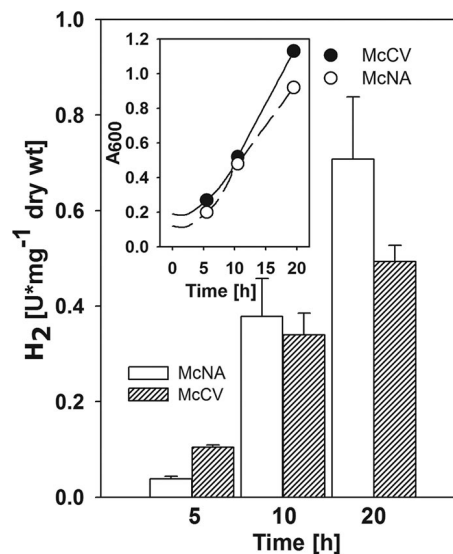


FIG. 3. Changes in formate-dependent  $H_2$  production by resting cells following growth under  $H_2$  limitation. The wild-type S2 was grown in minimal or McNA ( $\circ$ ) and rich or McCV ( $\bullet$ ) medium in 1-liter Wheaton bottles with 100 ml of broth. At the beginning of the experiment, the 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.

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  $\sim 0.4$ , 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  $\sim 0.8$ , 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  $0.12 \pm 0.03$  and  $0.41 \pm 0.04 U \cdot mg^{-1}$  (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



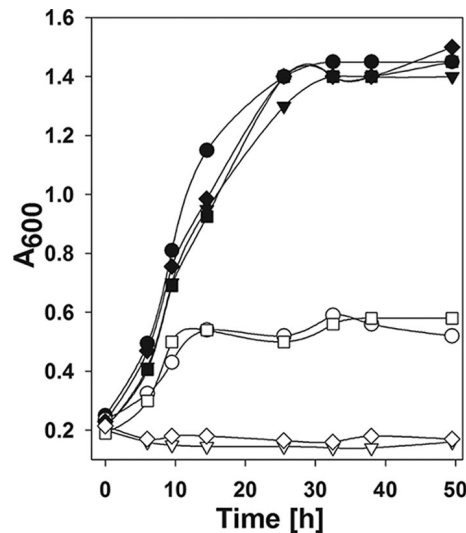


FIG. 4. Growth of the wild-type and *fdh* mutant strains with H<sub>2</sub> or formate. Solid symbols, growth with H<sub>2</sub> (276 kPa; H<sub>2</sub>-CO<sub>2</sub> 80:20 [vol/vol]); open symbols, growth with formate (100 mM). Circles, S2; inverted triangles,  $\Delta fdhA1$ ; squares,  $\Delta fdhA2$ ; diamonds,  $\Delta fdhA1\text{-}\Delta fdhA2$ . Each point represents the mean value of two replicates. Similar curves were obtained in a replicate experiment.

dehydrogenase (*fdh1*) and F<sub>420</sub>-reducing hydrogenase (*fru*) genes are significantly higher during H<sub>2</sub> limitation (17).

**Role of formate dehydrogenase.** To ascertain the importance of the two Fdh isozymes in H<sub>2</sub> production, the mutant strains  $\Delta fdhA1$ ,  $\Delta fdhA2$ , and  $\Delta fdhA1\text{-}\Delta fdhA2$  were tested for their ability to grow with formate, MV-dependent Fdh activity, and for H<sub>2</sub> production. The double mutant  $\Delta fdhA1\text{-}\Delta fdhA2$ , 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<sub>2</sub> production (Fig. 4, Table 2, and data not shown). In addition, in extracts of H<sub>2</sub>-grown cells, the MV-dependent Fdh activity was <0.02 U · mg<sup>-1</sup> (dry weight). Thus, H<sub>2</sub> production required Fdh. MV-dependent Fdh activities in cell extracts of the  $\Delta fdhA2$  mutant and the wild-type S2 strains were 1.0 ± 0.25 and 3.6 ± 1.2 U · mg<sup>-1</sup> (dry weight), respectively. In contrast, growth on formate of strain  $\Delta fdhA2$  was comparable to that of the wild type, and H<sub>2</sub> production was only slightly reduced. Therefore, the rate of formate oxidation did not appear to limit growth and H<sub>2</sub> production in this mutant. Notably, the  $\Delta fdhA1$  mutant failed to grow with formate without an extended incubation of 70 h and produced H<sub>2</sub> poorly (Fig. 4, Table 2, and data not

TABLE 2. Activities of formate-dependent H<sub>2</sub> production by *fdh* mutant strains<sup>a</sup>

Genotype	Sp act (U · mg <sup>-1</sup> [dry wt])
Wild type	0.32 ± 0.05
$\Delta fdhA1$	0.04 ± 0.01
$\Delta fdhA2$	0.29 ± 0.02
$\Delta fdhA1\text{-}\Delta fdhA2$	<0.02

<sup>a</sup> Cultures were grown with H<sub>2</sub> at 136 kPa of H<sub>2</sub>-CO<sub>2</sub> (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.

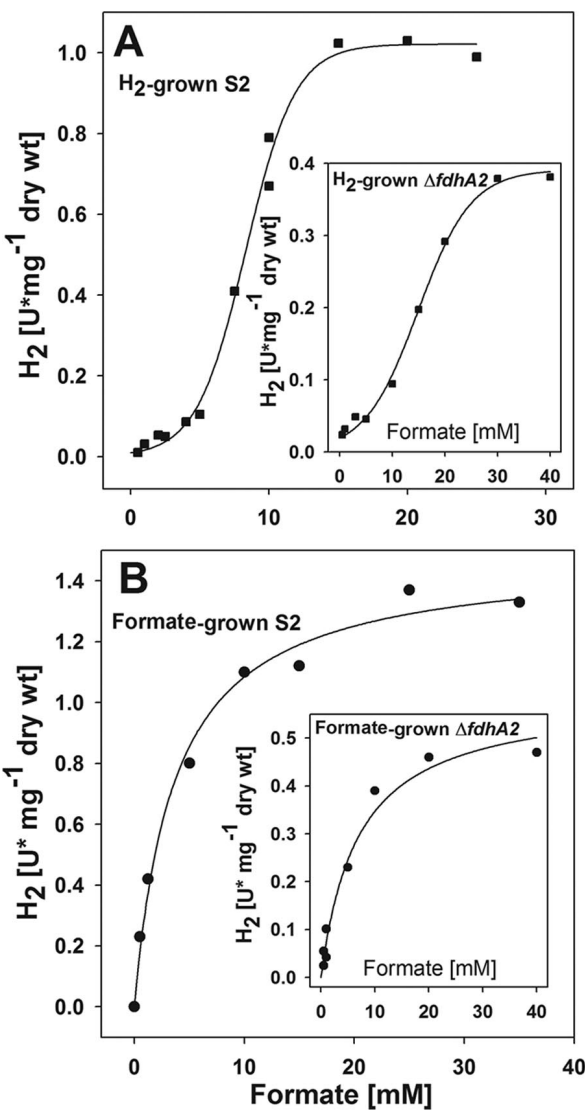


FIG. 5. Kinetics of formate-dependent H<sub>2</sub> production by resting cells of the wild-type strain S2 previously grown either with H<sub>2</sub> (A) or formate (B). The kinetics of the  $\Delta fdhA2$  mutant are shown in the insets. The S2 and  $\Delta fdhA2$  mutant strains were grown to absorbances of about 0.8 and 0.6, respectively.

shown). In addition, the MV-dependent Fdh activity was <0.02 U · mg<sup>-1</sup> (dry weight). Subsequent transfers of formate-grown cultures of the  $\Delta fdhA1$  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  $\Delta fdhA1$  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<sub>2</sub> production, and Fdh1 appeared to be the major isozyme in formate utilization under these conditions. In previous studies, the  $\Delta fdhA1$  mutant grew with formate at similar rates as the  $\Delta fdhA2$  strain (33). Presumably, this difference reflects the differences in the medium composition and the experimental design.

**Kinetics of formate-dependent H<sub>2</sub> production by whole cells.** The kinetics depended on the growth substrates of the cells.

TABLE 3. Activities of formate-dependent H<sub>2</sub> and CH<sub>4</sub> production for hydrogenase and methylene-H<sub>4</sub>MPT dehydrogenase mutant strains<sup>a</sup>

Genotype	Sp act (U · mg <sup>-1</sup> [dry wt])	
	H <sub>2</sub>	CH <sub>4</sub>
Wild type	0.56 ± 0.10	0.36 ± 0.03
$\Delta fruA$ - $\Delta frcA$	0.06 ± 0.02	0.20 ± 0.04
$\Delta hmd$	0.40 ± 0.08	0.12 ± 0.02
$\Delta mtd$	0.35 ± 0.05	0.13 ± 0.02

<sup>a</sup> Cells were grown with formate to an absorbance of about 0.6, except for  $\Delta mtd$ , for which the absorbance was about 0.4. H<sub>2</sub> and CH<sub>4</sub> 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<sub>2</sub> production followed Michaelis-Menten kinetics. The  $K_p$  or concentration of formate required for half-maximal activity, was  $3.6 \pm 0.5$  mM (mean ± 1 standard deviation), and the  $V_p$  or the maximal specific activity, was  $1.5 \pm 0.1$  U · mg<sup>-1</sup> (dry weight) (Fig. 5). For H<sub>2</sub>-grown cells, H<sub>2</sub> production followed sigmoidal kinetics, and the kinetic values were  $9 \pm 1$  mM and  $1.1 \pm 0.2$  U · mg<sup>-1</sup> (dry weight), respectively. The sigmoidal kinetics for the H<sub>2</sub>-grown cells were not due to the presence of two isozymes in the wild-type cells, because the  $\Delta fdhA2$  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<sub>2</sub> production. Expression of *fdhC* is reduced in H<sub>2</sub>-grown cells (33). Thus, at low formate concentrations, low levels of the transporter may limit the rate of formate uptake and H<sub>2</sub> 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<sub>2</sub> production.** Given the requirement for Fdh, two pathways of H<sub>2</sub> production were likely (Fig. 1). In selenium-grown cells, the major pathway was expected to include the oxidation of Fdh-generated F<sub>420</sub>H<sub>2</sub> by the F<sub>420</sub>-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<sup>-1</sup> 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<sup>-1</sup> (dry weight), values that were consistent with a role in whole-cell H<sub>2</sub> production. To examine this point further, the H<sub>2</sub> production activities of a series of in-frame deletion mutants in the F<sub>420</sub>-dependent hydrogenases,  $\Delta fruA$ ,  $\Delta frcA$ , and  $\Delta fruA$ - $\Delta frcA$ , were examined. The activity of the  $\Delta fruA$ - $\Delta frcA$  mutant was severely reduced (Table 3). The  $\Delta fruA$  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  $\Delta frcA$  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<sub>420</sub> is an intermediate in the process and provided evidence for an F<sub>420</sub>-dependent formate-hydro-

gen lyase system in *M. maripaludis*. Because the  $\Delta fruA$ - $\Delta frcA$  mutant retained about 10% of the wild-type H<sub>2</sub> 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<sub>4</sub>MPT-dependent pathway (Fig. 1).

**Is H<sub>2</sub> an intermediate of methanogenesis with formate?** The rates of H<sub>2</sub> generation were comparable to the rates of methanogenesis, which suggested that H<sub>2</sub> was produced in sufficient amounts to be an intermediate during methanogenesis with formate. During hydrogenotrophic growth, H<sub>2</sub> is proposed to be the electron donor for the Eha-dependent reduction of CO<sub>2</sub> 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<sub>420</sub> is initially reduced. If methanogenesis proceeds in a fashion similar to hydrogenotrophic growth, H<sub>2</sub> could be generated from F<sub>420</sub>H<sub>2</sub> to produce the reductant for the first and last steps of methanogenesis. To test this hypothesis, rates of formate-dependent H<sub>2</sub> and CH<sub>4</sub> 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

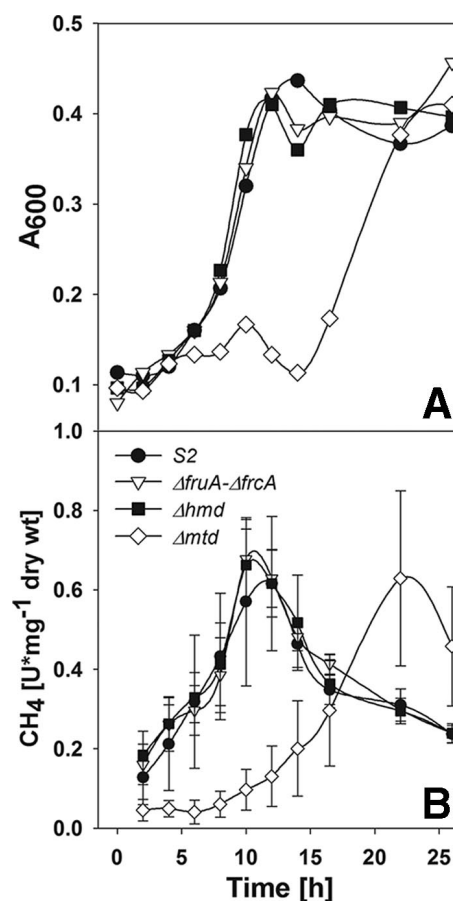


FIG. 6. Growth (A) and specific activity of CH<sub>4</sub> production (B) by the wild-type S2 (●),  $\Delta fruA$ - $\Delta frcA$  (▽),  $\Delta hmd$  (■), and  $\Delta mtd$  (◇) mutant strains grown with formate (100 mM). Each point represents the mean value of three replicates, and error bars in panel B represent 1 standard deviation. Similar curves were obtained in a replicate experiment.

experiments, there was little correlation between the rates of H<sub>2</sub> and CH<sub>4</sub> production in the wild type and  $\Delta fruA$ - $\Delta frcA$ ,  $\Delta hmd$ , and  $\Delta mtd$  mutants. In fact, for the  $\Delta fruA$ - $\Delta frcA$  mutant, the rate of methanogenesis exceeded the rate of H<sub>2</sub> production, which seemed to preclude the possibility that H<sub>2</sub> could be an obligate intermediate of methanogenesis.

Methanogenesis is very O<sub>2</sub> 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  $\Delta fruA$ - $\Delta frcA$  and  $\Delta hmd$  mutants, growth and methanogenesis were nearly the same as the wild type. Even though the growth and CH<sub>4</sub> production were delayed for the  $\Delta mtd$  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<sub>2</sub> 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<sub>2</sub> production measured with a H<sub>2</sub> probe is too low to be an obligatory intermediate for methanogenesis from formate, it is still formally possible that a H<sub>2</sub> cycle could still exist within the cell. In this case, the cellular H<sub>2</sub> levels would not equilibrate with the bulk H<sub>2</sub> dissolved in the medium. However, this possibility seems unlikely, given that inactivation of each of the major pathways of H<sub>2</sub> production, either in the  $\Delta fruA$ - $\Delta frcA$  or the  $\Delta hmd$  and  $\Delta mtd$  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<sub>2</sub>-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<sub>2</sub> reduction by flavin-mediated electron bifurcation. According to this model, one low-potential electron from H<sub>2</sub> 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<sub>2</sub> is not an obligate intermediate during growth with formate supports this model, it remains unclear how F<sub>420</sub>H<sub>2</sub> generated from formate donates electrons for the flavin-mediated electron bifurcation. If H<sub>2</sub> is not an intermediate, an enzymatic complex with the function of F<sub>420</sub>H<sub>2</sub>: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<sub>2</sub>, which is a clean and efficient energy source. Microbial H<sub>2</sub> production has involved a range of approaches (8, 13, 14, 24). Methanogens are very active H<sub>2</sub> consumers; if these systems could be used for H<sub>2</sub> production, high rates would be possible. To test this concept, H<sub>2</sub> production from formate by resting cells of methanococci was evaluated. Rates

of formate-dependent H<sub>2</sub> production have been previously examined in a few bacteria. The methylotrophs *Methylobacterium albus* and *Methylobacterium trichosporium* produced H<sub>2</sub> at rates of 1.6 and 0.4 mU · mg<sup>-1</sup> (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<sup>-1</sup> (dry weight) were obtained with genetically engineered *Escherichia coli* strains (35). The rates obtained from wild-type methanococci, up to 1.4 U · mg<sup>-1</sup> (dry weight), were comparable. However, because of the equilibrium constant, high concentrations of H<sub>2</sub> cannot be produced from formate regardless of the catalyst employed. Therefore, biotechnological applications would require an efficient means of harvesting H<sub>2</sub> at low levels. Because F<sub>420</sub>H<sub>2</sub> is a key intermediate in methanococcal H<sub>2</sub> production, one might speculate that the substrate range could be increased by genetically engineering methanococci to couple the reduction of F<sub>420</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> production. For wild-type cells, the rates of formate-dependent H<sub>2</sub> 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<sub>2</sub> from the relatively inexpensive chemical, which can be derived from biomass (35).

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