

# H<sub>2</sub>-Independent Growth of the Hydrogenotrophic Methanogen *Methanococcus maripaludis*

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**ABSTRACT** Hydrogenotrophic methanogenic *Archaea* require reduced ferredoxin as an anaplerotic source of electrons for methanogenesis. H<sub>2</sub> oxidation by the hydrogenase Eha provides these electrons, consistent with an H<sub>2</sub> requirement for growth. Here we report the identification of alternative pathways of ferredoxin reduction in *Methanococcus maripaludis* that operate independently of Eha to stimulate methanogenesis. A suppressor mutation that increased expression of the glycolytic enzyme glyceraldehyde-3-phosphate:ferredoxin oxidoreductase resulted in a strain capable of H<sub>2</sub>-independent ferredoxin reduction and growth with formate as the sole electron donor. In this background, it was possible to eliminate all seven hydrogenases of *M. maripaludis*. Alternatively, carbon monoxide oxidation by carbon monoxide dehydrogenase could also generate reduced ferredoxin that feeds into methanogenesis. In either case, the reduced ferredoxin generated was inefficient at stimulating methanogenesis, resulting in a slow growth phenotype. As methanogenesis is limited by the availability of reduced ferredoxin under these conditions, other electron donors, such as reduced coenzyme F<sub>420</sub>, should be abundant. Indeed, when F<sub>420</sub>-reducing hydrogenase was reintroduced into the hydrogenase-free mutant, the equilibrium of H<sub>2</sub> production via an F<sub>420</sub>-dependent formate:H<sub>2</sub> lyase activity shifted markedly toward H<sub>2</sub> compared to the wild type.

**IMPORTANCE** Hydrogenotrophic methanogens are thought to require H<sub>2</sub> as a substrate for growth and methanogenesis. Here we show alternative pathways in methanogenic metabolism that alleviate this H<sub>2</sub> requirement and demonstrate, for the first time, a hydrogenotrophic methanogen that is capable of growth in the complete absence of H<sub>2</sub>. The demonstration of alternative pathways in methanogenic metabolism suggests that this important group of organisms is metabolically more versatile than previously thought.

Received 24 January 2013 Accepted 30 January 2013 Published 26 February 2013

**Citation** Costa KC, Lie TJ, Jacobs MA, Leigh JA. 2013. H<sub>2</sub>-independent growth of the hydrogenotrophic methanogen *Methanococcus maripaludis*. mBio 4(2):e00062-13. doi:10.1128/mBio.00062-13.

**Editor** Dianne Newman, California Institute of Technology/HHMI

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Methanogenic *Archaea* can be grouped into two physiologically distinct groups, the methylotrophs and the hydrogenotrophs (1). Methylotrophic methanogens are relatively versatile, as their substrate repertoire for methanogenesis includes H<sub>2</sub> and CO<sub>2</sub>, acetate, methyl compounds, such as methanol and methylamines, and CO. Hydrogenotrophic methanogens are more restricted, using H<sub>2</sub>, formate, or for a few species, certain alcohols as electron donors for CO<sub>2</sub> reduction to CH<sub>4</sub>.

Hydrogenotrophic methanogens are distinct from methylotrophic methanogens in their use of electron bifurcation as an energy-conserving step in methanogenesis from CO<sub>2</sub> (1–4). Two pairs of electrons enter the methanogenic pathway at a protein complex that contains two key enzymes, heterodisulfide reductase (Hdr) and formylmethanofuran dehydrogenase (Fwd). One pair of electrons is used by Hdr in an exergonic reaction to reduce the heterodisulfide CoM-S-S-CoB to the sulfhydryl coenzymes HS-CoB and HS-CoM, which in turn serve as reductants for reduction of the methyl group on coenzyme M (CH<sub>3</sub>-S-CoM) to CH<sub>4</sub>. The other pair of electrons is used in an endergonic reaction by Fwd to reduce CO<sub>2</sub> to the formyl group of formyl-methanofuran (formyl-MFR) (for a diagram of the methanogenic pathway, see reference 4).

By coupling the final, methane-producing step to the initial, CO<sub>2</sub>-reducing step, electron bifurcation renders the pathway cyclic. The cyclic model of methanogenesis was recently named the Wolfe cycle in honor of the contributions of Ralph S. Wolfe (5). The cyclic nature of the pathway can explain in part why hydrogenotrophs have not evolved the metabolic versatility of the methylotrophs. The latter organisms use acetate by oxidizing the carbonyl to provide electrons for reduction of the methyl to methane, and they use methyl compounds by disproportionation where some of the substrate is oxidized and some is reduced to methane. However, in hydrogenotrophs, the stoichiometric coupling of the methane-producing step to the recruitment of CO<sub>2</sub> into the pathway prohibits the input of additional intermediates. Furthermore, in the case of acetate, only one pair of electrons is available from the oxidation of the carbonyl, yet electron bifurcation requires that two pairs of electrons must feed into the heterodisulfide reductase complex. Although many hydrogenotrophic methanogens contain acetyl coenzyme A (acetyl-CoA) synthase/CO dehydrogenase (ACS/CODH) (the essential enzyme for aceticlastic methanogenesis), they appear to use it only anabolically for CO<sub>2</sub> fixation and not catabolically for acetate utilization.

Either H<sub>2</sub> or formate donates electrons to all four reduction

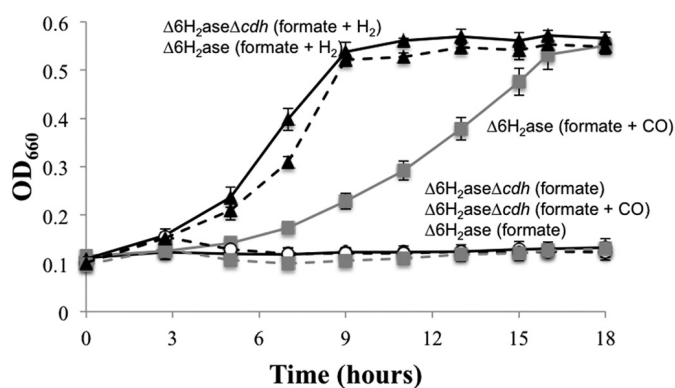
steps in methanogenesis from  $\text{CO}_2$ . Both substrates supply electrons directly to the Hdr complex via an Hdr-associated hydrogenase or an Hdr-associated formate dehydrogenase (Fdh) (2), thus providing for the first and last reduction steps in the pathway. The two intermediate reduction steps use the coenzyme  $\text{F}_{420}$  as the direct electron donor, and  $\text{H}_2$  or formate directly reduces  $\text{F}_{420}$  via an  $\text{F}_{420}$ -reducing hydrogenase or Fdh. Hence,  $\text{H}_2$  or formate alone can provide the stoichiometric reducing requirements for  $\text{CO}_2$  reduction to methane. However, as another consequence of the Wolfe cycle, the levels of intermediates in methanogenesis must be anaplerotically maintained in order to avoid decaying flux in the pathway (5). In the model species *Methanococcus maripaludis* we showed how this occurs by genetically eliminating six of the seven hydrogenases encoded in the genome (4). The resulting mutant, designated  $\Delta\text{H}_2\text{ase}$ , was unable to use  $\text{H}_2$  as the stoichiometric electron donor for methanogenesis. The mutant also lacked a formate- $\text{H}_2$  lyase activity found in the wild-type strain. As a result of these two deficiencies, the  $\Delta\text{H}_2\text{ase}$  mutant required both formate and  $\text{H}_2$  for growth and methanogenesis. While formate was needed stoichiometrically for the four reduction steps of methanogenesis,  $\text{H}_2$  was required only in small amounts sufficient to support the anaplerotic needs of methanogenesis. In the  $\Delta\text{H}_2\text{ase}$  mutant, only one hydrogenase remained: the membrane-bound energy-converting hydrogenase Eha, which harvests chemiosmotic energy to drive the reduction of a low-potential ferredoxin which in turn is thought to reduce  $\text{CO}_2$  to formyl-MFR. Hence, during growth on  $\text{H}_2$  or formate, Eha is essential in wild-type *M. maripaludis* to anaplerotically replenish methanogenesis at the first reduction step. Indeed, a knockout of the genes encoding Eha was not successful. Consistent with these results,  $\text{H}_2$  stimulated methanogenesis from formate in cell suspensions (4).

Here we show that the essentiality of  $\text{H}_2$  and Eha does not always hold, and that in *M. maripaludis*, there are at least two additional pathways by which the anaplerotic requirements of methanogenesis can be satisfied. We delete genes encoding Eha in a strain derived from the  $\Delta\text{H}_2\text{ase}$  mutant that expresses one of these pathways, thus eliminating all  $\text{H}_2$  metabolism in a hydrogenotrophic methanogen. By reintroducing a single hydrogenase—the  $\text{F}_{420}$ -reducing hydrogenase—into the hydrogenase-free strain and observing its effects in isolation from other  $\text{H}_2$ -metabolizing pathways, we find that substantial electron flux is diverted to  $\text{H}_2$  production. These findings show that hydrogenotrophic methanogens are metabolically more versatile than previously thought.

## RESULTS

**CO stimulates growth of the  $\Delta\text{H}_2\text{ase}$  mutant in the absence of  $\text{H}_2$ .** Eha provides anaplerotic input to the methanogenic pathway by acting as a supplement to electron bifurcation for the reduction of a ferredoxin that in turn is used to reduce  $\text{CO}_2$  to formyl-MFR (4). However, other pathways of ferredoxin reduction/oxidation exist in methanogenic *Archaea*. The ACS/CODH enzyme complex converts  $\text{CO}_2$  to CO, which ultimately becomes the carbonyl carbon of acetyl-CoA (6, 7). This reduction is dependent on reduced ferredoxin as an electron donor.

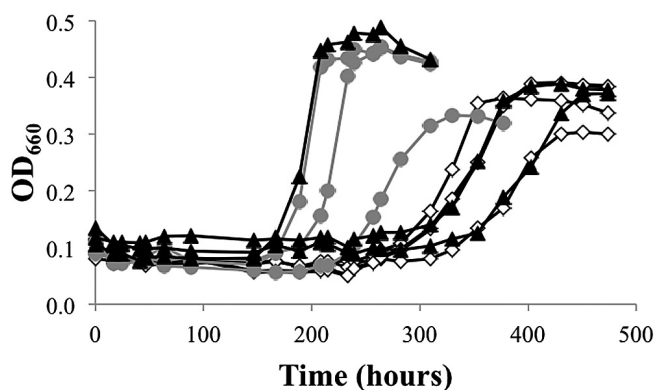
To test whether the reverse reaction of ACS/CODH—CO oxidation to  $\text{CO}_2$  with the production of reduced ferredoxin—can stimulate methanogenesis in an  $\text{H}_2$ -independent manner, the  $\Delta\text{H}_2\text{ase}$  mutant was grown on formate medium with or without the addition of  $\text{H}_2$  or CO to the culture headspace. As expected,  $\text{H}_2$



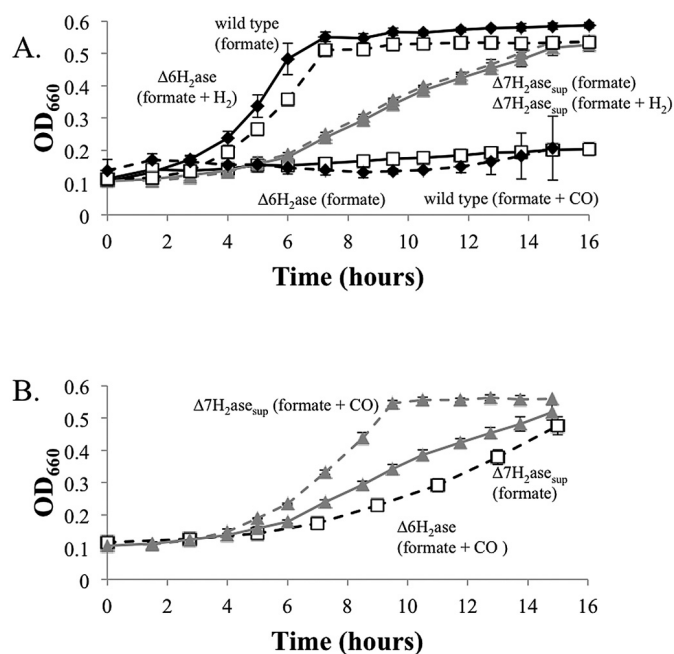
**FIG 1** Growth of the  $\Delta\text{H}_2\text{ase}$  mutant with CO and formate. The  $\Delta\text{H}_2\text{ase}$  mutant (solid black lines) and the  $\Delta\text{H}_2\text{ase}\Delta\text{cdh}$  mutant (broken lines) were grown with formate plus  $\text{H}_2$  (black symbols), formate plus 5% CO (gray symbols), or formate alone (white symbols). Data points are averages of three cultures, and error bars represent 1 standard deviation around the mean.

promoted robust growth. In addition, CO promoted growth in the absence of  $\text{H}_2$  (Fig. 1). Growth rates were much lower with CO in place of  $\text{H}_2$ , suggesting that ferredoxin reduction by Eha is preferred. Growth promoted by CO was directly attributable to ACS/CODH, since when the  $\text{CO}_2$ -reducing subunits were genetically eliminated ( $\Delta\text{H}_2\text{ase}\Delta\text{cdh}$ ), growth no longer occurred with CO and formate as the only electron donors (Fig. 1).

**Isolation of a suppressor mutation that allows growth of the  $\Delta\text{H}_2\text{ase}$  mutant on formate alone.** The growth of the  $\Delta\text{H}_2\text{ase}$  mutant with formate and CO suggests that ferredoxin reduction by Eha is not necessary for growth, provided alternative mechanisms to anaplerotically stimulate methanogenesis are present.  $\text{CH}_3\text{-S-CoM}$  addition to cell extracts stimulates methanogenesis (8, 9), and methanogenic *Archaea* are capable of  $\text{CH}_3\text{-S-CoM}$  uptake via a poorly characterized and very inefficient activity (10, 11). Therefore, we tried to grow the  $\Delta\text{H}_2\text{ase}$  mutant on formate in the absence of  $\text{H}_2$  in the presence and absence of  $\text{CH}_3\text{-S-CoM}$ . Surprisingly, regardless of the presence or absence of  $\text{CH}_3\text{-S-CoM}$ , after prolonged incubation of nine independent cultures, all nine grew (Fig. 2). Upon transfer to new medium, each strain



**FIG 2** Generation of suppressor strains of the  $\Delta\text{H}_2\text{ase}$  mutant capable of  $\text{H}_2$ -independent growth. The  $\Delta\text{H}_2\text{ase}$  mutant was grown in formate-containing medium without  $\text{H}_2$  or CO. The medium contained 1,000  $\mu\text{M}$  (black symbols), 100  $\mu\text{M}$  (gray symbols), or 0  $\mu\text{M}$  (white symbols)  $\text{CH}_3\text{-S-CoM}$  (three replicates each); however,  $\text{CH}_3\text{-S-CoM}$  had no stimulatory effect on growth. Each curve represents growth in a single tube.



**FIG 3** Growth of the  $\Delta 7H_2ase_{sup}$  mutant. (A) Growth of the  $\Delta 7H_2ase_{sup}$  mutant with formate alone or formate plus H<sub>2</sub>. (B) Growth of the  $\Delta 7H_2ase_{sup}$  mutant with formate + CO. The  $\Delta 7H_2ase_{sup}$  mutant grown on formate (from Fig. 3A) and the  $\Delta 6H_2ase$  mutant grown on formate plus CO (from Fig. 1) are shown for comparison. Wild-type strain MM901 (black symbols) and the  $\Delta 7H_2ase_{sup}$  (gray symbols) and  $\Delta 6H_2ase$  (white symbols) mutants were studied. Broken lines indicate that the cultures were grown with H<sub>2</sub> or 5% CO in the culture headspace. Data points are averages of three cultures, and error bars represent 1 standard deviation around the mean.

tested routinely grew to maximum optical density at 660 nm (OD<sub>660</sub>) within 24 h. These results suggested that independent suppressor mutations ( $\Delta 6H_2ase_{sup}$ ) were generated that allowed for growth on formate alone. Suppressor strains that were generated in the presence of CH<sub>3</sub>-S-CoM grew well in its absence. Hence, although CH<sub>3</sub>-S-CoM failed to stimulate growth, mutants developed that had a novel mechanism to anaerobically stimulate methanogenesis.

**Deletion of *eha* is possible due to a suppressor mutation.** A suppressor mutation that allows growth of  $\Delta 6H_2ase_{sup}$  on formate alone could have produced a novel H<sub>2</sub> production activity, or it could have generated a new ferredoxin reducing activity that is independent of H<sub>2</sub>. If a novel H<sub>2</sub> production pathway were responsible, *eha* would still be essential (4). We attempted deletion of the genes encoding the active site subunits of Eha (*ehaNO*) in one of the  $\Delta 6H_2ase_{sup}$  strains (12, 13). Deletion of *ehaNO* was indeed possible, suggesting that another ferredoxin reduction activity is present in the suppressor background. The new strain ( $\Delta 7H_2ase_{sup}$ ) lacks the genes encoding the active sites for all genomically encoded hydrogenases:  $\Delta vhuAU \Delta vhcA \Delta fruA \Delta frcA \Delta hmd \Delta ehbN \Delta ehaNO$ . The  $\Delta 7H_2ase_{sup}$  mutant grew in the absence of H<sub>2</sub>, and H<sub>2</sub> did not stimulate growth (Fig. 3A).

CO is a potent inhibitor of nickel-containing hydrogenases (14, 15). Five percent CO inhibited wild-type *M. maripaludis* grown on formate but had no inhibitory effect on the  $\Delta 7H_2ase_{sup}$  mutant. In fact, the  $\Delta 7H_2ase_{sup}$  mutant grew better on formate in the presence of 5% CO than it did on formate alone (Fig. 3B), suggesting that in the  $\Delta 7H_2ase_{sup}$  mutant, like the  $\Delta 6H_2ase$  mu-

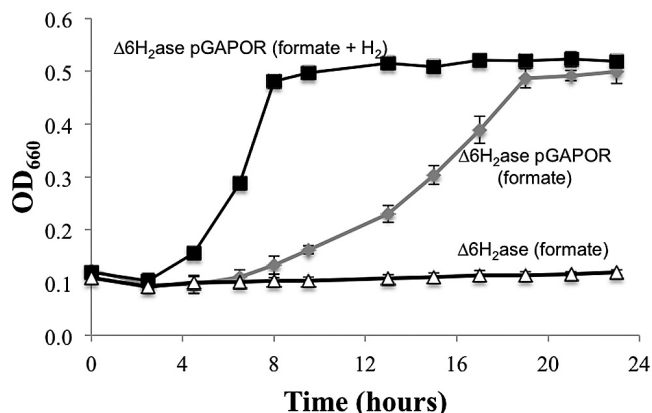
tant, CO was oxidized to CO<sub>2</sub>, leading to the production of reduced ferredoxin. The growth of the  $\Delta 7H_2ase_{sup}$  mutant is apparently limited by the availability of reduced ferredoxin, and CO oxidation partially relieves its slow growth phenotype. The reduced ferredoxins generated by the suppressor activity and ACS/CODH appear additive in stimulating methanogenesis. The  $\Delta 7H_2ase_{sup}$  mutant grew faster with formate and CO than either the  $\Delta 7H_2ase_{sup}$  mutant grown with formate alone or the  $\Delta 6H_2ase$  mutant grown with formate and CO.

**Genome sequencing reveals a suppressor mutation allowing for growth of the  $\Delta 6H_2ase_{sup}$  and  $\Delta 7H_2ase_{sup}$  mutants on formate alone.** To determine the genetic background that allowed growth of the  $\Delta 6H_2ase_{sup}$  and  $\Delta 7H_2ase_{sup}$  mutants on formate in the absence of H<sub>2</sub> or CO, we performed Illumina sequencing on the  $\Delta 7H_2ase_{sup}$  mutant and six of the isolated suppressors and compared the sequence to the  $\Delta 6H_2ase$  parent (see Table S1 in the supplemental material). Four of six of the  $\Delta 6H_2ase_{sup}$  mutants, as well as the  $\Delta 7H_2ase_{sup}$  mutant, shared a common insertion (AT at position 931341) in an intergenic region directly upstream of the gene for glyceraldehyde-3-phosphate (G3P):ferredoxin oxidoreductase (GAPOR) (see Fig. S1 in the supplemental material). The insertion generated the sequence AATATATA upstream of GAPOR which is very similar to the consensus methanogen promoter TTTA(T/A)ATA (16). Therefore, it appears that generation of a promoter upstream of GAPOR increases expression of this ferredoxin-reducing enzyme to allow for H<sub>2</sub>-independent growth. Sanger sequencing confirmed the presence of this mutation in the  $\Delta 7H_2ase_{sup}$  mutant and its absence in the  $\Delta 6H_2ase$  mutant. The nature of the suppressor mutations allowing growth in the other two  $\Delta 6H_2ase_{sup}$  strains was not readily apparent from the genome sequencing data.

**Overexpression of GAPOR in the  $\Delta 6H_2ase$  mutant allows growth without H<sub>2</sub>.** Although the generation of a putative promoter sequence upstream of GAPOR suggests that overexpression of this gene leads to growth, the nature of the mutation could also result in a promoter reading in the opposite direction (see Fig. S1 in the supplemental material). Therefore, instead of engineering the mutation on the chromosome of the  $\Delta 6H_2ase$  mutant to test its efficacy at stimulating growth, we chose to overexpress GAPOR on a replicative vector to recapitulate the effect and avoid possible overexpression of a second operon. GAPOR was placed under control of the *Methanococcus vanneilii* histone promoter on the replicative vector pLW40neo (17) and introduced into the  $\Delta 6H_2ase$  background. The resulting strain displayed moderate growth in the absence of H<sub>2</sub> and robust growth in the presence of H<sub>2</sub>, verifying that either GAPOR or Eha could be used to generate the reduced ferredoxin required for growth (Fig. 4).

**The  $\Delta 7H_2ase_{sup}$  mutant expressing F<sub>420</sub>-reducing hydrogenase can produce substantial amounts of H<sub>2</sub>.** The  $\Delta 7H_2ase_{sup}$  mutant grows more slowly than the wild type on formate, suggesting that reduced ferredoxin is limiting. Reduced ferredoxin limitation of methanogenesis implies that other reduced cofactors that feed into the pathway, such as F<sub>420</sub>H<sub>2</sub>, are present in excess. Wild-type *M. maripaludis* possesses an F<sub>420</sub>-dependent formate:H<sub>2</sub> lyase activity that is catalyzed by Fdh and F<sub>420</sub>-reducing hydrogenase. The wild-type strain grown on formate can accumulate H<sub>2</sub> in the culture headspace to a concentration of 0.16% ± 0.02% of the gas phase at 2 atm pressure (mean ± standard deviation [SD] for three biological replicates) (18, 19). An excess of F<sub>420</sub>H<sub>2</sub> should drive the equilibrium of this activity toward increased H<sub>2</sub> produc-





**FIG 4** Growth of the  $\Delta 6H_2ase$  mutant overexpressing GAPOR in the presence and absence of  $H_2$ . The  $\Delta 6H_2ase$  mutant overexpressing GAPOR grown on formate plus  $H_2$  (black symbols), the  $\Delta 6H_2ase$  mutant overexpressing GAPOR grown on formate alone (gray symbols), and the  $\Delta 6H_2ase$  mutant grown on formate alone (white symbols) were examined.

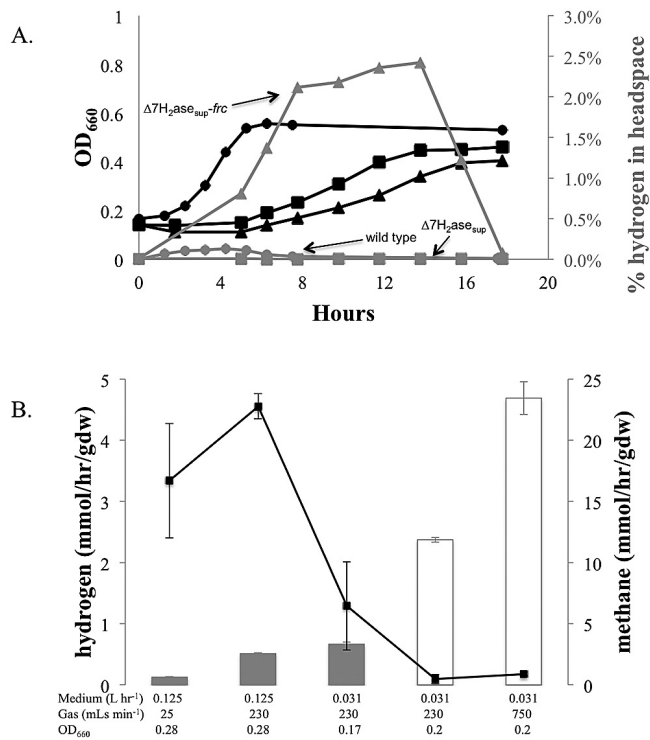
tion. *frc* encoding  $F_{420}$ -reducing hydrogenase was placed on the replicative vector pLW40 (17) and reintroduced into the  $\Delta 7H_2ase_{sup}$  mutant to restore formate: $H_2$  lyase activity. When grown with formate as the only electron donor for methanogenesis, the  $\Delta 7H_2ase_{sup}-frc$  mutant was capable of producing  $H_2$  up to a concentration of  $2.32\% \pm 0.79\%$  (Fig. 5A; see Fig. S2 in the supplemental material). When cultures entered stationary phase,  $H_2$  reuptake occurred, presumably due to depletion of formate and an equilibrium shift back toward  $F_{420}H_2$  production from  $H_2$ . The  $\Delta 7H_2ase_{sup}$  mutant, which lacks formate: $H_2$  lyase activity, was incapable of  $H_2$  production.

We also attempted continuous culture of the  $\Delta 7H_2ase_{sup}-frc$  mutant to assess how additional factors affect the equilibrium of formate: $H_2$  lyase activity. The  $\Delta 7H_2ase_{sup}-frc$  mutant was maintained at a low  $OD_{660}$  under conditions where the medium dilution rate ( $0.125 \text{ liters h}^{-1}$ ) slightly exceeded the growth rate to ensure that the culture was continuously growing. Under these conditions, the  $\Delta 7H_2ase_{sup}-frc$  mutant produced between 100 and  $500 \mu\text{mol } H_2 \text{ gdw}^{-1}$  (gdw stands for grams [dry weight]) (Fig. 5B). The higher production rates were observed when the gas flow rate was increased from 25 to  $230 \text{ ml min}^{-1}$ , demonstrating that increased removal of  $H_2$  results in an equilibrium shift toward even greater  $H_2$  production. When the growth rate was allowed to exceed the medium dilution rate ( $0.031 \text{ liters h}^{-1}$ ),  $H_2$  production was not greatly affected.

As reduced ferredoxin limitation of growth appears to lead to increased  $H_2$  production, we sought to take this to the extreme case of the absence of reduced ferredoxin. Metronidazole, an antibiotic capable of oxidizing ferredoxin (20), was added to the chemostat, and after 1 h,  $H_2$  production was found to have increased 5-fold to  $\sim 2.5 \text{ mmol gdw}^{-1} \text{ h}^{-1}$ . With increased gas flow, this rate approached  $5 \text{ mmol gdw}^{-1} \text{ h}^{-1}$ . Robust  $H_2$  production upon metronidazole addition verifies that limiting reduced ferredoxin leads to an excess of  $F_{420}H_2$ .

## DISCUSSION

**Hydrogenotrophic methanogens have unappreciated metabolic versatility.** Although they do not share pathways of methylotrophic methanogens, hydrogenotrophic methanogens have a



**FIG 5** Growth and  $H_2$  production by the  $\Delta 7H_2ase_{sup}$  mutant expressing  $F_{420}$ -reducing hydrogenase ( $\Delta 7H_2ase_{sup}-frc$ ). (A) Growth on formate (black symbols) and  $H_2$  production (gray symbols) by the wild-type strain MM901 (circles), the  $\Delta 7H_2ase_{sup}$  mutant (squares), and the  $\Delta 7H_2ase_{sup}-frc$  mutant (triangles) in batch culture. Data are from a single representative experiment, but two replicate experiments gave similar results (see Fig. S2 in the supplemental material). (B)  $CH_4$  (black curve) and  $H_2$  production (bars) of the  $\Delta 7H_2ase_{sup}-frc$  mutant in continuous culture. Actively growing cultures (gray bars) and cultures after metronidazole ( $50 \mu\text{g ml}^{-1}$ ) was added to completely oxidize ferredoxin (white bars) are shown. The medium dilution rate, gas flow rate, and culture optical density are shown on the x axis.

different kind of metabolic versatility. We have shown previously that this is due in part to the ability of hydrogenotrophs to use either formate or  $H_2$  as the electron donor to all four reduction steps of methanogenesis (2, 4). In addition, we have shown here that hydrogenotrophs also have versatility in their pathways of ferredoxin reduction for the anaplerotic electron input to methanogenesis. First, our results show clearly that  $H_2$  is not the only possible reductant for this purpose. The  $\Delta 6H_2ase$  mutant grows in the complete absence of  $H_2$  as long as CO is present along with formate. In the case of the  $\Delta 7H_2ase_{sup}$  strain, even CO was not needed, and formate was the sole electron donor.  $H_2$  addition to cultures of the  $\Delta 7H_2ase_{sup}$  mutant grown on formate had no stimulatory effect, confirming that  $H_2$  uptake did not occur (Fig. 3B). CO, a hydrogenase inhibitor, also had no inhibitory effect in the  $\Delta 7H_2ase_{sup}$  background but did in the wild type (14, 15). Additionally, the  $\Delta 7H_2ase_{sup}$  mutant lacked all formate: $H_2$  lyase activity (Fig. 5A). Taken together, these data confirm that all hydrogenase activity had been eliminated. The  $\Delta 6H_2ase$ ,  $\Delta 6H_2ase_{sup}$  and  $\Delta 7H_2ase_{sup}$  strains represent, to our knowledge, the first examples of hydrogenotrophic methanogens capable of growth in the complete absence of  $H_2$ .

**$H_2$ -independent growth occurs via novel electron flow pathways.** Here we have demonstrated two pathways by which  $H_2$  may

be replaced as the anaplerotic electron donor for methanogenesis. Like Eha with H<sub>2</sub>, both pathways reduce ferredoxin which then presumably reduces CO<sub>2</sub> to formyl-MFR. First, CO served this purpose in the  $\Delta 6H_2ase$  and  $\Delta 7H_2ase_{sup}$  strains. To our knowledge, only one other study reported the use of CO for methanogenesis in a hydrogenotrophic methanogen. In that report, CO as the sole substrate supported slow growth of *Methanothermobacter thermautotrophicus* by disproportionation to CO<sub>2</sub> and CH<sub>4</sub>, and an F<sub>420</sub>-reducing carbon monoxide dehydrogenase activity was found (21). This differs from our results in which formate as well as CO was present. In addition, our  $\Delta 6H_2ase$  and  $\Delta 7H_2ase_{sup}$  mutants are capable of producing F<sub>420</sub>H<sub>2</sub> directly from formate, so an F<sub>420</sub>-reducing carbon monoxide dehydrogenase activity is likely of little importance. Instead, the oxidation of CO leads to reduced ferredoxin. CO formation from H<sub>2</sub> and CO<sub>2</sub> with reduced ferredoxin as an intermediate has been observed in cell suspensions of hydrogenotrophic methanogens possessing ACS/CODH (7, 22), and the process we observed here, which depended on ACS/CODH, appears to be the reverse.

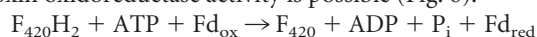
The CO utilization demonstrated here for *M. maripaludis* contrasts with methylotrophic methanogens. First, in methylotrophic methanogens, CO can be converted to methane and CO<sub>2</sub> or to formate and acetate with the concomitant generation of ATP (23, 24). In contrast, 5% CO, as was used in our growth experiments, is insufficient as a stoichiometric electron donor for the amount of growth observed and functioned only anaplerotically. In both kinds of methanogens, CO oxidation results in reduced ferredoxin, but only the methylotrophs are known to use reduced ferredoxin as a stoichiometric source of electrons for methanogenesis. Second, methylotrophic methanogens can carry out methanogenesis solely from acetate by using ACS/CODH to cleave acetyl-CoA. *M. maripaludis* can utilize acetate anabolically (25) and can also use ACS/CODH anabolically for CO<sub>2</sub> fixation to acetyl-CoA (6). Nevertheless, although CO utilization as an anaplerotic stimulant of methanogenesis in *M. maripaludis* occurred via ACS/CODH, *M. maripaludis* is apparently unable to use acetate for methanogenesis, even anaplerotically. Thus, our  $\Delta 6H_2ase$  mutant, which was always grown in the presence of acetate and Casamino Acids, would not grow on formate in the absence of H<sub>2</sub> without a suppressor mutation occurring.

As an additional novel pathway, overexpression of GAPOR could substitute for Eha and H<sub>2</sub>. GAPOR is found throughout the *Archaea* (26–29) and functions in glycolysis, catalyzing the oxidation of G3P to 3-phosphoglycerate (3-PG) with the concomitant reduction of ferredoxin (26, 29). The corresponding gluconeogenic reactions are catalyzed by G3P dehydrogenase (GAPDH), a NADPH-dependent enzyme, and phosphoglycerate kinase (PGK), an ATP-dependent enzyme (Fig. 6). Running both pathways simultaneously would result in:

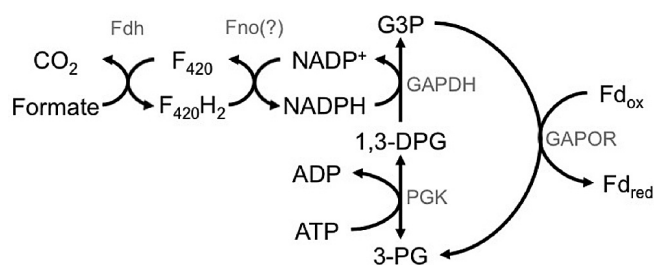


where Fd is ferredoxin, Fd<sub>ox</sub> is oxidized Fd, and Fd<sub>red</sub> is reduced Fd.

*M. maripaludis* also encodes an F<sub>420</sub>H<sub>2</sub>:NADP<sup>+</sup> oxidoreductase (Fno) and the F<sub>420</sub>-dependent Fdh (12, 30). When these activities are taken into account, an ATP-dependent F<sub>420</sub>H<sub>2</sub>:ferredoxin oxidoreductase activity is possible (Fig. 6):



This pathway evidently operates in the  $\Delta 6H_2ase_{sup}$  strains and the  $\Delta 7H_2ase_{sup}$  strain, as well as in the  $\Delta 6H_2ase$  strain with GAPOR overexpressed on a plasmid.



**FIG 6** Glyceraldehyde-3-phosphate:ferredoxin oxidoreductase cycle for ATP-dependent ferredoxin reduction. The GAPOR cycle of *M. maripaludis* as originally described by Park et al. (29) is shown with potential input from F<sub>420</sub>H<sub>2</sub>. Fdh, formate dehydrogenase; Fno, F<sub>420</sub>H<sub>2</sub>:NADP<sup>+</sup> oxidoreductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAPOR, glyceraldehyde-3-phosphate:ferredoxin oxidoreductase; PGK, phosphoglycerate kinase. G3P, glyceraldehyde-3-phosphate; 1,3-DPG, 1,3-diphosphoglycerate; 3-PG, 3-phosphoglycerate.

**Reduced ferredoxin abundance versus inefficient electron transfer.** The CO-dependent and GAPOR-dependent pathways of ferredoxin reduction appear less efficient than Eha in supplying anaplerotic electrons to methanogenesis, since the  $\Delta 6H_2ase$  strain with formate and CO and the  $\Delta 7H_2ase_{sup}$  strain with formate alone grew more slowly than Eha<sup>+</sup> strains with formate and H<sub>2</sub>. Indeed, combining the two pathways by including CO with formate for growth of the  $\Delta 7H_2ase_{sup}$  strain increased growth in an additive manner. The low efficiency of the alternative pathways may be due to low concentrations of reduced ferredoxin produced compared to what can be produced by Eha, or the reduced ferredoxin could be abundant but inefficient at transferring electrons to Fwd. The latter interpretation is consistent with the proposal that the ferredoxin pools for anabolism and catabolism are normally separated in *M. maripaludis* (4), since the anabolic ferredoxin-reducing hydrogenase Ehb and the anaplerotic ferredoxin-reducing Eha reduce ferredoxins that substitute inefficiently for each other (4, 25).

**Robust H<sub>2</sub> production by the  $\Delta 7H_2ase_{sup}$ -frc mutant suggests abundant F<sub>420</sub>H<sub>2</sub>.** A low availability of reduced ferredoxin, limiting methanogenesis and growth, should result in a buildup of F<sub>420</sub>H<sub>2</sub>. The equilibrium between F<sub>420</sub>H<sub>2</sub> and H<sub>2</sub> should then result in significant H<sub>2</sub> production upon reintroducing F<sub>420</sub>-reducing hydrogenase into the  $\Delta 7H_2ase_{sup}$  strain. This proved to be the case. Furthermore, the enhancement of H<sub>2</sub> production by the addition of metronidazole supported the notion that an abundance of F<sub>420</sub>H<sub>2</sub> resulted from a depletion of reduced ferredoxin.

## MATERIALS AND METHODS

**Growth conditions.** All strains were grown as described previously in McCas medium containing 200 mM sodium formate and 200 mM morpholinepropanesulfonic acid (MOPS) (pH 7) buffer (19, 31). H<sub>2</sub>-CO<sub>2</sub> (20:80; 40 lb/in<sup>2</sup>) or N<sub>2</sub>-CO<sub>2</sub> (20:80; 15 lb/in<sup>2</sup>) was added to the culture headspace unless otherwise indicated. Antibiotics (neomycin sulfate [5 mg ml<sup>-1</sup>], puromycin [2.5 μg ml<sup>-1</sup>], or metronidazole [50 μg ml<sup>-1</sup>]) were used where appropriate. For growth with CO in the culture headspace, 50% CO was injected to a final concentration of 5% (vol/vol). For growth curves, triplicate cultures were inoculated with a 10% inoculum, and the optical density at 660 nm (OD<sub>660</sub>) was monitored.

For growth under continuous culture, a modified version of a previously established chemostat system was employed (32–34). The  $\Delta 7H_2ase_{sup}$ -frc mutant was grown under steady-state conditions with 380 mM sodium formate. NaCl was removed to maintain osmotic bal-

TABLE 1 *Methanococcus maripaludis* strains used in this study

| Strain | Description  | Relevant locus   | Reference  |
|--------|--|------------------|------------|
| MM901  | Wild-type <i>M. maripaludis</i> with an in-frame deletion of <i>upt</i>                            | See reference    | 2          |
| MM1284 | $\Delta 6H_2ase$ , MM901 $\Delta vhuAU \Delta vhcA \Delta fruA \Delta frcA \Delta hmd \Delta ehbN$ | See reference    | 4          |
| MM1310 | $\Delta 7H_2ase_{sup}$ , MM1316 $\Delta ehaNO$   | Mmp1461, Mmp1462 | This study |
| MM1315 | $\Delta 6H_2ase$ mutant with a suppressor allowing for growth without $H_2$                        |                  | This study |
| MM1316 | $\Delta 6H_2ase$ mutant with a suppressor allowing for growth without $H_2$                        |                  | This study |
| MM1317 | $\Delta 6H_2ase$ mutant with a suppressor allowing for growth without $H_2$                        |                  | This study |
| MM1318 | $\Delta 6H_2ase$ mutant with a suppressor allowing for growth without $H_2$                        |                  | This study |
| MM1319 | $\Delta 6H_2ase$ mutant with a suppressor allowing for growth without $H_2$                        |                  | This study |
| MM1320 | $\Delta 6H_2ase$ mutant with a suppressor allowing for growth without $H_2$                        |                  | This study |
| MM1327 | MM1284 with a deletion of CO dehydrogenase ( <i>cdh</i> )  | Mmp0983-0985     | This study |
| MM1338 | MM1284 with GAPOR overexpressed on pLW40neo  | Mmp0945          | This study |
| MM1339 | MM1310 with $F_{420}$ -reducing hydrogenase ( <i>frc</i> ) on pLW40                                | Mmp0817-0820     | This study |

ance. Casamino Acids (0.2% [wt/vol]) and ampicillin ( $100 \mu\text{g ml}^{-1}$ ) were included in the growth medium. A gas mixture containing  $\text{Ar}-\text{CO}_2$ -1%  $\text{H}_2\text{S}$  (29:8:3) was used. Medium and gas flow rates are indicated in the text. The pH of steady-state samples was monitored and maintained at 6.95 by the automated addition of 10%  $\text{H}_2\text{SO}_4$ . Agitation in the vessel was maintained at 50 rpm throughout steady-state growth.

For continuous culture, the  $\Delta 7H_2ase$ -*frc* mutant was grown to a maximum  $\text{OD}_{660}$  of 0.7 with a medium dilution rate of  $0.083 \text{ liters h}^{-1}$  and a gas flow rate of  $26 \text{ ml} \cdot \text{min}^{-1}$ . Medium dilution was changed to  $0.125 \text{ liters h}^{-1}$  until the culture  $\text{OD}_{660}$  dropped to 0.28 at which point the first sample was taken. The low  $\text{OD}_{660}$  ensured the culture was constantly growing with excess formate. When the medium dilution rate was changed throughout the course of sampling, culture was given at least 24 h to equilibrate before samples were collected. For changes in the gas flow rate, the gas phase was allowed at least 1 h to equilibrate before sample collection.

**Generation of mutants and plasmids.** Strains used in this study are listed in Table 1. Plasmids and primers can be found in Table S2 in the supplemental material. Strain MM901, a derivative of *M. maripaludis* S2 that lacks uracil phosphoribosyl transferase (*upt*), was used as the wild-type strain (2, 35). Mutants were constructed using methods described in reference 31 and modified in reference 2. Briefly, to generate deletion mutants, a PCR product containing an in-frame deletion of the gene of interest was ligated into the vector pCRuptneo (neo stands for neomycin) (2). This was then transformed into strain MM901. Neomycin sulfate ( $5 \text{ mg ml}^{-1}$ ) was used to select for a merodiploid, and 6-azauracil ( $250 \mu\text{g ml}^{-1}$ ) was used as negative selection to isolate the mutant of interest. To place genes onto a replicative vector, PCR products were cloned directly into pLW40 or pLW40neo and transformed into the strain of interest. Either neomycin sulfate ( $5 \text{ mg ml}^{-1}$ ) or puromycin ( $2.5 \mu\text{g ml}^{-1}$ ) was included as appropriate. Suppressor strains of the  $\Delta 6H_2ase$  mutant capable of growth in the absence of  $\text{H}_2$  were grown in formate medium either with or without the addition of  $100 \mu\text{M}$  or  $1 \text{ mM}$  ammonium 2-(methylthio)ethanesulfonate ( $\text{CH}_3\text{-S-CoM}$ ); however,  $\text{CH}_3\text{-S-CoM}$  was found to have no stimulatory effect on growth and was excluded from all subsequent experiments. All mutants were confirmed by PCR screen, and deletion of *ehaNO* was also confirmed by Southern blotting and Illumina sequencing (see below).

**Genome sequencing.** Genome sequencing was carried out for the  $\Delta 6H_2ase$  mutant (MM1284), the  $\Delta 7H_2ase_{sup}$  mutant (MM1310), and six of the  $\Delta 6H_2ase$  strains that had developed suppressor mutations (MM1315, MM1316, MM1317, MM1318, MM1319, and MM1320).  $\Delta 6H_2ase$  strains were isolated by dilution to extinction before processing. High-molecular-weight DNA for sequencing was extracted using the Qia-gen PureGene kit according to the manufacturer's instructions. Genomes were sequenced as described in reference 36, using unpaired 36-bp reads with the Illumina Genome Analyzer GA IIX according to the manufacturer's instructions (Illumina, San Diego, CA). For each genome, a random-fragment library was constructed using a custom protocol. Briefly,

genomic DNA (gDNA) samples were sheared using a Bioruptor UCD-200 (Diagenode Inc., Denville, NJ), and end repaired using an End-It DNA end repair kit (Epicentre). Repaired fragments were subjected to A tailing using *Taq* DNA polymerase (Roche Inc., USA, Chicago, IL), and custom adaptors ligated to A-tailed fragments using T4 DNA ligase (New England Biolabs, Beverly, MA). Libraries were size selected using automated electrophoresis on a Pippin Prep (Sage Science, Beverly, MA) and assessed for size range and concentration using a Qubit (Invitrogen Inc., Carlsbad, CA) and a Bioanalyzer (Agilent Inc., San Diego, CA).

Raw sequence data were compared directly to the *M. maripaludis* strain S2 reference genome (NCBI reference NC\_005791.1) to identify possible suppressor mutations allowing for  $\text{H}_2$ -independent growth (see Table S1 in the supplemental material). Putative mutations upstream of the gene encoding glyceraldehyde-3-phosphate:ferredoxin oxidoreductase (GAPOR) were identified and verified in  $\Delta 6H_2ase$  and  $\Delta 7H_2ase_{sup}$  mutants with Sanger sequencing (GeneWiz, South Plainfield, NJ).

**$\text{CH}_4$  and  $\text{H}_2$  measurements.** To measure  $\text{H}_2$  from batch culture, 2.5 ml of culture headspace was collected and stored for no longer than 24 h in a 5-ml serum vial preflushed with 100%  $\text{N}_2$ . To measure gas concentration from chemostat-grown cells, gas was collected directly into 5-ml serum vials after flushing with at least 350 ml of chemostat gas out-flow. For batch culture measurements, the headspace contained a pressure of 2 atm.  $\text{H}_2$  and  $\text{CH}_4$  concentrations were measured by gas chromatography as described previously (4). To completely oxidize ferredoxin, metronidazole ( $50 \mu\text{g ml}^{-1}$ ) was used. Upon the addition of metronidazole, gas flow through the chemostat vessel was allowed to equilibrate for 1 h prior to collection and analysis. Rates of  $\text{H}_2$  production were calculated assuming a liter of cell material at an  $\text{OD}_{660}$  of 1.0 yields 0.34 gram (dry weight) (18).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00062-13/-/DCSupplemental>.

Figure S1, PDF file, 1 MB.

Figure S2, PDF file, 0.1 MB.

TABLE S1, PDF file, 0.1 MB.

TABLE S2, PDF file, 0.1 MB.

## ACKNOWLEDGMENTS

We thank David Stahl for use of the gas chromatograph for  $\text{H}_2$  measurements.

This work was supported by grant DE-FG02-05ER15709 from the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Science, U.S. Department of Energy. K.C.C. was supported in part by the Public Health Service National Research Service award T32 GM07270 from the National Institute of General Medical Sciences.



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