H₂-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. H₂ oxidation by the hydrogenase Eha provides these electrons, consistent with an H₂ 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 H₂-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 F₄₂₀ should be abundant. Indeed, when F₄₂₀-reducing hydrogenase was reintroduced into the hydrogenase-free mutant, the equilibrium of H₂ production via an F₄₂₀-dependent formate:H₂ lyase activity shifted markedly toward H₂ compared to the wild type.

IMPORTANCE  Hydrogenotrophic methanogens are thought to require H₂ as a substrate for growth and methanogenesis. Here we show alternative pathways in methanogenic metabolism that alleviate this H₂ requirement and demonstrate, for the first time, a hydrogenotrophic methanogen that is capable of growth in the complete absence of H₂. The demonstration of alternative pathways in methanogenic metabolism suggests that this important group of organisms is metabolically more versatile than previously thought.

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 H₂ and CO₂, acetate, methyl compounds, such as methanol and methylamines, and CO. Hydrogenotrophic methanogenic enzymes are more restricted, using H₂, formate, or for a few species, certain methylamines, and CO. Hydrogenotrophic methanogens are distinct from methylotrophic methanogens in their use of electron bifurcation as an energy-conserving step in methanogenesis from CO₂ (1–4). Two pairs of electrons enter the methanogenic pathway at a protein complex, the 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 (CH₃-S-CoM) to CH₄. The other pair of electrons is used in an endergonic reaction by Fwd to reduce CO₂ 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, CO₂-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 CO₂ 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 CO₂ fixation and not catabolically for acetate utilization.

Either H₂ or formate donates electrons to all four reduction
steps in methanogenesis from CO₂. 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 F₄₂₀ as the direct electron donor, and H₂ or formate directly reduces F₄₂₀ via an F₄₂₀-reducing hydrogenase or Fdh. Hence, H₂ or formate alone can provide the stoichiometric reducing requirements for CO₂ 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 ΔH₂ₐse, was unable to use H₂ as the stoichiometric electron donor for methanogenesis. The mutant also lacked a formate-H₂ lyase activity found in the wild-type strain. As a result of these two deficiencies, the ΔH₂ₐse mutant required both formate and H₂ for growth and methanogenesis. While formate was needed stoichiometrically for the four reduction steps of methanogenesis, H₂ was required only in small amounts sufficient to support the anaplerotic needs of methanogenesis. In the ΔH₂ₐse 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 CO₂ to formyl-MFR. Hence, during growth on H₂ 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, H₂ stimulated methanogenesis from formate in cell suspensions (4).

Here we show that the essentiality of H₂ 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 ΔH₂ₐse mutant that expresses one of these pathways, thus eliminating all H₂ metabolism in a hydrogenotrophic methanogen. By reintroducing a single hydrogenase—the F₄₂₀-reducing hydrogenase—into the hydrogenase-free strain and observing its effects in isolation from other H₂-metabolizing pathways, we find that substantial electron flux is diverted to H₂ production. These findings show that hydrogenotrophic methanogens are metabolically more versatile than previously thought.

RESULTS
CO stimulates growth of the ΔH₂ₐse mutant in the absence of H₂. 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 CO₂ to formyl-MFR (4). However, other pathways of ferredoxin reduction/oxidation exist in methanogenic Archaea. The ACS/CODH enzyme complex converts CO₂ 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 CO₂, with the production of reduced ferredoxin—can stimulate methanogenesis in an H₂-independent manner, the ΔH₂ₐse mutant was grown on formate medium with or without the addition of H₂ or CO to the culture headspace. As expected, H₂ promoted robust growth. In addition, CO promoted growth in the absence of H₂ (Fig. 1). Growth rates were much lower with CO in place of H₂, suggesting that ferredoxin reduction by Eha is preferred. Growth promoted by CO was directly attributable to ACS/CODH, since when the CO₂-reducing subunits were genetically eliminated (ΔH₂ₐse-Δcdh), 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 ΔH₂ₐse mutant on formate alone. The growth of the ΔH₂ₐse 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. CH₃-S-CoM addition to cell extracts stimulates methanogenesis (8, 9), and methanogenic Archaea are capable of CH₃-S-CoM uptake via a poorly characterized and very inefficient activity (10, 11). Therefore, we tried to grow the ΔH₂ₐse mutant on formate in the absence of H₂ in the presence and absence of CH₃-S-CoM. Surprisingly, regardless of the presence or absence of CH₃-S-CoM, after prolonged incubation of nine independent cultures, all nine grew (Fig. 2). Upon transfer to new medium, each strain

FIG 1 Growth of the ΔH₂ₐse mutant with CO and formate. The ΔH₂ₐse mutant (solid black lines) and the ΔH₂ₐse Δcdh mutant (broken lines) were grown with formate plus H₂ (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.

FIG 2 Generation of suppressor strains of the ΔH₂ₐse mutant capable of H₂-independent growth. The ΔH₂ₐse mutant was grown in formate-containing medium without H₂ or CO. The medium contained 1,000 M (black symbols), 100 µM (gray symbols), or 0 µM (white symbols) CH₃-S-CoM (three replicates each); however, CH₃-S-CoM had no stimulatory effect on growth. Each curve represents growth in a single tube.
tested routinely grew to maximum optical density at 660 nm (OD$_{660}$) within 24 h. These results suggested that independent suppressor mutations ($\Delta$6H$_2$ase$_{sup}$) were generated that allowed growth for formate alone. Suppressor strains that were generated in the presence of CH$_3$-S-CoM grew well in its absence. Hence, although CH$_3$-S-CoM failed to stimulate growth, mutants developed that had a novel mechanism to anaplerotically stimulate methanogenesis.

**Deletion of eha is possible due to a suppressor mutation.** A suppressor mutation that allows growth of $\Delta$6H$_2$ase$_{sup}$ on formate alone could have produced a novel H$_2$ production activity, or it could have generated a new ferredoxin reducing activity that is independent of H$_2$. If a novel H$_2$ 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$6H$_2$ase$_{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$7H$_2$ase$_{sup}$) lacks the genes encoding the active sites for all three biological replicates (18, 19). An excess of F$_{420}$H$_2$ should lead to the production of reduced ferredoxin. The growth of the $\Delta$7H$_2$ase$_{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$7H$_2$ase$_{sup}$ mutant grew faster with formate and CO than either the $\Delta$7H$_2$ase$_{sup}$ mutant grown with formate alone or the $\Delta$6H$_2$ase$_{mutant}$ grown with formate and CO.

**Genome sequencing reveals a suppressor mutation allowing for growth of the $\Delta$6H$_2$ase$_{sup}$ and $\Delta$7H$_2$ase$_{sup}$ mutants on formate alone.** To determine the genetic background that allowed growth of the $\Delta$6H$_2$ase$_{sup}$ and $\Delta$7H$_2$ase$_{sup}$ mutants on formate in the absence of H$_2$ or CO, we performed Illumina sequencing on the $\Delta$7H$_2$ase$_{sup}$ mutant and six of the isolated suppressors and compared the sequence to the $\Delta$6H$_2$ase parent (see Table S1 in the supplemental material). Four of six of the $\Delta$6H$_2$ase$_{sup}$ mutants, as well as the $\Delta$7H$_2$ase$_{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 TTAT(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$_2$-independent growth. Sanger sequencing confirmed the presence of this mutation in the $\Delta$7H$_2$ase$_{sup}$ mutant and its absence in the $\Delta$6H$_2$ase mutant. The nature of the suppressor mutations allowing growth in the other two $\Delta$6H$_2$ase$_{sup}$ strains was not readily apparent from the genome sequencing data.

**Overexpression of GAPOR in the $\Delta$6H$_2$ase mutant allows growth without H$_2$.** 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$6H$_2$ase 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$6H$_2$ase background. The resulting strain displayed moderate growth in the absence of H$_2$ and robust growth in the presence of H$_2$, verifying that either GAPOR or Eha could be used to generate the reduced ferredoxin required for growth (Fig. 4).

**The $\Delta$7H$_2$ase$_{sup}$ mutant expressing F$_{420}$-reducing hydrogenase can produce substantial amounts of H$_2$.** The $\Delta$7H$_2$ase$_{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$_{420}$H$_2$, are present in excess. Wild-type M. maripaludis possesses an F$_{420}$-dependent formate:H$_2$ lyase activity that is catalyzed by Fdh and F$_{420}$-reducing hydrogenase. The wild-type strain grown on formate can accumulate H$_2$ 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$_{420}$H$_2$ should drive the equilibrium of this activity toward increased H$_2$ produc-
tion. frc encoding F420-reducing hydrogenase was placed on the replicative vector pLW40 (17) and reintroduced into the ΔH2ase mutant to restore formate:H2 lyase activity. When grown with formate as the only electron donor for methanogenesis, the ΔH2ase mutant was capable of producing H2 up to a concentration of 2.32% ± 0.79% (Fig. 5A; see Fig. S2 in the supplemental material). When cultures entered stationary phase, H2 reuptake occurred, presumably due to depletion of formate and an equilibrium shift back toward F420H2 production from H2. The ΔH2ase mutant, which lacks formate:H2 lyase activity, was incapable of H2 production.

We also attempted continuous culture of the ΔH2ase sup mutant to assess how additional factors affect the equilibrium of formate:H2 lyase activity. The ΔH2ase sup frc mutant was maintained at a low OD660 under conditions where the medium dilution rate (0.125 liters h–1) slightly exceeded the growth rate to ensure that the culture was continuously growing. Under these conditions, the ΔH2ase sup frc mutant produced between 100 and 230 ml min–1, demonstrating that increased removal of H2 results in an equilibrium shift toward F420H2 production from H2.

As reduced ferredoxin limitation of growth appears to lead to increased H2 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, H2 production was found to have increased 5-fold to ~2.5 mmol gdw–1 h–1. With increased gas flow, this rate approached 5 mmol gdw–1 h–1. Robust H2 production upon metronidazole addition verifies that limiting reduced ferredoxin leads to an excess of F420H2.

**DISCUSSION**

Hydrogenotrophic methanogens have unappreciated metabolic versatility. Although they do not share pathways of methylotrophic methanogens, hydrogenotrophic methanogens have a 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 H2 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 H2 is not the only possible reductant for this purpose. The ΔH2ase mutant grows in the complete absence of H2 as long as CO is present along with formate. In the case of the ΔH2ase sup strain, even CO was not needed, and formate was the sole electron donor. H2 addition to cultures of the ΔH2ase sup frc mutant grown on formate had no stimulatory effect, confirming that H2 uptake did not occur (Fig. 3B). CO, a hydrogenase inhibitor, also had no inhibitory effect in the ΔH2ase sup background but did in the wild type (14, 15). Additionally, the ΔH2ase sup mutant lacked all formate:H2 lyase activity (Fig. 5A). Taken together, these data confirm that all hydrogenase activity had been eliminated. The ΔH2ase, ΔH2ase sup, and ΔH2ase sup strains represent, to our knowledge, the first examples of hydrogenotrophic methanogens capable of growth in the complete absence of H2.

H2-independent growth occurs via novel electron flow pathways. Here we have demonstrated two pathways by which H2 may
be replaced as the anaplerotic electron donor for methanogenesis. Like Eha with H₂, both pathways reduce ferredoxin which then presumably reduces CO₂ to formyl-MFR. First, CO served this purpose in the ΔH₄₅αse and ΔH₄₅αse⁺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 CO₂ and CH₄, and an F₄₂₀-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 ΔH₄₅αse and ΔH₄₅αse⁺sup mutants are capable of producing F₄₂₀H₂ directly from formate, so an F₄₂₀-reducing carbon monoxide dehydrogenase activity is likely of little importance. Instead, the oxidation of CO leads to reduced ferredoxin. CO formation from H₂ and CO₂ 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 CO₂ 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 methyloths are known to use reduced ferredoxin as a stoichiometric source of electrons for methanogenesis. Second, methyloths 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 CO₂ 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 ΔH₄₅αse mutant, which was always grown in the presence of acetate and Casamino Acids, would not grow on formate in the absence of H₂ without a suppressor mutation occurring.

As an additional novel pathway, overexpression of GAPOR could substitute for Eha and H₂. 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:

\[ \text{NADPH} + \text{ATP} + \text{Fd}_{\text{ox}} \rightarrow \text{NADP}^+ + \text{ADP} + \text{P}_i + \text{Fd}_{\text{red}} \]

where Fd is ferredoxin, Fd_{ox} is oxidized Fd, and Fd_{red} is reduced Fd.

*M. maripaludis* also encodes an F₄₂₀H₂-NADP⁺ oxidoreductase (Fno) and the F₁₂₀-dependent Fdh (12, 30). When these activities are taken into account, an ATP-dependent F₄₂₀H₂-ferredoxin oxidoreductase activity is possible (Fig. 6):

\[ \text{F₄₂₀H₂} + \text{ATP} + \text{Fd}_{\text{ox}} \rightarrow \text{F₄₂₀} + \text{ADP} + \text{P}_i + \text{Fd}_{\text{red}} \]

This pathway evidently operates in the ΔH₄₅αse⁺sup strains and the ΔH₄₅αse⁺mutant strain, as well as in the ΔH₄₅αse strain with GAPOR overexpressed on a plasmid.

**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 ΔH₄₅αse strain with formate and CO and the ΔH₄₅αse⁺mutant strain alone grew more slowly than Eha⁺ strains with formate and H₂. Indeed, combining the two pathways by including CO with formate for growth of the ΔH₄₅αse⁺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 H₂ production by the ΔH₄₅αse⁺sup-frc mutant suggests abundant F₄₂₀H₂.** A low availability of reduced ferredoxin, limiting methanogenesis and growth, should result in a buildup of F₄₂₀H₂. The equilibrium between F₄₂₀H₂ and H₂ should then result in significant H₂ production upon reintroducing F₄₂₀⁻ reducing hydrogenase into the ΔH₄₅αse⁺sup strain. This proved to be the case. Furthermore, the enhancement of H₂ production by the addition of metronidazole supported the notion that an abundance of F₄₂₀H₂ 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). H₂-CO₂ (20:80; 40 lb/in²) or N₂-CO₂ (20:80; 15 lb/in²) was added to the culture headspace unless otherwise indicated. Antibiotics (neomycin sulfate [5 mg ml⁻¹], puromycin [2.5 µg ml⁻¹], or metronidazole [50 µg ml⁻¹]) 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 (OD₆₆₀) was monitored.

For growth under continuous culture, a modified version of a previously established chemostat system was employed (32–34). The ΔH₄₅αse⁺sup-frc mutant was grown under steady-state conditions with 380 mM sodium formate. NaCl was removed to maintain osmotic bal-

![FIG 6 Glyceraldelyde-3-phosphate:ferredoxin oxidoreductase cycle for ATP-dependent ferredoxin reduction.](mbio.asm.org)
The automated addition of 10% H2SO4. Agitation in the vessel was main-
The pH of steady-state samples was monitored and maintained at 6.95 by
H2S (29:8:3) was used. Medium and gas flow rates are indicated in the text.
The maximum OD660 of 0.7 with a medium dilution rate of 0.083 liters h
interest. To place genes onto a replicative vector, PCR products were
ammonium 2-(methylthio)ethanesulfonate (CH3-S-CoM); however,
tained at 50 rpm throughout steady-state growth.
For continuous culture, the ΔH2ase-frc mutant was grown to a max-
imum OD660 of 0.7 with a medium dilution rate of 0.083 liters h -1 and a
gas flow rate of 26 ml - min -1. Medium dilution was changed to 0.125 li-
ters h -1 until the culture OD660 dropped to 0.28 at which point the first
sample was taken. The low OD660 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
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 S1. 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 pCRuppneo (neo stands for neomycin)
(2). This was then transformed into strain MM901. Neomycin sulfate
(5 mg ml -1) was used to select for a merodiploid, and 6-azauracil
1) was used as negative selection to isolate the mutant of
M. maripaludis. To measure H2 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 prefilled with 100% N2. To measure gas
collection. Rates of H2 production were calcu-
ized assuming a liter of cell material at an OD660 of 1.0 yields 0.34 gram
1 h prior to collection and analysis. Rates of H2 production were calcu-
ulated assuming a liter of cell material at an OD660 of 1.0 yields 0.34 gram
(dry weight) (18).

**CH4 and H2 measurements.** To measure H2 from batch culture,
and a Bioanalyzer (Agilent Inc., San Diego, CA).

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

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ences.

### TABLE 1 Methanococcus maripaludis strains used in this study

| Strain       | Description                                      | Relevant locus | Reference |
|--------------|--------------------------------------------------|----------------|-----------|
| MM901        | Wild-type *M. maripaludis* with an in-frame deletion of *upt* | See reference | 2         |
| MM1284       | ΔH2ase, MM901 Δalu/ΔH2al ΔAfrΔ2 ΔfrcAΔfrcAΔflmd ΔhblN | See reference | 4         |
| MM1310       | ΔH2ase sup, MM1316 ΔhblNO                         | Mmp1461, Mmp1462 | This study |
| MM1315       | ΔH2ase sup mutant with a suppressor allowing for growth without H2 | This study     |           |
| MM1316       | ΔH2ase sup mutant with a suppressor allowing for growth without H2 | This study     |           |
| MM1317       | ΔH2ase sup mutant with a suppressor allowing for growth without H2 | This study     |           |
| MM1318       | ΔH2ase sup mutant with a suppressor allowing for growth without H2 | This study     |           |
| MM1319       | ΔH2ase sup mutant with a suppressor allowing for growth without H2 | This study     |           |
| MM1320       | ΔH2ase sup mutant with a suppressor allowing for growth without H2 | This study     |           |
| MM1327       | MM1284 with a deletion of CO dehydrogenase (cduh) | Mmp0983-0985   | This study |
| MM1328       | MM1284 with GAPOR overexpressed on pLW40neo       | Mmp0945       | This study |
| MM1339       | MM1310 with F420-reducing hydrogenase (frc) on pLW40 | Mmp0817-0820   | This study |

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REFERENCES

1. Thauer RK, Kaster AK, Seedorf H, Buckel W, Hedderich R. 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. Nat. Rev. Microbiol. 6:579–591.

2. Costa KC, Wong PM, Wang T, Lie TJ, Dowskiath JA, Swanson I, Burn JA, Hackett M, Leigh JA. 2010. Protein complexing in a methanogen suggests electron bifurcation and electron delivery from formate to heterodisulfide reductase. Proc. Natl. Acad. Sci. U. S. A. 107:11050–11055.

3. Kaster AK, Moll J, Parey K, Thauer RK. 2011. Coupling of ferredoxin and heterodisulfide reduction via electron bifurcation in hydrogennonrophic methanogenic archaea. Proc. Natl. Acad. Sci. U. S. A. 108: 2981–2986.

4. Lie TJ, Costa KC, Lupa B, Korpole S, Whitman WB, Leigh JA. 2012. Essential anaplerotic role for the energy-converting hydrogenase Eha in hydrogenotrophic methanogenesis. Proc. Natl. Acad. Sci. U. S. A. 109:15473–15478.

5. Thauer RK. 2012. The Wolfe cycle comes full circle. Proc. Natl. Acad. Sci. U. S. A. 109:15084–15085.

6. Berg IA, Kockelkorn D, Ramos-Vera WH, Say RF, Zarzycki J, Hugler M, Alber BE, Fuchs G. 2010. Auto-otrophic carbon fixation in archaea. Nat. Rev. Microbiol. 8:447–460.

7. Eikmanns B, Fuchs G, Thauer RK. 1985. Formation of carbon monoxide from CO₂ and H₂ by Methanobacterium thermautotrophicum. Eur. J. Biochem. 146:149–154.

8. Bobik TA, Wolfe RS. 1989. Activation of formylmethanofuran synthase in cell extracts of Methanothermarche thermotrophicum. J. Bacteriol. 171:1423–1427.

9. Gunsalus RP, Wolfe RS. 1977. Stimulation of CO₂ reduction to methane by methylcoenzyme M in extracts Methanothermarche. Biophys. Res. Commun. 76:790–795.

10. Dybas M, Konisky J. 1989. Transport of coenzyme M (2-mercaptoethanesulfonic acid) and methylcoenzyme M [(2-methylthio)ethanesulfonic acid] in Methanothermarche voltae: identification of specific and general uptake systems. J. Bacteriol. 171:5866–5871.

11. Balch WE, Wolfe RS. 1979. Transport of coenzyme M (2-mercaptoethanesulfonic acid) in Methanothermarche ruminitum. J. Bacteriol. 137:264–273.

12. Hendrickson EL, Kaul R, Zhou Y, Bovee D, Chapman P, Chung J, Conway de Macario E, Dowskiath JA, Gillet W, Graham DE, Hackett M, Haydock AK, Kang A, Land ML, Levy R, Lie TJ, Major TA, Moore BC, Porat I, Palmeiri A, Rejtar T, Andrews VP, Reichlen M, Hill K, Morar JJ, Karger BL, Ferry JG. 2006. An unconventional pathway for reduction of CO₂ to methane in co-grown Methanosarcina acetivorans revealed by proteomics. Proc. Natl. Acad. Sci. U. S. A. 103:17921–17926.

13. Porat I, Kim W, Hendrickson EL, Xia Q, Zhang Y, Wang T, Taub F, Moore BC, Anderson IJ, Hackett M, Leigh JA, Whitman WB. 2006. Disruption of the operon encoding Ehb hydrogenase limits anabolic CO₂ assimilation in the archaeon Methanothermarche maripaludis. J. Bacteriol. 188: 1373–1380.

14. Mukund S, Adams MW. 1995. Glyceraldehyde-3-phosphate ferredoxin oxidoreductase, a novel tungsten-containing enzyme with a potential glycolytic role in the hyperthermophilic archaeon Pyrococcus furiosus. J. Biol. Chem. 270:8389–8392.

15. Selig M, Xavier KB, Santos H, Schönheit P. 1997. Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in hyperthermophilic archaea and the bacterium Thermotoga. Arch. Microbiol. 167:217–232.

16. van der Oost J, Schut G, Kengen SW, Hagen WR, Thomm M, de Vos WM. 1998. The ferredoxin-dependent conversion of glyceraldehyde-3-phosphate in the hyperthermophilic archaeon Pyrococcus furiosus represents a novel site of glycolytic regulation. J. Biol. Chem. 273:28149–28154.

17. Park MO, Mizutani T, Jones PR. 2007. Glyceraldehyde-3-phosphate ferredoxin oxidoreductase from Methanothermarche maripaludis. J. Bacteriol. 189:7281–7289.

18. Berk H, Thauer RK. 1998. FADH₂: NADP oxidoreductase from Methanothermarche thermotrophicum: identification of the encoding gene via functional overexpression in Escherichia coli. FEBS Lett. 458:124–126.

19. Moore BC, Leigh JA. 2005. Markerless mutagenesis in Methanothermarche maripaludis demonstrates roles for alanine dehydrogenase, alanine racemase, and alanine permease. J. Bacteriol. 187:972–979.

20. Haydock AK, Porat I, Whitman WB, Leigh JA. 2004. Continuous culture of Methanothermarche maripaludis under defined nutrient conditions. FEMS Microbiol. Lett. 238:85–91.

21. Hendrickson EL, Haydock AK, Moore BC, Whitman WB, Leigh JA. 2007. Functionally distinct genes regulated by hydrogen limitation and growth rate in methanogenic Archaea. Proc. Natl. Acad. Sci. U. S. A. 104:8930–8934.

22. Leigh JA. 2011. Growth of methanogens under defined hydrogen conditions. Methods Enzymol. 494:111–118.

23. Whitman WB, Shieh J, Sohn S, Caras DS, Premachandran U. 1986. Isolation and characterization of 22 mesophilic methanococci. Syst. Appl. Microbiol. 7:235–240.

24. Hayden HS, Lim R, Brittnachner MJ, Sima EH, Ramage ER, Fong C, Wu Z, Crist E, Chang J, Zhou Y, Radey M, Rohmer I, Haugen E, Gillett W, Wuthiekanun V, Peacock SJ, Kaul R, Miller SI, Manoil C, Jacobs MA. 2012. Evolution of Burkholderia pseudomallei in recurrent melioidosis. PLoS One 7:e56507. dx.doi.org/10.1371/journal.pone.0036507.