Essential anaplerotic role for the energy-converting hydrogenase Eha in hydrogenotrophic methanogenesis

Thomas J. Lie[a,1], Kyle C. Costa[a,1], Boguslaw Lupa[b], Suresh Korpole[c], William B. Whitman[b], and John A. Leigh[a,2]

[a]Department of Microbiology, University of Washington, Seattle WA 98195; [b]Department of Microbiology, University of Georgia, Athens, GA 30602; and [c]Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Sector 39A, Chandigarh, India

Despite decades of study, electron flow and energy conservation in methanogenic Archaea are still not thoroughly understood. For methanogens without cytochromes, flavin-based electron bifurcation has been proposed as an essential energy-conserving mechanism that couples exergonic and endergonic reactions of methanogenesis. However, an alternative hypothesis posits that the energy-converting hydrogenase Eha provides a chemiosmosis-driven electron input to the endergonic reaction. In vivo evidence for both hypotheses is incomplete. By genetically eliminating all nonessential pathways of H_2 metabolism in the model methanogen *Methanococcus maripaludis* and using formate as an additional electron donor, we isolate electron flow for methanogenesis from flux through Eha. We find that Eha does not function stoichiometrically for methanogenesis, implying that electron bifurcation must operate in vivo. We show that Eha is nevertheless essential, and a substoichiometric requirement for H_2 suggests that its role is anaplerotic. Indeed, H_2 via Eha stimulates methanogenesis from formate when intermediates are not otherwise replenished. These results fit the model for electron bifurcation, which renders the methanogenic pathway cyclic, and as such requires the replenishment of intermediates. Defining a role for Eha and verifying electron bifurcation provide a complete model of methanogenesis where all necessary electron inputs are accounted for.

hydrogenotrophs | H_2:F_{420} oxidoreductase | ferredoxin | formate dehydrogenase

Methanogenesis is an anaerobic respiration carried out by a phylogenetically related group of Archaea within the phylum Euryarchaeota. Methanogens are divided into two metabolic types, those without and those with cytochromes (1). Methanogens without cytochromes use H_2 as an electron donor and are termed hydrogenotrophic. Some species can substitute H_2 with formate, and a few can use secondary alcohols. CO_2 is the electron acceptor and is reduced to methane. Methanogens with cytochromes reduce certain methyl compounds or the methyl carbon of acetate to methane and are called methylotrophic. Many can also use H_2 and CO_2, as can hydrogenotrophic methanogens.

Although the pathways of methanogenesis have long been known, an understanding of energy conservation has been slower to emerge. Methanogens with and without cytochromes both export Na^+ when a methyl group is transferred from the carrier tetrahydromethanopterin (H_4MPT) to coenzyme M (CoM) (Fig. 1). The Na^+ gradient across the membrane is used directly for ATP synthesis or is converted by an antiporter to a proton gradient. However, for methanogenesis from CO_2, the initial reduction of CO_2 to a formyl group attached to methanofuran (MFR) is endergonic. How energy is provided to drive this reaction is not well understood. Methanogens with and without cytochromes have membrane-associated energy-converting hydrogenases that couple the reduction of low-potential ferredoxins (Fd) to a chemiosmotic membrane gradient (2). If such a Fd donates electrons for CO_2 reduction, an energy-converting hydrogenase is the conduit of energy for this reaction. Indeed, for methanogens with cytochromes, an energy-converting hydrogenase is required for CO_2 reduction (3). However, the energy requirement for the first step in the pathway results in a need for additional energy conservation. This could be provided by the final step of methanogenesis, which involves an exergonic reduction of a heterodisulfide of two methanogenic cofactors (CoM-S-S-CoB) by heterodisulfide reductase (Hdr). Methanogens with cytochromes harvest the energy yielded in heterodisulfide reduction with a proton-exporting electron transport chain. However, methanogens without cytochromes lack this electron transport chain and an alternative explanation is required.

Here we present results supporting an emerging view of methanogenesis without cytochromes. The emerging model diverges from the conventional picture of a linear pathway of CO_2 reduction to methane. Instead, a cyclical pathway involving electron bifurcation has been proposed (1) (Fig. 1). The reductions of the heterodisulfide and CO_2 are coupled in the flavin-containing enzyme complex centered around Hdr. For each pair of electrons accepted, one electron is used for the exergonic reduction of CoM-S-S-CoB, and one is used to reduce a low-potential ferredoxin that in turn donates electrons for the reduction of CO_2 to formyl-MFR. Hence, electron bifurcation, a nonchemiosmotic form of energy conservation, couples the exergonic and endergonic steps of methanogenesis and allows for the net availability of chemiosmotic energy for ATP synthesis. The electron bifurcation model renders methanogenesis a cyclic process, in which late steps are coupled by electron flow to the initial step, and explains why in cell extracts, CH_4 production from CO_2 requires an input of C-1 intermediates (4). Electron bifurcation is supported by experiments with whole cells (5), with purified enzymes (6), and by the characterization of an enzyme complex in which it could take place (7). However, these studies do not explain the presence in most methanogens without cytochromes of the energy-converting hydrogenase Eha that is apparently linked to the first step (2). Electron flux from this hydrogenase would appear to compete with flux from electron bifurcation as well as to consume chemiosmotic energy, leaving a deficit for ATP synthesis.

Whatever the correct model for energy conservation, it likely centers around reactions that reduce low-potential ferredoxins. Three such reactions are proposed to occur in methanogens without cytochromes. Two of these reactions are those mentioned above, the concomitant reduction of Fd and CoM-S-S-CoB that occurs in electron bifurcation, and the H_2-dependent reduction of Fd by the energy-converting hydrogenase Eha, both of which are proposed to lead to the endergonic reduction of CO_2 to formyl-MFR. A third such reaction, which reduces a Fd with another energy-converting hydrogenase, Ehb, functions in anabolic CO_2 fixation reactions and does not appear to be involved in methanogenesis (8, 9).
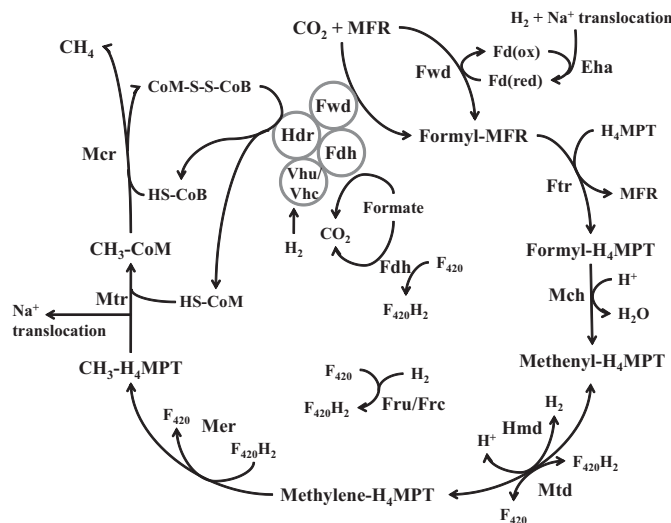
Fig. 1. The methanogenic pathway. Eha, energy-converting hydrogenase A; Fdh, formate dehydrogenase; Fru and Frc, F_{420}-reducing hydrogenases; Ftr, formyl-MFR:H_4MPT formyltransferase; Fwd, formyl-MFR dehydrogenase; Hdr, heterodisulfide reductase; Hmd, H_2-dependent methylene-H_4MPT dehydrogenase; Mch, methenyl-H_4MPT cyclohydrolase; Mcr, methyl-CoM reductase; Mer, methylene-H_4MPT reductase; Mtd, F_{420}-dependent methylene-H_4MPT dehydrogenase; Mtr, methyl-H_4MPT-CoM methyltransferase; Vhu and Vhc, F_{420}-nonreducing (Hdr-associated) hydrogenases.

Here we present an analysis of electron flow in methanogens without cytochromes, focusing on the role of H_2 when formate is the electron donor for methanogenesis. We show that there are two pools of electrons that are distinguished by their substrate origins, their carriers, and their functions. One pool of electrons feeds into methanogenesis via coenzyme F_{420} as well as directly to Hdr from electron-donating growth substrates. Surprisingly, these electrons need not come from H_2, even in hydrogenotrophic methanogens, but instead can come directly from formate. Another pool of electrons supports critical biosynthetic or anaplerotic steps, are carried by low-potential ferredoxins, and come only from H_2. We show that only one hydrogenase, Eha, is the essential conduit of electrons from H_2 and that Eha supports methanogenesis, but it does so in an anaplerotic and not a stoichiometric manner. Eha is needed only to replenish intermediates that are removed from the methanogenesis cycle by diversion to biosynthetic pathways, dilution of intermediates due to growth, or imperfect coupling in electron bifurcation as proposed previously (1). Electron bifurcation still accounts for the stoichiometric flow of electrons for methanogenesis. Our results therefore support the electron bifurcation model in vivo as well as demonstrating the function of Eha.

Results

Identification of an Additional H_2:F_{420} Oxidoreductase Activity and Demonstration of a H_2 Requirement for Growth. Our initial question was whether H_2 is a necessary substrate or intermediate for growth of hydrogenotrophic methanogens. *Methanococcus maripaludis* was an ideal species for addressing this question because it can substitute formate for H_2 and mutations are easily generated (10). In the conventional view, during growth on formate, H_2 generated from formate serves as the electron donor. Indeed, H_2 is generated from formate and recycled in a poorly understood manner (5, 11). However, if there is no direct requirement for H_2 in methanogenesis (5, 7), most of the hydrogenases encoded in the genome ought to be dispensable during growth on formate. The Hdr-associated hydrogenases (Vhu and Vhc) (Fig. 1), which provide electrons to the last reductive step of methanogenesis and

potentially the first step via electron bifurcation (1, 6, 7), can be substituted by formate dehydrogenase (Fdh) during growth on formate (7). The F_{420}-reducing hydrogenases (Fru and Frc) generate $F_{420}H_2$ for the second and third reductive steps of methanogenesis, but the Fdh is also F_{420} reducing (12, 13). The hydrogenase Hmd catalyzes the second reductive step directly with H_2, but its function is redundant with Mtd, which uses reduced F_{420} for the same purpose (11). Finally, the anabolic energy-converting hydrogenase Ehb is nonessential in the presence of fixed carbon and is not required for methanogenesis (8, 9). Only Eha remains as possibly essential.

Based on the above considerations, we expected that H_2 would not be needed as an intermediate for methanogenesis from formate. Indeed, experiments with cell suspensions have already shown that rates of methanogenesis can substantially exceed rates of H_2 production from formate (5). As a further test, our approach here was to genetically remove formate-hydrogen lyase activity, so that H_2 would not be produced from formate. If growth still occurred on formate without added H_2, then H_2 was not a required intermediate. Because Fdh is F_{420} reducing, removal of formate-hydrogen lyase activity amounts to the removal of F_{420}:H_2 oxidoreductase activity. Two such activities are known, the direct Fru or Frc activity and the Hmd-Mtd cycle (Fig. 1) (11). Therefore, deletion of *fru*, *frc*, and *hmd* should eliminate both modes of formate-hydrogen lyase activities. However, cell suspensions of a $\Delta fru\Delta frc\Delta hmd$ mutant (MM1290, henceforth designated $\Delta 3H_2$ase) still produced substantial H_2 from formate (Fig. 2). Furthermore, the mutant grew not only on formate as predicted, but also on H_2, albeit poorly (Fig. 3A). Because $F_{420}H_2$ is essential for methanogenesis, a third pathway must exist for F_{420} reduction by H_2.

In a further attempt to remove F_{420}:H_2 oxidoreductase activity, *vhu* and *vhc* were deleted in the $\Delta 3H_2$ase background, resulting in strain MM1289 containing deletions in five hydrogenases ($\Delta fru\Delta frc\Delta hmd\Delta vhu\Delta vhc$, $\Delta 5H_2$ase). This strain required both formate and H_2 for growth (Fig. 3B). This result suggested that F_{420}:H_2 oxidoreductase activity had been reduced to below the level needed to support growth on H_2 alone. The low level of F_{420}:H_2 oxidoreductase activity in $\Delta 5H_2$ase was verified by low H_2 production from formate compared with wild type and $\Delta 3H_2$ase in cell suspensions (Fig. 2). The third F_{420}:H_2 oxidoreductase activity is evidently Vhu/Vhc dependent and represents a previously uncharacterized electron flow pathway in methanogenic Archaea. Although further experiments are needed to characterize this pathway, it could involve Vhu/Vhc, Hdr, and
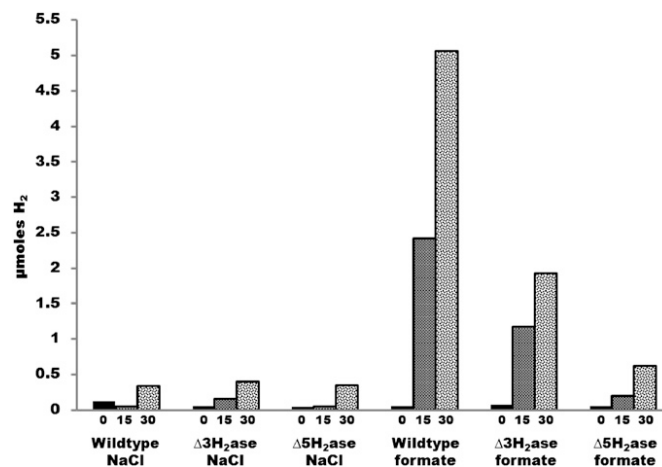

Fig. 2. H_2 production by cell suspensions in the absence or presence of formate. Values in *x* axis are in minutes.
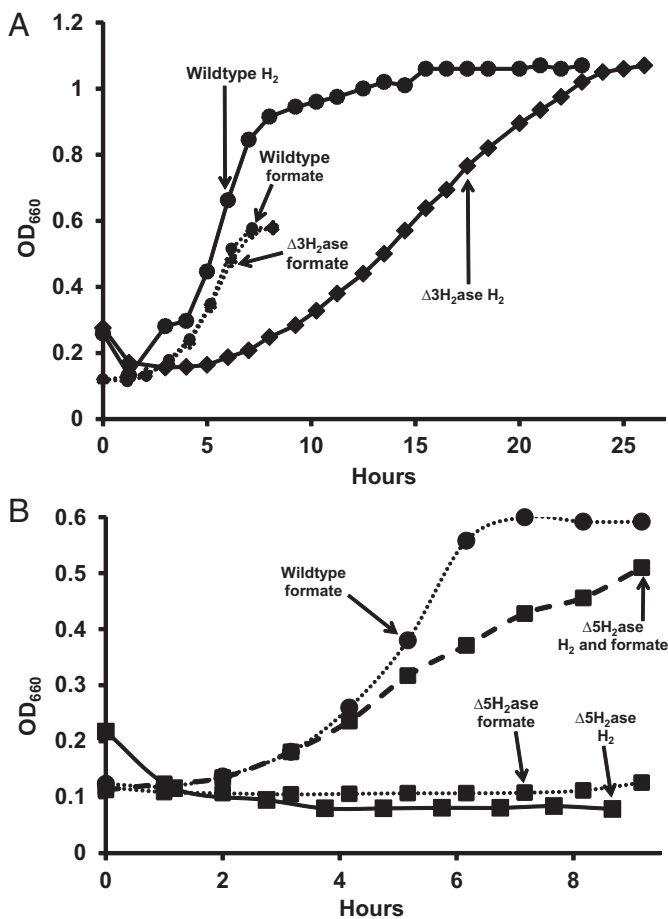
Fig. 3. Requirements of the $\Delta 3H_2$ase (A) and $\Delta 5H_2$ase (B) mutants for H_2 and formate for growth. For growth of the $\Delta 5H_2$ase mutant on H_2 and formate, 14.3 µmoles of H_2 was added.

Fdh, which is F_{420} reducing and like Vhu/Vhc is Hdr associated. The H_2 requirement of the $\Delta 5H_2$ase mutant demonstrates that, contrary to our initial expectation, H_2 is a required intermediate during growth on formate and H_2 is indeed required for growth of hydrogenotrophic methanogens.

H_2 Requirement Is Quantitatively Low. It was unclear whether the H_2 that is required for growth of the $\Delta 5H_2$ase mutant supports the catabolic process of methanogenesis or the anabolic process of CO_2 fixation. Cultures of *M. maripaludis* grown on our formate medium use 1 mmole of formate (11). Therefore, if H_2 were required for just one reductive step of methanogenesis, the amount of H_2 needed would be ~0.33 mmoles. However, we observed maximum growth with H_2 as low as 10–15 µmoles (Fig. 4). At this level, H_2 cannot be a substantial electron donor for methanogenesis. Instead, the H_2 requirement may be anabolic and/or anaplerotic (see below). In fact, during autotrophic growth, about 35 µmol of H_2 was required for each mg of cell dry weight formed in the $\Delta 5H_2$ase mutant (Fig. 4) (for *M. maripaludis*, milligrams of dry weight/OD_{660}/milliliter = 0.34) (5). Under these conditions, cells require 10.7 µmol of pyruvate and 3.9 µmol of acetate per milligram of cell dry weight for autotrophic growth (14). In methanogens, acetate biosynthesis requires two pairs of low potential electrons, one for formation of formyl-MFR and one for the acetyl-CoA synthase step. Pyruvate is formed from acetyl-CoA and requires one additional pair of low potential electrons. Thus, about 40 µmol of low potential electron pairs per milligram of cell dry weight are required for autotrophic growth,

close to the value observed. If the H_2 was required for generation of low potential electrons for anabolism, the addition of carbon sources to the medium should decrease the amount of H_2 needed for growth. In fact, in the presence of acetate and acetate plus casamino acids, the amount of H_2 required decreased to 17 and 8 µmol of H_2 per milligram of cell dry weight, respectively (Fig. 4). Therefore, the H_2 requirement appears at least partially anabolic.

H_2 Is Not Required for Methanogenesis in Vitro but Stimulates Methanogenesis in Cell Suspensions. We performed two additional experiments to further examine the nature of the H_2 requirement. For both experiments, first Ehb was genetically eliminated from the $\Delta 5H_2$ase mutant background to generate strain MM1284, which contained deletions in six hydrogenases ($\Delta fruA\Delta frcA\Delta hmd\Delta$-$vhuAU\Delta vhcA\Delta ehbN$, $\Delta 6H_2$ase). Eha was the sole remaining hydrogenase. Similar to the $\Delta 5H_2$ase mutant, $\Delta 6H_2$ase required H_2 as well as formate for growth (Fig. S1). In the first experiment, in vitro CH_4 production assays were performed (Fig. 5). These assays followed published reports (4, 15), which show that CH_4 production from CO_2 in vitro requires stimulation by the intermediate CH_3-S-CoM and that the yield of CH_4 is limited by the CH_3-S-CoM added. In our assays, CH_3-S-CoM was added and CH_4 production continued presumably until CH_3-S-CoM was depleted. CH_4 production was measured in extracts of $\Delta 6H_2$ase mutant or wild-type cells with either H_2 or formate as electron donor. Extract of wild-type *M. maripaludis* produced substantial CH_4 from CO_2 with either electron donor. In contrast, the mutant extract produced substantial CH_4 only from formate. H_2 had no stimulatory effect on CH_4 production from formate (Fig. S2).

Next, we assayed CH_4 production by cell suspensions of the $\Delta 6H_2$ase mutant. With cell suspensions, stimulation by an intermediate in the pathway was not needed and CH_3-S-CoM was not added. Methanogenesis occurred with formate but not with H_2. Significantly, methanogenesis was greatly enhanced by H_2, either present initially or added during the course of the assay (Fig. 6). Hence, H_2 did not contribute to methanogenesis in vitro where the pathway intermediate CH_3-S-CoM was added, but H_2, presumably acting through Eha, stimulated methanogenesis in cell suspensions.

Eha Is Essential for Growth of *M. maripaludis*. To test whether Eha is essential, mutagenesis of *ehaHIJ* was attempted. The genes *ehaH*, *I*, and *J* encode the presumed cation translocator of the enzyme complex. Each is homologous to a portion of *ehbF*, for
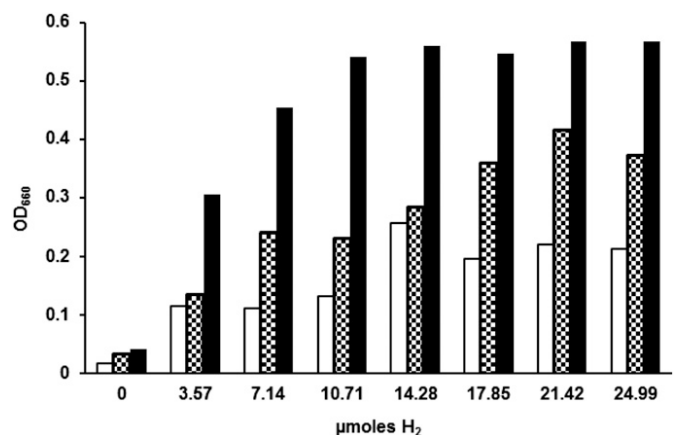

Fig. 4. H_2 dose–response of $\Delta 5H_2$ase mutant. Clear bars, mineral medium; checkered bars, 10 mM acetate added; solid bars, 10 mM acetate and casamino acids (0.2% wt/vol) added. All cultures contained 200 mM formate. Five-milliliter cultures were incubated until stationary phase and OD_{660} was measured.
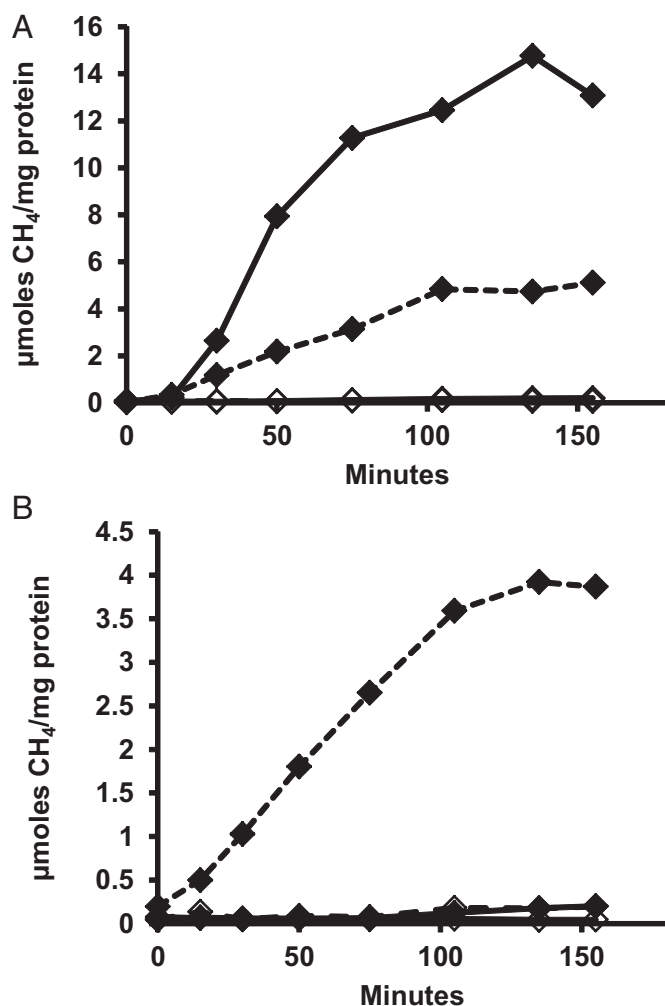
Fig. 5. Methanogenesis in cell extracts from CH_3-S-CoM and CO_2 using H_2 (solid line) or formate (dashed line) as the electron donor in cell extracts from (*A*) wild-type or (*B*) $\Delta 6H_2$ase mutant. Each reaction contained 300 nmols of CH_3-S-CoM and 200–350 µg of protein. Extracts with no CH_3-S-CoM added are represented by open diamonds.

which a null allele has a strong phenotype (9). In preliminary experiments, the construction of an *ehaHIJ* allelic replacement with a puromycin resistance cassette was unsuccessful. An additional test of essentiality was sought. As before (11), our strategy was to determine whether negative selection to resolve a merodiploid would result in a deletion allele. All other things equal, if there is no growth disadvantage for a null allele, deletion mutants should arise with roughly the same frequency as wild-type alleles, and this occurred in a control experiment where a *ehbF*⁺-Δ*ehbF* merodiploid was resolved (Table 1). However, resolution of the *ehaHIJ*⁺-Δ*ehaHIJ* merodiploid in standard medium with H_2 resulted in only wild-type clones. Similar results were obtained when formate rather than H_2 was used. Eha could be involved in 2-ketoglutarate biosynthesis, because 2-ketoglutarate oxidoreductase depends on Fd (9), and high levels of glutamate in the medium (10 mM) could provide sufficient 2-ketoglutarate and remove the requirement for Eha. Alternatively, Eha might be involved in NAD^+ reduction, and alanine dehydrogenase in methanococci generates NADH (16). However, when glutamate or alanine was added, still no mutations were obtained. In contrast, when the *ehaHIJ* mutagenesis experiment was performed in the presence of *trans*-complementation (P*nif-ehaHIJ*), the majority of clones contained the deletion.

These results strongly suggest that Eha is essential, consistent with the H_2 requirement for growth of the $\Delta 6H_2$ase mutant.

Discussion

Distinct Electron Pools Function in Hydrogenotrophic Methanogens. Until recently, it was not known whether any of the hydrogenase activities in hydrogenotrophic methanogens could be eliminated. However, in past work we reported that some of these hydrogenases were unnecessary under some conditions. Thus, in separate strains we deleted genes encoding the F_{420}-reducing hydrogenases (11), the Hdr-associated hydrogenases (7), and the hydrogen-using methylene-H_4MPT dehydrogenase (11). Here we eliminated all three of these hydrogenase activities in a single strain ($\Delta 5H_2$ase) and found that both formate and H_2 were required for growth, the former in quantities stoichiometrically sufficient for methanogenesis, and the latter in much smaller quantities. The mutant effectively separates two pools of electrons that ordinarily exchange via H_2. One pool of electrons provides a stoichiometric supply of electrons for methanogenesis and flows through F_{420} and Hdr (Fig. 1). In the wild-type strain, either formate or H_2 functions as electron donor for this pool. The $\Delta 5H_2$ase and $\Delta 6H_2$ase mutants, by eliminating H_2:F_{420} oxidoreductase activities, disrupt electron flow from H_2 and, as a result, formate is required as the stoichiometric electron donor for methanogenesis. The other pool of electrons supports biosynthesis, and as demonstrated here, anaplerotically replenishes methanogenesis (see below). This pool is carried by ferredoxins, and only H_2 functions as electron donor. In the wild-type strain, H_2 produced from formate allows the latter to function as sole electron donor. However, in the $\Delta 5H_2$ase and $\Delta 6H_2$ase mutants where electron flow between the two pools is blocked, H_2 must be provided.

Function of Eha Is Essential, Anaplerotic, and Ancillary to Electron Bifurcation. In the $\Delta 5H_2$ase mutant, two hydrogenases remain, Eha and Ehb. Previous work has suggested that the role of Ehb is the reduction of Fd for anabolic CO_2 fixation via acetyl-CoA synthase, pyruvate oxidoreductase, 2-ketoglutarate oxidoreductase, indole-pyruvate oxidoreductase, and 2-oxoisovalerate oxidoreductase (8, 9). Eha may play a role analogous to a related energy converting hydrogenase, Ech, which in methanogens with cytochromes generates reduced Fd for CO_2 reduction to formyl-MFR (2). Because Eha and Ehb have different functions, they may reduce different ferredoxins. Although not yet proven biochemically, Eha could have specificity for a polyferredoxin associated with formylmethanofuran dehydrogenase (Fwd), the

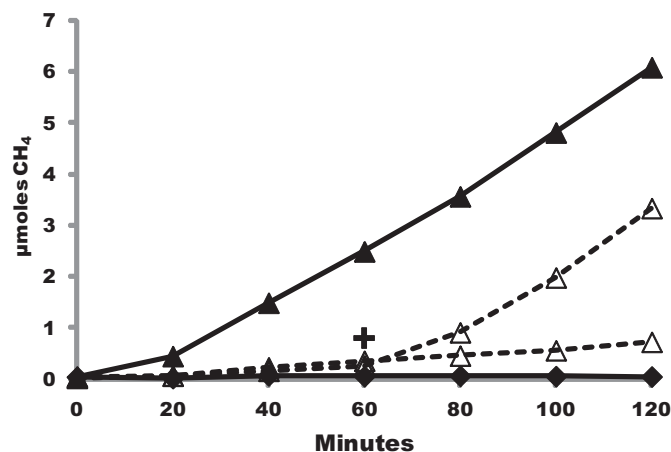**Fig. 6.** CH_4 production by cell suspensions of the $\Delta 6H_2$ase mutant. Black diamonds, H_2 alone; open triangles, formate alone with (+) or without H_2 addition [8% (vol/vol) final concentration] at 60 min; black triangles, H_2 and formate.

Table 1. Resolution of merodiploids of *ehb* or *eha*

Merodiploid	No. of clones	
	WT	Mutant
Δ*ehbF*	26	34
Δ*ehaHIJ*	108	0
Δ*ehaHIJ*, formate	24	0
Δ*ehaHIJ*, glu	12	0
Δ*ehaHIJ*, ala	38	0
Δ*ehaHIJ* + P$_{nif}$-*ehaHIJ*	5	52

Number of clones with the wild-type (WT) or mutant allele following resolution of the merodiploids is given. Resolution was performed in complex medium with the indicated additions or *trans*-complementation by the plasmid expressing *ehaHIJ* from the P$_{nif}$ promoter.

enzyme that catalyzes CO_2 reduction to formyl-MFR (17), whereas Ehb could reduce a Fd associated with anabolic CO_2 fixation reactions.

In methanogens without cytochromes, if the role of Eha is to reduce CO_2 to formyl-MFR, how is this reconciled with electron bifurcation as the main pathway for delivery of electrons for the same step? Our data show that Eha needs to provide only a small portion of the electrons for this reaction. In the Δ5H$_2$ase mutant, formate provided nearly all electrons for methanogenesis and the H$_2$ requirement accounted for only up to 4% of the electrons for growth, much of this apparently for anabolic purposes. We propose that Eha functions in the reduction of CO_2 to formyl-MFR, but does so anaplerotically. In the electron bifurcation model, hydrogenotrophic methanogenesis is a cyclic pathway where the first step is dependent on the last step (1). However, this model presents a dilemma. A constant pool of CoM-S-S-CoB is required, yet intermediates in methanogenesis will inevitably be diluted by growth and cell division, or lost due to a leaky electron bifurcating Hdr complex (1, 6). In addition, intermediates will diminish when methyl-H$_4$MPT is diverted from methanogenesis to generate acetyl-CoA for autotrophic CO_2 fixation (18). Our results show that Eha solves this dilemma by priming or recharging the cycle: it anaplerotically restores intermediates to the methanogenic pathway at the level of formyl-MFR. This model accounts for all of the following observations. First, only small amounts of H$_2$ are required for growth. Second, Eha is essential even though electron bifurcation can account for a stoichiometric supply of electrons for methanogenesis. Third, even though H$_2$ and Eha are essential, there is no need for H$_2$ for methanogenesis in an in vitro assay where the intermediate CH$_3$-S-CoM is added. Finally, for methanogenesis in cell suspensions where no intermediate is provided, H$_2$ is stimulatory.

Why do most hydrogenotrophic methanogens, including *M. maripaludis*, maintain two ion-translocating energy-converting hydrogenases that reduce Fd and provide electrons to fix CO_2? One such hydrogenase could suffice, and indeed, Eha can apparently recognize the Ehb-type ferredoxin and substitute for Ehb, albeit inefficiently (8, 9). Having both hydrogenases separates the recharging of methanogenesis from other anabolic activities and may optimize control over the separate processes. When conditions limit growth, anabolic CO_2 fixation is unimportant but CO_2 reduction to formyl-MFR for methanogenesis and ATP synthesis are still necessary for survival. Under these conditions, functional Eha is essential, and a functional Ehb could be detrimental.

Electron Flow and Energy Conservation in Hydrogenotrophic Methanogens.
Electron bifurcation at Hdr explains a decades-old dilemma regarding methanogenesis: How is net energy conservation achieved in hydrogenotrophic methanogens (1)? The results presented here verify that electron bifurcation must

function in vivo and elaborate on the mechanisms that allow this to be the case, filling in the known gaps of a pathway that has been incomplete since methanogens were first discovered and grown in culture (19). A complete model for electron flow in methanogens without cytochromes can now be described: Methanogenesis is dependent upon F$_{420}$-reducing enzymes and enzymes that feed electrons to Hdr for electron bifurcation. During growth on H$_2$, these enzymes are the F$_{420}$-reducing hydrogenases Fru and Frc and the Hdr-associated hydrogenases Vhu and Vhc. During growth on formate, both kinds of hydrogenases are unnecessary and Fdh performs both functions. Biochemical experiments have demonstrated CoM-S-S-CoB-dependent reduction of a clostridial Fd with H$_2$ (6), but are still needed to prove that electrons flow from H$_2$ or formate to Fwd concurrent with flow to Hdr. Nevertheless, in previous work, we showed that in *M. maripaludis*, Fdh as well as Vhu exist in a complex with Hdr, and that Fwd is in this complex as well (7). Hence, an enzyme complex exists that is suited for electron bifurcation with either H$_2$ or formate. Fdh, either complexed or existing in an isolated form, also generates F$_{420}$H$_2$. Through electron bifurcation at Hdr and F$_{420}$ reduction, formate provides the reducing equivalents to all four reductive steps of methanogenesis. A separate electron pool supports anabolism, which depends on electrons from H$_2$ entering through Ehb and its associated Fd (8, 9). These isolated inputs keep the electron pools for catabolism and anabolism separated and under different regulatory control. Overlap between the two electron pools occurs when electrons from H$_2$ enter methanogenesis through Eha. When low CoM-S-S-CoB concentrations limit the reduction of CO_2 to formyl-MFR by electron bifurcation, Eha recharges methanogenesis.

Materials and Methods

Construction and Growth of Strains. Unless otherwise stated, strains were grown in medium containing casamino acids and acetate, with H$_2$ or formate as the electron donor (11). Strains and plasmids are shown in Table S1. Strain Mm901 was used as the wild-type strain unless otherwise stated. To construct plasmids pCRuptΔ*fru*neo, pCRuptΔ*frc*neo, and pCRuptΔ*hmd*neo, insert DNA from plasmids pCRprtΔ*fru*neo, pCRprtΔ*frc*neo, and pCRprtΔ*hmd*neo (11) was recloned into pCRuptneo (7). To construct plasmids pCRuptΔ*fruGB*neo, pCRuptΔ*frcGB*neo, and pCRuptΔ*ehbN*neo, ~0.5 kb of DNA upstream and downstream of the designated loci was obtained by PCR from genomic DNA and cloned into pCRuptneo. Primers are shown in Table S2. In each case, the resulting plasmid contained an in-frame deletion consisting of a start codon and a stop codon with intervening codons contained within an AscI site. Plasmid DNA was used to make deletions using a markerless mutagenesis method with neomycin for positive selection and 6-azauracil for negative selection as described (7, 20). When formate was present as electron donor, neomycin was increased to 5 mg/mL on liquid or solid medium (7). MM1290 (Mm901Δ*fruAGB*Δ*frcAGB*Δ*hmd*, Δ3H$_2$ase) was constructed by sequential deletion of *frcA*, *hmd*, *fruA*, *fruG* and *B*, and *frcG* and *B*. The first deletion was constructed using H$_2$ as the growth substrate, and the remaining deletions used formate. MM1313 (MM901Δ*fruA*Δ*frcA*Δ*vhuAU*Δ*vhcA*, Δ4H$_2$ase) was constructed from Mm1272 (MM901Δ*vhuAU*Δ*vhcA*) (7) by deletion of *fruA* and *frcA* using formate. MM1289 (MM901Δ*fruA*Δ*frcA*Δ*hmd*Δ*vhuAU*Δ*vhcA*, Δ5H$_2$ase) was then constructed by deleting *hmd*. Finally, MM1284 (MM901Δ*fruA*Δ*frcA*Δ*hmd*Δ*vhuAU*Δ*vhcA*Δ*ehbN*, Δ6H$_2$ase) was constructed by deleting *ehbN*. During construction of the Δ5H$_2$ase and Δ6H$_2$ase mutants, cultures were grown with formate and H$_2$. All deletions were confirmed by PCR.

Essentiality of Eha. Merodiploids were constructed containing deletions of the *ehaHIJ* genes and *ehbF* (Fig. S3 and Table S1), and the generation of deletion mutants by recombination-based resolution of the merodiploids was attempted using wild-type strain Mm900 as described (20). Among the resulting clones, those containing deletion mutations and wild-type alleles were distinguished by PCR. A *trans*-complementing plasmid (pMEV1*nif*::*ehaHIJ*) (Table S1) was constructed on a replicative vector with *ehaHIJ* under control of the nitrogen-regulated *nif* promoter (21, 22). To test the effect of *trans*-complementation, merodiploids containing this plasmid were plated with alanine or ammonia, and the merodiploids were resolved. In preliminary

experiments, *ehaHIJ* deletion strains could be obtained with either nitrogen source, and ammonia was used henceforth.

H₂ and CH₄ Production. To measure H_2 production by cell suspensions, cultures (5 mL) were grown on formate to OD_{660} between 0.5 and 0.6, and cells were pelleted, washed, and resuspended anaerobically in the same volume of assay buffer (modified from ref. 5, 50 mM Mops pH 7.0, 400 mM NaCl, 20 mM KCl, 20 mM MgCl₂, 1 mM CaCl₂, 5 mM DTT, and 1 mM bromoethanesulfonate). Tubes were then flushed with N_2 for 10–30 min to remove residual H_2. At time = 0 min, an initial gas sample was taken, then the assay was initiated by addition of 40 mM (final concentration) sodium formate (pH 7) or NaCl and incubated at 37 °C with shaking. At 15-min and 30-min time points, the headspace was sampled and transferred to butyl rubber stoppered 5-mL vials, mouth ID 13 mm × OD 20 mm (Wheaton; catalog no. 223685) preflushed with N_2. H_2 was analyzed with a SRI Instruments gas chromatography (GC) model 8610C equipped with a 6 foot × one-eighth inch stainless steel Molecular Sieve 5A packed column and a reduced gas detector. The carrier gas was He (20 psi), oven temperature was 130 °C, and detector temperature was 290 °C. CH_4 production by cell suspensions was measured as above except cells were grown to mid-log phase (OD_{660} ~0.25), bromoethanesulfonate was omitted from the assay buffer, and the head-

space was directly analyzed on a Buck Scientific model 910 GC equipped with a flame ionization detector provided with air (16 psi) and H_2 (26 psi). The carrier gas was He (24 psi). To measure CH_4 production by cell-free extracts, cells were washed and suspended in buffer (modified from ref. 4) 100 mM Trizma base, 15 mM MgCl₂, 5 mM ATP, 2 mM 2-mercaptoethanol, 500 μM FAD^+, with or without 50 mM formic acid, and pH adjusted to 7.1 with 1 M HCl). The suspension was sonicated with a Misonix XL-2000 series sonicator to disrupt the cells. Debris was removed by centrifugation at $16{,}000 \times g$, and 200 μL supernatant was placed in a 5-mL serum vial and preincubated for 10 min at room temperature. To start the assay, the headspace was flushed with N_2:CO_2 (80:20, for formate) or H_2:CO_2 (80:20), and 1.5 mM CH_3-S-CoM was added (4). Methanogenesis was monitored as described above.

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. Hedderich R (2004) Energy-converting [NiFe] hydrogenases from archaea and extremophiles: Ancestors of complex I. *J Bioenerg Biomembr* 36:65–75.
3. Meuer J, Kuettner HC, Zhang JK, Hedderich R, Metcalf WW (2002) Genetic analysis of the archaeon *Methanosarcina barkeri* Fusaro reveals a central role for Ech hydrogenase and ferredoxin in methanogenesis and carbon fixation. *Proc Natl Acad Sci USA* 99:5632–5637.
4. Gunsalus RP, Wolfe RS (1977) Stimulation of CO_2 reduction to methane by methylcoenzyme M in extracts Methanobacterium. *Biochem Biophys Res Commun* 76: 790–795.
5. Lupa B, Hendrickson EL, Leigh JA, Whitman WB (2008) Formate-dependent H_2 production by the mesophilic methanogen *Methanococcus maripaludis*. *Appl Environ Microbiol* 74:6584–6590.
6. Kaster AK, Moll J, Parey K, Thauer RK (2011) Coupling of ferredoxin and heterodisulfide reduction via electron bifurcation in hydrogenotrophic methanogenic archaea. *Proc Natl Acad Sci USA* 108:2981–2986.
7. Costa KC, et al. (2010) Protein complexing in a methanogen suggests electron bifurcation and electron delivery from formate to heterodisulfide reductase. *Proc Natl Acad Sci USA* 107:11050–11055.
8. Major TA, Liu Y, Whitman WB (2010) Characterization of energy-conserving hydrogenase B in *Methanococcus maripaludis*. *J Bacteriol* 192:4022–4030.
9. Porat I, et al. (2006) Disruption of the operon encoding Ehb hydrogenase limits anabolic CO_2 assimilation in the archaeon *Methanococcus maripaludis*. *J Bacteriol* 188: 1373–1380.
10. Leigh JA, Albers SV, Atomi H, Allers T (2011) Model organisms for genetics in the domain Archaea: Methanogens, halophiles, Thermococcales and Sulfolobales. *FEMS Microbiol Rev* 35:577–608.
11. Hendrickson EL, Leigh JA (2008) Roles of coenzyme F_{420}-reducing hydrogenases and hydrogen- and F_{420}-dependent methylenetetrahydromethanopterin dehydrogenases in reduction of F_{420} and production of hydrogen during methanogenesis. *J Bacteriol* 190:4818–4821.
12. Widdel F, Wolfe R (1989) Expression of secondary alcohol dehydrogenase in methanogenic bacteria and purifcation of the F_{420}-specific enzyme from *Methanogenium thermophilum* strain TC1. *Arch Microbiol* 152:322–328.
13. Schauer NL, Ferry JG, Honek JF, Orme-Johnson WH, Walsh C (1986) Mechanistic studies of the coenzyme F_{420} reducing formate dehydrogenase from *Methanobacterium formicicum*. *Biochemistry* 25:7163–7168.
14. Yang YL, Glushka JN, Whitman WB (2002) Intracellular pyruvate flux in the methane-producing archaeon *Methanococcus maripaludis*. *Arch Microbiol* 178:493–498.
15. Bobik TA, Wolfe RS (1988) Physiological importance of the heterodisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate in the reduction of carbon dioxide to methane in *Methanobacterium*. *Proc Natl Acad Sci USA* 85:60–63.
16. Yu JP, Ladapo J, Whitman WB (1994) Pathway of glycogen metabolism in *Methanococcus maripaludis*. *J Bacteriol* 176:325–332.
17. Hochheimer A, Schmitz RA, Thauer RK, Hedderich R (1995) The tungsten formylmethanofuran dehydrogenase from *Methanobacterium thermoautotrophicum* contains sequence motifs characteristic for enzymes containing molybdopterin dinucleotide. *Eur J Biochem* 234:910–920.
18. Berg IA, et al. (2010) Autotrophic carbon fixation in archaea. *Nat Rev Microbiol* 8: 447–460.
19. Barker H (1940) Studies upon the methane fermentation. IV. The isolation and culture of *Methanobacterium omelianskii*. *Antonie van Leeuwenhoek J. Microbiol. Serol.* 6: 201–220.
20. Moore BC, Leigh JA (2005) Markerless mutagenesis in *Methanococcus maripaludis* demonstrates roles for alanine dehydrogenase, alanine racemase, and alanine permease. *J Bacteriol* 187:972–979.
21. Dodsworth JA, Cady NC, Leigh JA (2005) 2-Oxoglutarate and the PII homologues Nifl1 and Nifl2 regulate nitrogenase activity in cell extracts of *Methanococcus maripaludis*. *Mol Microbiol* 56:1527–1538.
22. Lie TJ, Wood GE, Leigh JA (2005) Regulation of *nif* expression in *Methanococcus maripaludis*: roles of the euryarchaeal repressor NrpR, 2-oxoglutarate, and two operators. *J Biol Chem* 280:5236–5241.

Supporting Information

Lie et al. 10.1073/pnas.1208779109

Fig. S1. Growth curves of ∆H₂ase mutants.

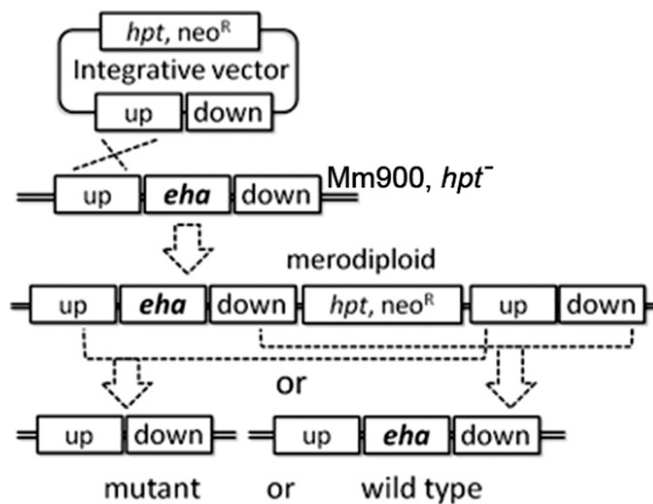**Fig. S2.** CH₄ production in vitro by wild type and ∆6H₂ase mutant.

Fig. S3. In-frame deletion method for generation of the *ehaHIJ* mutation (1). The integrative vector contained DNA fragments upstream and downstream of *ehaHIJ*, fused together deleting the *ehaHIJ*. After transformation into strain Mm900, homologous recombination and selection with neomycin resulted in the merodiploid, which contained both mutated and wild-type copies of the genes (confirmed by Southern blot). Depending on which homologous fragment undergoes recombination, two different merodiploids can be generated and only one is shown for clarity. A second homologous recombination event results in removal of the plasmid backbone, and the resulting clone is obtained by negative selection for *hpt*. Depending on which fragment undergoes re-combination, either wild type or mutant is produced. Dashed lines indicate possible homologous recombination sites. In the merodiploid, the downstream genes in the *eha* operon are transcribed from the neo promoter. Even though the continuity of the *eha* operon was disrupted, the genes were expressed at sufficient levels to sustain cell viability. The mRNA levels estimated by RT-PCR of *ehaH* and *ehaN* in merodiploid, which were located downstream of the vector were comparable to their levels in WT strain Mm900. The mRNA levels of *ehaH* compared with a DNA primase gene standard were 0.5 ± 0.05 (means of triplicates of two independent cultures $\pm$ 1 SD) for both merodiploid and the WT. Corresponding values for *ehaN* were 0.6 ± 0.3 and 1.0 ± 0.1. These results clearly demonstrated that genes downstream of vector in the merodiploid were expressed and that these mutations do not necessarily yield loss of function.

1. Moore BC, Leigh JA (2005) Markerless mutagenesis in *Methanococcus maripaludis* demonstrates roles for alanine dehydrogenase, alanine racemase, and alanine permease. *J Bacteriol* 187:972–979.

Table S1. Plasmids and strains used in this study

Name	Features	Source
Plasmids/constructs		
pCRupt∆*frc*neo	Deletion of *frcA*	This study
pCRupt∆*fru*neo	Deletion of *fruA*	This study
pCRupt∆*hmd*neo	Deletion of *hmd*	This study
pCRupt∆*frcGB*neo	Deletion of *frcGB*	This study
pCRupt∆*fruGB*neo	Deletion of *fruGB*	This study
pCRupt∆*ehbN*neo	Deletion of *ehbN*	This study
pCRuptneo	Methanococcal integration vector, *upt*+, Neo^R, Amp^R, Kan^R	This study
pIJA03	Methanococcal integration vector, Pur^R, Amp^R	W.B.W. and (1)
p IJA03*ehaHIJ*-up::*pac*::down	Flanking regions of *ehaHIJ* for recombination	This study
pIJAO3::*hpt-pac*	*hpt* cassette next to *pac* in pIJAO3	This study
pIJAO3:: *ehaHIJ*-up::*hpt-pac*::down	*ehaHIJ* flanking regions in the integration vector	This study
pCRprtneo	Methanococcal integration vector, *hpt*+, Neo^R, Amp^R, Kan^R	(2)
pCRprtneo::*ehaHIJ*-up::down-IF	Flanking regions fused together deleting *ehaHIJ*	This study
pCRprtneo::*ehbF*-up::down-IF	Flanking regions fused together deleting *ehbF*	
pMEV1::*lacZ* (pWLG40::*lacZ*)	Replicative vector for M. maripaludis; lacZ with M. voltae histone promoter; and pac cassette; Amp^r, Pur^r	W.B.W.
pMEV1::*ehaHIJ*	*ehaHIJ* in expression vector	This study
pMEV1*nif*::*lacZ*	*lacZ* in replicative vector for *M. maripaludis* with *nif* promoter; Amp^R, Pur^R	J.A.L.
pMEV1*nif*::*ehaHIJ*	*ehaHIJ* in expression vector with regulated promoter	This study
Strains		
S2	*M. maripaludis* wild type	(3)
Mm900	'Wild type'; ∆*hpt*	(2)
Mm901	'Wild type'; ∆*upt*	(4)
MM1272	MM901∆*vhuAU*∆*vhcA*	(4)
MM1290	MM901∆*fruAGB*∆*frcAGB*∆*hmd*	This study
MM1313	MM901∆*fruA*∆*frcA*∆*vhuAU*∆*vhcA*	This study
MM1289	MM901∆*fruA*∆*frcA*∆*hmd*∆*vhuAU*∆*vhcA*	This study
MM1284	MM901∆*fruA*∆*frcA*∆*hmd*∆*vhuAU*∆*vhcA*∆*ehbN*	This study
Merodiploid Mm900*eha*up-down IF	Mm900 with integrated vector harboring *ehaHIJ*+ and *ehaHIJ*- in frame deletion, Neo^R	This study
Merodiploid Mm900*eha*up-down IF + pMEV*nif*::*ehaHIJ*	Merodiploid for in-frame deletion with the complementation plasmid, Neo^R, Pur^R	This study
∆*ehbF*	In-frame deletion of *ehbF* in Mm900	This study
∆*ehaHIJ* + pMEV1::*ehaHIJ*	*ehaHIJ* deletion with the complementation vector, Pur^R	This study
∆*ehaHIJ* + pMEV1*nif*::*ehaHIJ*	*ehaHIJ* deletion with the complementation vector with regulated promoter, Pur^R	This study

1. Lie TJ, Dodsworth JA, Nickle DC, Leigh JA (2007) Diverse homologues of the archaeal repressor NrpR function similarly in nitrogen regulation. *FEMS Microbiol Lett* 271:281–288.
2. Moore BC, Leigh JA (2005) Markerless mutagenesis in *Methanococcus maripaludis* demonstrates roles for alanine dehydrogenase, alanine racemase, and alanine permease. *J Bacteriol* 187:972–979.
3. Whitman WB, Shieh J, Sohn S, Caras DS, Premachandran U (1986) Isolation and characterization of 22 mesophilic methanococci. *Syst Appl Microbiol* 7:235–240.
4. Costa KC, et al. (2010) Protein complexing in a methanogen suggests electron bifurcation and electron delivery from formate to heterodisulfide reductase. *Proc Natl Acad Sci USA* 107:11050–11055.

Table S2. Oligonucleotides used in this study

Primer sequence (5′→3′)	Restriction site	Rationale
AAGCGGCCGCCGGAAATATTCTATTTGGGGATG	NotI	Amplify upstream *frcG*
AAGGCGCGCCTCTTACCACTATATCACCATTATTCG	AscI	Amplify upstream *frcG*
AAGGCGCGCCATACTAATCCATTCCTTTAATTTTTG	AscI	Amplify downstream *frcB*
AATCTAGAAAGGAGTTTCACTTAATTTTGGCC	XbaI	Amplify downstream *frcB*
AAGCGGCCGCAGGAAATATGGTTCTTGCATGCGG	NotI	Amplify upstream *fruG*
AAGGCGCGCCCATGGTATTCTCCTCCCTTAGTTG	AscI	Amplify upstream *fruG*
AAGGCGCGCCAGGATTACCTGTTCCATACTAACTTC	AscI	Amplify downstream *fruB*
AATCTAGAAAATTCCAATAGATGCAACAACCGCAGC	XbaI	Amplify downstream *fruB*
AAGCGGCCGCTGATCAGTTATTTTTACTTCCCTTGG	NotI	Amplify upstream *ehbN*
AAGGCGCGCCGAAGCGTACATGATTTTTCCC	AscI	Amplify upstream *ehbN*
AAGGCGCGCCGAACCCTTTAAAAAAATAATCAC	AscI	Amplify downstream *ehbN*
AATCTAGACTTAATTTATAGTATATCTC	XbaI	Amplify downstream *ehbN*
CTAATAGCTAGCCAACCCCTGGGGGAATA	NheI	*ehaH* up region for cloning into pIJAO3
GGCTTATAGCATTCATTGTGGCACTAGTCTTGAT	SpeI	
ATAACGTCTAGAGCCCGGTATTGACTTTGCTTG	XbaI	*ehaJ* down region for cloning into pIJAO3
AGTAATCTCGAGGCCTGTTCAACGTATGCG	XhoI	
CCCCCCAGATCTTTGCATATATCATTGTTAGACC	BglII	*hpt* cassette for cloning into pIJAO3
CCCCCCTCTAGATTATTCTAAAATGTTTACTTTTCC	XbaI	
CCCCCCGGATCCGGAATCACTGACTTTGCTCCTC	BamHI	*ehaH* up region for cloning into pIJAO3:: *hpt-pac*
CCCCCCAGATCTAACGAGCATTTTAATCACCTTTG	BglII	
CCCCCCGGTACCGCCACTACTTGCTCCAAATCAC	KpnI	*ehaJ* down region for cloning into pIJAO3::*hpt-pac*
CCCCCCGCTAGCGACGGTAAGTAGCCTGCCTTC	NheI	
ACAATAGGGCCCGGAATCACTGACTTTGCTCCT	ApaI	*ehaH* up region for in-frame deletion fusion and cloning into pCRprtneo
CCCCCCAGATCTAACGAGCATTTTAATCACCTTTG	BglII	
CCCCCCAGATCTGCCACTACTTGCTCCAAATCAC	BglII	*ehaJ* down region for in-frame deletion fusion and cloning into pCRprtneo
CCCCCCCTCTAGAGACGGTAAGTAGCCTGCCTTC	XhoI	
CCCCATGCATGCTCGTTGAATATATCGCAGGAAACTTT	NsiI	*ehaHIJ* for cloning into pMEV1*nif*
GGGGGTCTAGACTAATGGATGATTCTGTATGCCAGATCAATA	XbaI	
GTTTAACGGACTTATGT*ATGCGT*TGTATGCCTTTTTTAGTTGGCGG		Site-directed mutagenesis, removal of NsiI site from *ehaJ*
CCGCCAACTAAAAAGGCATACA*ACGCAT*ACATAAGTCCGTTAAAC		
CAACTTGGATCCGGAATCACTGACTTT GCTCCTC		
GCCGCATAAAACGGAGCAACGCCT		Determination of presence of *ehaH*
CAAAAGATGCGATACAGGGCCAGG		*eha* fragment with flanking regions external of cloning sites
TTTCCCAAGCTCCGGTTACAGGAC		
GCACCTGATTTTCCGATAATGCCCAA		Detects the presence of *ehbF*
CACCGACTATTCCCTGACGATGTAGTC		