Hydrogen is a preferred intermediate in the energy-conserving electron transport chain of *Methanosarcina barkeri*

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Methanogens use an unusual energy-conserving electron transport chain that involves reduction of a limited number of electron acceptors to methane gas. Previous biochemical studies suggested that the proton-pumping $F_{420}H_2$ dehydrogenase (Fpo) plays a crucial role in this process during growth on methanol. However, *Methanosarcina barkeri* Δ*fpo* mutants constructed in this study display no measurable phenotype on this substrate, indicating that Fpo plays a minor role, if any. In contrast, Δ*frh* mutants lacking the cytoplasmic F_{420}-reducing hydrogenase (Frh) are severely affected in their ability to grow and make methane from methanol, and double Δ*fpo*/Δ*frh* mutants are completely unable to use this substrate. These data suggest that the preferred electron transport chain involves production of hydrogen gas in the cytoplasm, which then diffuses out of the cell, where it is reoxidized with transfer of electrons into the energy-conserving electron transport chain. This hydrogen-cycling metabolism leads directly to production of a proton motive force that can be used by the cell for ATP synthesis. Nevertheless, *M. barkeri* does have the flexibility to use the Fpo-dependent electron transport chain when needed, as shown by the poor growth of the Δ*frh* mutant. Our data suggest that the rapid enzymatic turnover of hydrogenases may allow a competitive advantage via faster growth rates in this freshwater organism. The mutant analysis also confirms the proposed role of Frh in growth on hydrogen/carbon dioxide and suggests that either Frh or Fpo is needed for aceticlastic growth of *M. barkeri*.

hydrogen electron transport | F_{420} | H_2 cycling | methanogenesis

Methanogenesis is the terminal step in biomass degradation in many anaerobic environments and plays a central role in the global carbon cycle. Although most of the methane (CH_4) produced is oxidized to carbon dioxide (CO_2) by methane-consuming organisms, substantial quantities (*ca.* 10^{14} g/year) escape into the atmosphere where it acts as a potent greenhouse gas (1). Most methanogens produce CH_4 by reducing CO_2 with hydrogen gas (H_2) (2). However, some *Methanosarcina* species such as *M. barkeri* and *M. mazei* also are capable of using a variety of other substrates, including acetate, which accounts for *ca.* 2/3 of global CH_4 production (3), and C1 compounds such as methanol, methylsulfides, and methylamines (4).

Methanogenic organisms produce CH_4 as a byproduct of anaerobic respiration involving a unique energy-conserving electron transport chain found only in Archaea. At least 2 distinct types of methanogenic respiration exist: 1 found in methanogens, including *Methanosarcina* species that synthesize cytochromes, and the other in those that lack cytochromes (1). The penultimate step of both respiratory pathways involves the reduction of methyl-coenzyme M (CoM-SH, mercaptoethanesulfonic acid) to CH_4 using coenzyme B (CoB-SH, *N*-7-mercaptoheptanoyl-*O*-phospho-L-threonine) and CoM-SH as electron donors. The other product of this reaction is the heterodisulfide of CoM-SH and CoB-SH (CoM-S-S-CoB), which serves as the terminal electron acceptor in the energy-conserving electron transport chain. In methanogenic Archaea that lack cytochromes, the means by which energy is conserved is poorly understood but probably involves a cytoplasmic electron bifurcation pathway similar to that recently characterized in *Clostridium* (1, 5). In contrast, the energy-conserving electron transport chain of cytochrome-containing methanogens, exemplified by *Methanosarcina* species, has been studied in detail, including the reconstitution of 2 distinct proton-translocating electron transport systems in vitro (6–9).

In *Methanosarcina* species, a membrane-bound electron transport chain that terminates with the reduction of the CoM-S-S-CoB heterodisulfide generates ion-motive force that can be used by ATP synthase to form ATP (Fig. 1). Either H_2 or reduced coenzyme F_{420} ($F_{420}H_2$) can donate electrons for reduction of CoM-S-S-CoB (2, 4). In the H_2:heterodisulfide oxidoreductase system, a methanophenazine-dependent hydrogenase (Vht or Vhx) (10) oxidizes H_2 in the periplasm with transfer of 2 electrons to the membrane-soluble electron carrier methanophenazine. The reduced methanophenazine (MPH_2) subsequently is oxidized by the enzyme heterodisulfide reductase (Hdr) with concomitant reduction of CoM-S-S-CoB. In the $F_{420}H_2$:heterodisulfide oxidoreductase system, the $F_{420}H_2$ dehydrogenase (Fpo) catalyzes electron transfer from $F_{420}H_2$ to methanophenazine, concomitantly pumping 2 protons out of the cell. As in the H_2:heterodisulfide oxidoreductase system, MPH_2 passes electrons to CoM-S-S-CoB, leading to translocation of 2 additional protons. In vitro measurements suggest that the magnitude of proton motive force generated is the same for both oxidoreductase systems, probably $4H^+/2e^-$ (9, 11).

The $F_{420}H_2$:heterodisulfide oxidoreductase system shares features with the aerobic electron transport chain found in many bacteria. Fpo was purified from *M. mazei* Gö1 as a 5-subunit complex (FpoBCDIF) capable of oxidizing $F_{420}H_2$ with a variety of artificial electron acceptors (12). Most of these proteins were shown later to be encoded by a 13-gene operon, *fpoABCDHIJJKLMNO* (*fpo*), with *fpoF* being located elsewhere on the genome (supporting information (SI) Fig. S1) (11). Fpo shares significant homology with NADH dehydrogenase I (Nuo) of *Escherichia coli*, and the nomenclature reflects that similarity (Table S1). Accordingly, both Fpo and Nuo are composed of similar membrane-integral modules (the A, H, J, K, L, M, and N subunits) and membrane-associated modules (the B, C, D, and I subunits). However, the enzymes differ in their substrates

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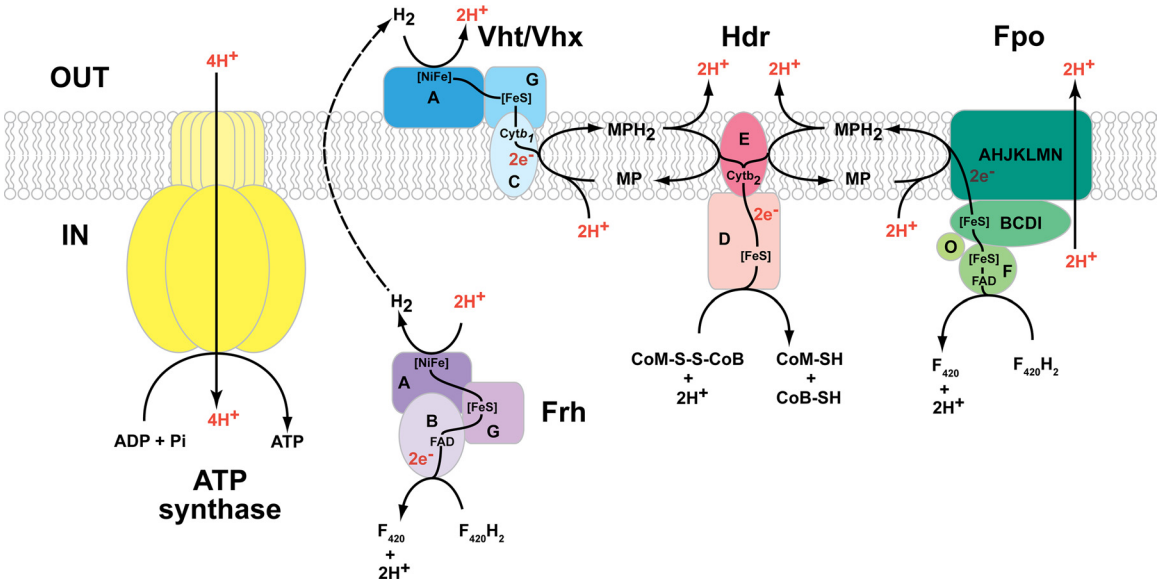

Fig. 1. The electron transport chain of *M. barkeri* has been proposed to comprise 2 energy-conserving systems, the $F_{420}H_2$:heterodisulfide oxidoreductase and the H_2:heterodisulfide oxidoreductase. In the former system, $F_{420}H_2$ is oxidized by FpoF releasing 2 electrons that are transferred through FpoBCDI and then FpoAHJKLMN to membrane-soluble methanophenazine. This reaction is coupled to the pumping of 2 protons outside the cell. Reduction of methanophenazine consumes 2 protons from the cytoplasm, which subsequently are released outside the cell upon oxidation of MPH_2. The electrons then are transferred through HdrED to reduce CoM-S-S-CoB with 2 protons from the cytoplasm. Alternatively, in the H_2:heterodisulfide oxidoreductase, H_2 is oxidized by Vht/Vhx to produce 2 protons outside the cell and 2 electrons that are transferred to MPH_2, which then is used to reduce CoM-S-S-CoB. The dashed arrow represents a third possible energy-conserving mechanism that is proposed in this study. In this pathway, $F_{420}H_2$ is oxidized by the cytoplasmic hydrogenase Frh to generate H_2. The H_2 then diffuses outside the cell to the active site of membrane-bound hydrogenase Vht/Vhx, where it is reoxidized, resulting in the translocation of 2 protons via a H_2-cycling mechanism. The electrons are passed through methanophenazine to CoM-S-S-CoB, as in the other 2 systems. In all 3 systems, the entire electron transport process leads to the net translocation of 4 protons (highlighted in red) outside the cell per 2 electrons transferred from $F_{420}H_2$ or H_2 to the CoM-S-S-CoB. The electrochemical gradient generated is coupled to ATP synthesis via an A-type ATPase. Abbreviations: CoB-SH, coenzyme B; CoM-SH, coenzyme M; CoM-S-S-CoB, mixed disulfide of CoM-SH and CoB-SH; Cytb_2, cytochrome b_2; $F_{420}/F_{420}H_2$, oxidized and reduced Factor 420; FAD, flavin adenine dinucleotide; [FeS], iron-sulfur cluster; Fpo, $F_{420}H_2$:phenazine oxidoreductase; Frh, F_{420}-reducing hydrogenase; Hdr, heterodisulfide reductase; MP/MPH_2, oxidized and reduced methanophenazine; [NiFe], bimetallic catalytic center; Vht/Vhx, methanophenazine-dependent hydrogenase.

and hence in their input modules, so that NuoEFG oxidizes NADH, whereas FpoF oxidizes $F_{420}H_2$, which is a hydride carrier analogous to NADH (2).

The source of electrons for the 2 disparate *Methanosarcina* electron transport chains varies with the growth substrate (Fig. S2). During growth on H_2/CO_2 (the hydrogenotrophic pathway), or on H_2 in combination with a C1 electron acceptor (the methyl-respiration pathway), electrons are derived from H_2 oxidation and are used via the H_2:heterodisulfide oxidoreductase system (2). Genetic and biochemical experiments in *M. barkeri* suggest that use of acetate (the aceticlastic pathway) also involves the obligate intermediacy of H_2 and the H_2:heterodisulfide oxidoreductase system (13, 14). In contrast, metabolism of compounds such as methanol or methylamines (the methylotrophic pathway) produces 2 equivalents of $F_{420}H_2$ per C1 molecule oxidized. Thus, it has been suggested that $F_{420}H_2$:heterodisulfide oxidoreductase system plays a central, perhaps essential, role in the energy-conserving electron transport chain of the methylotrophic pathway in all *Methanosarcina* species (reviewed in refs. 2, 4, 15–18).

Surprisingly, the in vivo genetic data presented here tell a different story. Contrary to expectations, Fpo is not required for growth under any condition tested, including during growth on methanol. Instead, reducing equivalents from methanol oxidation seem to be preferentially passed to molecular H_2 by the cytoplasmic F_{420}-reducing hydrogenase (Frh). Subsequently, H_2 may enter the electron transport chain via the H_2:heterodisulfide oxidoreductase system (Fig. 1). These data are reminiscent of the hydrogen-cycling model for electron transport first proposed by Odom and Peck for sulfate-reducing bacteria (19) and suggest that a reanalysis of energy-conservation mechanisms in freshwater *Methanosarcina* species is warranted.

Results

The $F_{420}H_2$:Heterodisulfide Oxidoreductase Is Conserved in All Sequenced *Methanosarcina* Genomes. The genomes of *M. barkeri* Fusaro (20) and *M. acetivorans* C2A (21) encode *fpoABCDHIJJKLMNO* and *fpoF* operons that are nearly identical to those found in *M. mazei* (Fig. S1 and Table S1) (22). All known structural and catalytic amino acid residues are conserved in the predicted protein sequences of the Fpo subunits from all 3 *Methanosarcina* species (Fig. S3) (2, 4, 11). Further, each sequenced genome carries homologues of the *hdrDE* operon, which encodes the methanophenazine-linked Hdr (Fig. S1) (20–23). Thus, although biochemical activity has been examined only in *M. mazei*, functional $F_{420}H_2$:heterodisulfide oxidoreductase systems probably are present in each of the *Methanosarcina* species examined to date.

$F_{420}H_2$ Dehydrogenase Is Not Required for Methanogenesis or Growth in *M. barkeri*. As described in earlier sections, in vitro biochemical experiments led to the suggestion that the $F_{420}H_2$:heterodisulfide oxidoreductase system should be essential for growth on C1 compounds via the methylotrophic pathway. To test this hypothesis in vivo, we constructed *M. barkeri* mutants lacking $F_{420}H_2$ dehydrogenase by deleting the *fpoA-O* operon or the *fpoF* gene (Table S2).

The Δ*fpoA-O* and Δ*fpoF* mutants were tested for their ability to grow on various methanogenic substrates (Table 1). As expected, both mutants grow on H_2/CO_2, on methanol plus H_2/CO_2, and on acetate with growth rates and yields similar to

Table 1. Generation time (h) and relative growth yield (%)* of *M. barkeri* Fusaro strains† in various media

Strain	Substrates			
	CH_3OH	$CH_3OH/H_2/CO_2$	H_2/CO_2	CH_3COOH
Δ*hpt*‡	7.3 ± 0.3 (100%)	5.7 ± 0.1 (100%)	13.7 ± 2.5 (100%)	37 ± 3.4 (100%)
Δ*fpo*	7.3 ± 0.2 (101%)	6.1 ± 0.5 (100%)	11.9 ± 0.9 (100%)	36 ± 2.6 (100%)
Δ*fpoF*	7.3 ± 0.2 (96%)	5.8 ± 0.5 (96%)	12.4 ± 1.2 (90%)	39 ± 1.9 (84%)
Δ*fre*	7.7 ± 0.3 (116%)	5.5 ± 0.4 (125%)	8.8 ± 0.8 (95%)	41 ± 3.1 (84%)
Δ*frh*	13.7 ± 0.6 (52%)	6.5 ± 0.3 (80%)	NG (NA)	55 ± 7.0 (84%)
Δ*fpo* Δ*frh*	NG (NA)	5.0 ± 0.3 (80%)	NG (NA)	NG (NA)
Δ*fpoF* Δ*frh*	NG (NA)	6.4 ± 0.4 (73%)	NG (NA)	76 ± 0.8 (66%)

*Growth rate and yield were measured as described in *Materials and Methods*; growth yield is relative to the parental strain on the same substrate. Values represent the average and standard deviation of at least triplicate measurements.
†Strains used were WWM85 (Δ*hpt*), WWM71 (Δ*fpo*), WWM123 (Δ*fpoF*), WWM122 (Δ*frh*), WWM108 (Δ*fpo* Δ*frh*), and WWM145 (Δ*fpoF* Δ*frh*).
‡*M. barkeri* Fusaro parent strain in which all deletions were constructed.
NG, no growth for at least 6 months of incubation; NA, not applicable.

the isogenic parental strain. However, we were surprised to discover that growth of the mutants on methanol also was unaffected. Further, CH_4 and CO_2 were produced by the Δ*fpoA-O* and Δ*fpoF* mutants in the expected 3:1 stoichiometry in amounts and at rates similar to the parent on all substrates (Tables 2 and 3). Thus, loss of $F_{420}H_2$ dehydrogenase, and therefore of the $F_{420}H_2$:heterodisulfide oxidoreductase system, does not measurably affect methanogenesis or growth in *M. barkeri*.

Frh Is Essential for Growth of *M. barkeri* on H_2/CO_2 and Plays an Important Role in the Methylotrophic Pathway. The dispensability of Fpo raises the question of which enzyme(s) transfers electrons from $F_{420}H_2$ to methanophenazine in the methylotrophic pathway. Because *M. barkeri* possesses a highly active F_{420}-reducing hydrogenase (24), we considered the possibility of electrons being channeled from $F_{420}H_2$ into the H_2:heterodisulfide oxidoreductase system via production of H_2 by Frh (Fig. 1).

The F_{420}-reducing hydrogenase couples oxidation of H_2 to F_{420} reduction in vitro and is fully reversible (24–26). *M. barkeri* has 2 operons, *frhADGB* and *freAEGB*, with the potential to encode F_{420}-reducing hydrogenases (Fig. S1) (27). It is unclear whether *freAEGB* encodes an active hydrogenase, because it lacks the required maturation protease encoded by *frhD* in the homologous operon. Nevertheless, the putative *fre*-encoded hydrogenase shares all important catalytic and structural amino acid residues with the *frh*-encoded enzyme. Thus, *freAEGB* could encode a functional F_{420}-reducing hydrogenase if the FrhD protease can function *in trans* (10). The F_{420}-reducing hydrogenase is proposed to provide $F_{420}H_2$, which is needed for CO_2 reduction in the hydrogenotrophic pathway (Fig. S2) (24); however, *frhADGB* and *freAEGB* are expressed during growth on both H_2/CO_2 and methanol (10, 27). Thus, it seems possible that these genes play a role in both the hydrogenotrophic and methylotrophic pathways.

To test this possibility, the *freAEGB* or *frhADGB* operons were deleted from the chromosome of *M. barkeri* (Table S2), and the resulting mutants were characterized. The Δ*fre* mutant is indistinguishable from its parent with respect to growth rate and yield on all substrates tested (Table 1). Further, deletion of *fre* from *M. barkeri* does not affect the amount and rate of CH_4 produced in resting cell suspensions from the various substrates, nor does the mutation change the expected 3:1 ratio of CH_4 to CO_2 on methanol (Tables 2 and 3). These data indicate that Fre is not required for growth of *M. barkeri* on any of the substrates tested.

In contrast, the Δ*frh* mutation has severe phenotypic consequences on several of the growth media examined (Tables 1–3). Although the Δ*frh* mutant is indistinguishable from its parent when grown on methanol plus H_2/CO_2 and acetate, it is unable to grow on H_2/CO_2. Moreover, the Δ*frh* mutant has a 2-fold slower growth rate and a 50% reduction in growth yield when methanol alone is used as a substrate. The Δ*frh* mutant exhibits a very long lag phase of 911 ± 21 h, as compared with 31 ± 0.4 h for wild-type *M. barkeri* on methanol. The growth defects of the Δ*frh* mutant are reflected further in CH_4 production. Resting cell suspensions of the Δ*frh* mutant produce negligible amounts of CH_4 from H_2/CO_2 and although they disproportionate methanol in the expected 3:1 ratio, they consistently produce only half as

Table 2. Methane (μmoL) and carbon dioxide (μmoL) production* from resting cell suspensions of *M. barkeri* Fusaro strains†

Strain	Substrates‡							
	N_2		CH_3OH		CH_3OH/H_2		H_2/CO_2	
	CH_4	CO_2	CH_4	CO_2	CH_4	CO_2	CH_4	CO_2
Δ*hpt*§	<1	2 ± 0.1	343 ± 4	108 ± 2	465 ± 17	<1	298 ± 21	NA
Δ*fpo*	<1	1 ± 0.1	365 ± 3	115 ± 1	494 ± 11	<1	356 ± 6	NA
Δ*fpoF*	<1	1 ± 0.1	332 ± 7	106 ± 2	439 ± 27	<1	359 ± 9	NA
Δ*fre*	<1	1 ± 0.1	399 ± 14	133 ± 4	508 ± 66	<1	381 ± 21	NA
Δ*frh*	<1	2 ± 0.9	149 ± 3	42 ± 1	481 ± 14	<1	7 ± 2	NA
Δ*fpo* Δ*frh*	<1	2 ± 0.7	47 ± 2	9 ± 1	480 ± 59	<1	10 ± 1	NA
Δ*fpoF* Δ*frh*	<1	4 ± 0.1	54 ± 3	16 ± 1	465 ± 9	<1	11 ± 5	NA

*Values are the average and standard deviation of at least 3 trials.
†Strains used were WWM85 (Δ*hpt*), WWM71 (Δ*fpo*), WWM123 (Δ*fpoF*), WWM122 (Δ*frh*), WWM108 (Δ*fpo* Δ*frh*), and WWM145 (Δ*fpoF* Δ*frh*).
‡ Assays were conducted as described in *Supplementary Information*.
§*M. barkeri* Fusaro parent strain in which all deletions were constructed.
NA, not applicable (CO_2 produced could not be measured because it was added to headspace).

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Table 3. Rate (nmol min^{-1} mg^{-1}) of methane production* from resting cell suspensions of *M. barkeri* Fusaro strains†

Strain	Substrate ‡		
	N_2	CH_3OH	CH_3OH/H_2
Δ*hpt*§	<1	70 ± 6	121 ± 35
Δ*fpo*	<1	93 ± 13	127 ± 29
Δ*fpoF*	<1	82 ± 2	143 ± 7
Δ*fre*	<1	95 ± 13	134 ± 32
Δ*frh*	<1	14 ± 0.5	97 ± 3
Δ*fpo* Δ*frh*	<1	4 ± .5	151 ± 3
Δ*fpoF* Δ*frh*	<1	4 ± 0.1	135 ± 15

*Values are the average and standard deviation of at least three trials.
†Strains used were WWM85 (Δ*hpt*), WWM71 (Δ*fpo*), WWM123 (Δ*fpoF*), WWM122 (Δ*frh*), WWM108 (Δ*fpo* Δ*frh*), and WWM145 (Δ*fpoF* Δ*frh*).
‡Assays were conducted as described in *SI Text*.
§*M. barkeri* Fusaro parent strain in which all deletions were constructed.

much CH_4 and CO_2 as the parent. Finally, the Δ*frh* mutation lowers the rate of CH_4 production from methanol in resting cells by *ca.* 4-fold relative to the parent.

Taken together, these data indicate that Frh is essential for growth by the hydrogenotrophic pathway and plays an important but dispensable role in the methylotrophic pathway, the latter potentially being the delivery of electrons from $F_{420}H_2$ into the H_2:heterodisulfide oxidoreductase system. Importantly, Fre is not able to substitute for the role of Frh under the conditions tested, suggesting that it does not encode a functional F_{420}-reducing hydrogenase in the absence of *frhADGB*.

***M. barkeri* Possesses Two Functional Pathways for Electron Transfer from $F_{420}H_2$ to Methanophenazine.** The fact that the Δ*frh* mutant retains the ability to grow and produce CH_4 from methanol, although at reduced rates, suggests that the cell has an alternative, less efficient route to deliver electrons from $F_{420}H_2$ to the CoM-S-S-CoB heterodisulfide. The most obvious candidate for this electron transfer pathway is the $F_{420}H_2$:heterodisulfide oxidoreductase system. To test this hypothesis, we constructed and characterized double mutants lacking *frhADGB* and either *fpoA-O* or *fpoF* (Tables S2 and Tables 1, 2, and 3).

Like the single Δ*frh* mutant, the Δ*fpoA-O*/Δ*frh* and Δ*fpoF*/Δ*frh* double mutants are unable to grow on H_2/CO_2 and produce negligible amounts of CH_4 in resting cell suspensions from this substrate. Both mutants are able to grow and produce CH_4 at levels and rates comparable to the parent on methanol plus H_2/CO_2. However, in contrast to the Δ*frh,* Δ*fpoA-O*, and Δ*fpoF* single mutants, the double mutants are incapable of growth on methanol. Thus, Frh and Fpo fulfill a similar role during growth on this substrate that is lost in the absence of both enzymes. Quantitative RT-PCR experiments show that although *fpoA-O* transcripts levels are slightly higher in the Δ*frh* mutant (3.9 ± 1.5-fold), they are easily detectable in the parental strain. Therefore, Fpo is expressed and has the potential to contribute to the methylotrophic electron transport chain in wild-type *M. barkeri*. Interestingly, the Δ*fpoA-O*/Δ*frh* and Δ*fpoF*/Δ*frh* mutants still produce small amounts of CH_4 and CO_2 from methanol despite their inability to grow on this substrate. Hence, an additional minor electron transport pathway(s) exists in *M. barkeri* that is incapable of supporting growth.

Surprisingly, the double mutants display different phenotypes when grown on acetate. The Δ*fpoF*/Δ*frh* mutant grows slowly on acetate, whereas the Δ*fpoA-O*/Δ*frh* mutant is unable to use acetate. It is unclear how the absence of both Fpo and Frh affects growth on acetate, because neither the Δ*fpoA-O* nor the Δ*frh* single mutant exhibits a growth defect on this substrate. These data seem to suggest that the input module of Fpo (FpoF) can be dispensable under conditions in which the proton-pumping methanophenazine oxidoreductase (FpoABCDHIJKLMNO) is not.

Discussion

Methanosarcina species have proven to be exceptional model organisms for genetic analysis of methanogenesis (14, 28–30), an approach that modified our concept of energy conservation in *M. barkeri*. The data presented here indicate that *M. barkeri* has 2 distinct energy-conserving electron transport pathways during growth via the methylotrophic methanogenic pathway (Fig. 1). Contrary to expectations, *M. barkeri* apparently prefers to transfer electrons obtained from C1 compound oxidation to the H_2:heterodisulfide oxidoreductase system via H_2 rather than to transfer them directly into the $F_{420}H_2$:heterodisulfide oxidoreductase system. Our results suggest that the cytoplasmic F_{420}-reducing hydrogenase mediates electron transfer to H_2 via oxidation of $F_{420}H_2$, which, along with reduced ferredoxin (Fd_{red}), is the direct product of C1 compound oxidation (24, 26). When the cells lose the ability to produce H_2 via this route, growth on methanol is severely affected, with reduction in the rates of growth and CH_4 production, in total growth yield, and in the amount of CH_4 produced. We suggest that the H_2 diffuses out of the cell and enters the H_2:heterodisulfide oxidoreductase system via methanophenazine-dependent hydrogenase (Vht or Vhx) (10), whose active site is known to be in the periplasm (4). Because production of H_2 by Frh consumes protons within the cytoplasm, whereas oxidation of H_2 by Vht/Vhx releases protons outside the cell, this electron transport chain is capable of establishing a proton gradient across the membrane that can be used to generate ATP by the ATP synthase (31), thus conserving energy via a H_2-cycling mechanism (19). *M. barkeri* genome harbors 2 operons, *vhtGACD* and *vhxGAC*, that potentially can encode methanophenazine-dependent hydrogenases. Analogous to the *fre* operon, the *vhx* operon lacks gene *D* that encodes the hydrogenase maturation protease and therefore may not encode a functional hydrogenase. However, Vhx shares all important catalytic and structural amino acid residues with Vht and could encode an active methanophenazine-dependent hydrogenase if the VhtD protease can function *in trans* (10).

H_2 cycling, as a mechanism of energy conservation, was first proposed in sulfate-reducing bacteria (19). Later, it also was proposed in other anaerobic organisms such as *Acetobacterium woodii* (32) and *Geobacter sulfurreducens* (33). Based on the production of H_2 during growth of *Methanosarcina* species on methylated substrates such as methanol, trimethylamine, and acetate, H_2 cycling also was suggested to occur in *Methanosarcina* (34). Experimental support for H_2 cycling has been provided in *Desulfovibrio vulgaris*, wherein simultaneous production and consumption of H_2 were detected during metabolism of pyruvate and sulfate (35). Also, suppression of a *D. vulgaris* H_2-evolving hydrogenase led to reduced growth rates on lactate and sulfate, suggesting the importance of H_2 production in growth (36). Nevertheless, the H_2-cycling theory is not accepted universally, and several lines of evidence have been used to argue that this mechanism is unlikely (reviewed in ref. 37). For example, high concentrations of H_2 do not inhibit lactate oxidation in sulfate-reducing bacteria (38), nor does a mutation that blocks use of H_2 prevent growth on lactate (39). Further, the idea that the cell would transfer electrons preferentially to a molecule that can diffuse freely away from the cell seems highly problematic.

The data presented here suggest a way to reconcile these conflicting views. Although *M. barkeri* apparently prefers to transfer electrons via H_2, they remain capable of using the $F_{420}H_2$:heterodisulfide oxidoreductase system when the ability to produce cytoplasmic H_2 is lost. Thus, *M. barkeri* employs a branched electron transport chain with most electrons flowing from $F_{420}H_2$ into the H_2:heterodisulfide oxidoreductase system via H_2 but with some fraction flowing directly into the

$F_{420}H_2$:heterodisulfide oxidoreductase system. A similar branched electron transport chain in sulfate reducers would explain the results cited as arguing against H_2 cycling without invalidating the model. It should be noted that a recently proposed metabolic model for *D. vulgaris* suggests just such a branched electron transport chain (37).

Why, then, is the H_2-cycling pathway the preferred electron transport chain in *M. barkeri*? Because the initial electron donor ($F_{420}H_2$) and final electron acceptor (methanophenazine) are the same, the amount of energy available from the 2 electron transport schemes must be identical. Experimental measurements suggest that electron transfer from $F_{420}H_2$ to CoM-S-S-CoB via Fpo and Hdr is accompanied by translocation of 4 protons across the membrane (11). Similarly, the flow of electrons from $F_{420}H_2$ to methanophenazine via Frh and Vht/Vhx leads to the translocation of 2 protons across the membrane by virtue of the H_2-cycling mechanism, whereas electron transfer from MPH_2 to CoM-S-S-CoB translocates another 2 protons (9). Thus, the magnitude of proton motive force generated should be identical via either route (Fig. 1). In contrast, the rate of CH_4 production from methanol in resting cell suspensions of Δ*frh* mutant is *ca.* 4 times slower than that of Δ*fpoA-O* and Δ*fpoF* mutants, indicating that H_2 cycling is a much faster mechanism of energy conservation and, as observed in our mutant strains, allows correspondingly faster growth rates. The biochemical properties of the enzymes involved are remarkably consistent with our in vivo data. Thus, FpoBCDIF [molecular weight (MW) = 135.1 kDa] (11) purified from *M. mazei* catalyzes $F_{420}H_2$ oxidation with a turnover number (K_{cat}) of 38 s^{-1} and catalytic efficiency [K_{cat}/Michaelis constant (K_m)] of 5.4 × 10^6 $M^{-1}s^{-1}$ (12); FrhAGB (MW = 194.4 kDa) (27) purified from *M. barkeri* catalyzes H_2 oxidation using F_{420} as the electron acceptor with a K_{cat} of 134 s^{-1} and K_{cat}/K_m of 4.4 × 10^7 $M^{-1}s^{-1}$ (24). Because methanol-metabolizing *M. barkeri* cells have been shown to maintain ratios of $F_{420}H_2$ and F_{420} in thermodynamic equilibrium with the H_2 partial pressure (25), it is reasonable to assume that Frh catalyzes the reverse reaction ($F_{420}H_2$ oxidation) with similar efficiency as the forward reaction (H_2 oxidation). Therefore, the K_{cat} of Frh is *ca.* 4 times higher than that of Fpo for $F_{420}H_2$ oxidation, suggesting that Frh is faster than Fpo in catalyzing this reaction.

A variety of data indicate that H_2 cycling is important to many, but not all, *Methanosarcina* species. Growth of *M. barkeri* on acetate involves the obligate production of H_2 (14). Thus, in combination with the data presented here, it seems likely that this species prefers to use H_2 cycling for all soluble substrates. The situation probably is similar in *M. mazei* because it has functional Frh, Vht, and Fpo enzymes as well (11). However, methylotrophic species such as *M. acetivorans* (10, 21, 28), *Methanolobus tindarius* (40, 41), and *Methanococcoides burtonii* (42) do not encode functional hydrogenases (22). Hence, these organisms probably rely exclusively on the $F_{420}H_2$:heterodisulfide oxidoreductase system for energy conservation. Moreover, we made an intensive effort to delete the *fpo* genes from *M. acetivorans* without success, suggesting that $F_{420}H_2$ dehydrogenase is essential in this organism. It has been suggested previously that these organisms forego H_2-dependent electron transport pathways because of their high-salt marine habitat, wherein they exist as disaggregated single cells and would be prone to lose the freely diffusible H_2 gas to competing organisms. In contrast, freshwater organisms such as *M. barkeri* that exist as large multicellular aggregates have a higher chance of retaining H_2 gas within the aggregates, enabling them to use it as an electron carrier (28).

Finally, the phenotypes of the mutants constructed here also provide insight into the metabolism of H_2/CO_2 and acetate by *M. barkeri*. Because of its ability to catalyze F_{420} reduction with H_2, F_{420}-reducing hydrogenase was proposed to provide $F_{420}H_2$ for reduction of methenyl-tetrahydrosarcinapterin (H_4SPT) and methylene-H_4SPT in the hydrogenotrophic pathway (Fig. S2) (24, 43). The inability of the *frh* mutants to grow on H_2/CO_2 provides direct experimental support for this proposal. Interestingly, the putative *fre*-encoded hydrogenase cannot substitute for the *frh*-encoded hydrogenase. This inability may be caused by low expression of *fre*, absence of posttranslational processing, mutations in structural or catalytic residues, or some combination of these factors (10). Nevertheless, the dispensability of *fre* in *M. barkeri* is not surprising because *M. mazei* lacks the *fre* operon and is able to grow via all 4 methanogenic pathways (4, 10). Thus, the role of Fre in *M. barkeri* remains mysterious.

Methanogenesis from acetate does not require either Frh or Fpo; however, our results show that 1 of the 2 enzymes, but not both, is needed for growth on this substrate. We previously showed that mutations in the C1 oxidation pathway prevent growth on acetate, presumably by blocking the production of reducing equivalents needed for biosynthetic reactions (30). The lack of growth of the Δ*frh*/Δ*fpo* double mutant on acetate medium suggests that these reducing equivalents must flow through either Frh or Fpo to allow growth. Interestingly, the Δ*frh*/Δ*fpoF* double mutant is able to grow on acetate, clearly suggesting that the membrane-bound proton-pumping module has the ability to accept electrons from input modules other than FpoF. In this regard, FpoF is homologous to the β-subunit (B) of F_{420}-reducing hydrogenases and shares a common substrate, coenzyme F_{420} (11, 27). It is conceivable that in the absence of FpoF and FrhB, FreB serves as the input module, thus channeling electrons from $F_{420}H_2$ to Fpo and allowing growth of Δ*frh*/Δ*fpoF* double mutant on acetate; however, this conjecture remains to be tested.

Materials and Methods

Sequence Analysis. All sequence data are from publicly available genomes (11, 16, 20, 21). The Integrated Microbial Genome (IMG) system was used to identify orthologs and assess the genomic context of genes (22).

Strains, Media, and Growth Conditions. The construction and genotype of all *Methanosarcina* strains is presented in Table S2. *Methanosarcina* strains were grown as single cells at 37 °C in high-salt broth medium (44) or on agar-solidified medium as described (45). Standard conditions were used for growth of *E. coli* strains DH5α/λ-*pir* and DH10B (Stratagene) (46), which were used as hosts for plasmid constructions.

DNA Methods, Plasmid, and Strain Construction. Standard methods were used for plasmid DNA isolation and manipulation in *E. coli* (47). Liposome-mediated transformation was used for *Methanosarcina* as described (48). Genomic DNA isolation and DNA hybridization were as described (44, 45, 49). DNA sequences were determined from double-stranded templates by the W.M. Keck Center for Comparative and Functional Genomics, University of Illinois. Plasmid constructions are described in the *SI Text* (Tables S3 and S4).

Characterization of Mutants in Terms of Growth Characteristics and CH_4 and CO_2 Production in Cell Suspensions. Growth was quantified by measuring OD_{600}. Generation times were calculated during exponential growth phase, and growth yield was determined by measuring the maximal OD_{600} of the culture. Growth curve and cell suspension experiments were performed as described in *SI Text*.

Quantitative RT-PCR. Gene-specific primers (Table S3) were designed using Primer Express Software v2.0 (Applied Biosystems). A 1-step qRT-PCR was performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit with ROX (Invitrogen) in the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The relative standard curve method was used to quantify expression of *fpo* (ABI PRISM 7700 Sequence Detection System User Bulletin #2) using *rpoA1* as the reference gene (described in *Supporting Information*).

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