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Differences in Hydrogenase Gene Expression between *Methanosarcina acetivorans* and *Methanosarcina barkeri*▽†

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Methanosarcina acetivorans* C2A encodes three putative hydrogenases, including one cofactor F_{420}-linked (*frh*) and two methanophenazine-linked (*vht*) enzymes. Comparison of the amino acid sequences of these putative hydrogenases to those of *Methanosarcina barkeri* and *Methanosarcina mazei* shows that each predicted subunit contains all the known residues essential for hydrogenase function. The DNA sequences upstream of the genes in *M. acetivorans* were aligned with those in other *Methanosarcina* species to identify conserved transcription and translation signals. The *M. acetivorans vht* promoter region is well conserved among the sequenced *Methanosarcina* species, while the second *vht*-type homolog (here called *vhx*) and *frh* promoters have only limited similarity. To experimentally determine whether these promoters are functional in vivo, we constructed and characterized both *M. acetivorans* and *M. barkeri* strains carrying reporter gene fusions to each of the *M. acetivorans* and *M. barkeri* hydrogenase promoters. Generally, the *M. acetivorans* gene fusions are not expressed in either organism, suggesting that *cis*-acting mutations inactivated the *M. acetivorans* promoters. The *M. barkeri* hydrogenase gene fusions, on the other hand, are expressed in both organisms, indicating that *M. acetivorans* possesses the machinery to express hydrogenases, although it does not express its own hydrogenases. These data are consistent with specific inactivation of the *M. acetivorans* hydrogenase promoters and highlight the importance of testing hypotheses generated by using genomic data.

Methanogens are a phylogenetically diverse group of archaea, but the number of substrates that they use for growth and methanogenesis is limited. Using very similar central metabolic pathways, some methanogens utilize H_2-CO_2, while others use acetate or methylated compounds. *Methanosarcina* is the only genus that contains members capable of utilizing all these substrates, whereas most methanogens can use only one substrate. Nevertheless, not all *Methanosarcina* species are capable of using all methanogenic substrates. Instead, there is significant diversity within the genus *Methanosarcina* with respect to which substrates are utilized. To our knowledge, every *Methanosarcina* species isolated to date is capable of growth on methanol and other methylated compounds, and most species can use acetate; however, the ability to use H_2-CO_2 is less widespread. Interestingly, the ability to utilize H_2 seems to correlate with the environments from which individual species were isolated. Accordingly, the majority of *Methanosarcina* species isolated from freshwater environments, such as *Methanosarcina barkeri*, are capable of utilizing H_2-CO_2 (18–20, 29, 32, 44). The majority of marine *Methanosarcina* isolates, such as isolates of *Methanosarcina acetivorans*, do not grow on H_2-CO_2 (9, 23, 24, 30, 38, 39, 45, 51).

The ability of methanogens to utilize H_2 as a reductant requires a class of phylogenetically related enzymes called hydrogenases. In *Methanosarcina*, all hydrogenases are Ni-Fe enzymes that contain two core subunits, termed the large and small subunits (50). The large subunit contains two motifs that coordinate the Ni-Fe active site, RXCGXCXXXH and DPC XXCXXH. The small subunit contains 4 to 10 conserved Cys residues that coordinate Fe-S clusters.

The characterized Ni-Fe hydrogenases require posttranslational modification to become enzymatically active (3). While this modification has not been examined in *Methanosarcina*, it has been extensively studied in *Escherichia coli*, where the gene products of *hypABCDE* and *hypF* are responsible for insertion of Ni and Fe into the hydrogenase active site and coordination of —C≡O and —C≡N groups to the Fe. Homologs of these genes are found in each of the sequenced *Methanosarcina* genomes, suggesting that posttranslational activation occurs by similar mechanisms in these organisms.

The biochemical and physiological roles of hydrogenases have been studied in some detail in *M. barkeri* and *Methanosarcina mazei*. The Ech hydrogenase of *M. barkeri* has been purified and characterized. This enzyme is ferredoxin dependent and involved in coupling an electrochemical gradient to the reduction of CO_2 to formyl-methanofuran (35, 47). Deletion of *ech* eliminates growth on acetate and H_2-CO_2 due to blocks in methanogenesis and prevents growth on methanol-H_2-CO_2 due to a biosynthetic block (36).

The Frh hydrogenase, which has also been purified from *M. barkeri*, couples oxidation of H_2 to reduction of cofactor F_{420} (11). During growth on H_2-CO_2, Frh provides the reducing equivalents (via $F_{420}H_2$) for reduction of methenyl tetrahydromethanopterin (methenyl-H_4MPT) to methylene-H_4MPT

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and for reduction of methylene-H_4MPT to methyl-H_4MPT. *M. barkeri frh* is expressed during growth on H_2-CO_2, as expected. However, it is also expressed during growth on methanol (49), where its function is unknown. Molecular analysis revealed the presence of a second F_{420}-reducing hydrogenase operon (encoding a protein with ca. 86 to 88% amino acid identity to *M. barkeri* Frh) in *M. barkeri*, designated *fre*, but the physiological role of this second operon is also unknown (49).

A third type of hydrogenase has been characterized from *M. mazei*. The so-called F_{420}-nonreducing hydrogenase (also called viologen-reducing hydrogenase) transfers electrons from H_2 to methanophenazine, which is the electron donor for reduction of the heterodisulfide of coenzyme M and coenzyme B to the free thiols of coenzyme M and coenzyme B (48). The *M. mazei* genome was found to contain operons encoding two copies of this hydrogenase, named *vho* and *vht* (whose products exhibited ca. 95% amino acid sequence identity). Transcription of *vho* occurs during growth on methanol, trimethylamine (TMA), H_2-CO_2, and acetate, while *vht* is transcribed during growth on methanol, TMA, and H_2-CO_2 but not during growth on acetate (6, 7). The relative role of each methanophenazine-reducing hydrogenase during methanogenesis has yet to be addressed.

Recent genome sequencing demonstrated that *M. acetivorans* has genes encoding three hydrogenases in its chromosome (*frh* and two *vht*-type hydrogenase genes), but the role of these hydrogenases in an organism incapable of growth on H_2-CO_2 is unclear (12). Furthermore, *M. acetivorans* does not produce detectable levels of hydrogenase during growth on methanol or methanol-H_2-CO_2 despite the fact that the operons are present in the chromosome (13). The reason for this discrepancy has not been addressed yet. Consistent with this observation, early proteomic studies failed to detect any of the hydrogenase subunits or maturation proteins, while nonquantitative microarray experiments revealed the presence of transcripts only for *vhtG* and *hypA* and not for other genes in these putative transcriptional units (26–28). A later study found a single peptide that could be attributed to VhtA but, again, no peptide from any other hydrogenase subunit or maturation protein (25). These data may explain previous studies showing that *M. acetivorans* produces barely detectable levels of hydrogenase activity in crude cell extract during growth on acetate (37). The reason that *M. acetivorans* displays hydrogenase activity during growth on acetate but not during growth in the presence of H_2 is also unclear.

The availability of the *M. acetivorans*, *M. mazei*, and *M. barkeri* genome sequences (8, 12, 31) allows in silico analysis of the underlying reasons for the lack of H_2 metabolism in *M. acetivorans*. Hypotheses generated from genomic data can then be tested in vivo using recently developed genetic techniques (43). In this study, *M. acetivorans* predicted hydrogenase amino acid sequences and promoters were compared to analogous regions in *M. barkeri* and *M. mazei* to gain insight into possible mechanisms of *M. acetivorans* hydrogenase inactivation. Reporter gene fusions to each hydrogenase promoter from *M. barkeri* and *M. acetivorans* were then examined to test the expression of the operons in vivo.

MATERIALS AND METHODS

Sequence analysis. All sequence data used are data from previously published or publicly available genomes (8, 10, 12, 21, 31). DNA sequences were aligned using default ClustalW settings from Biology Workbench (http://workbench.sdsc.edu). Protein sequences were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/). Phylogeny was analyzed in PAUP* 4.0b10 using the maximum parsimony method with exhaustive sampling of tree topologies and 1,000 bootstrap replicates.

TABLE 1. Plasmids used

Plasmid	Purpose	Reference or source
pAMG82	Parent plasmid for promoter fusions with ATG start site	14
pAMG84	*M. barkeri vht* promoter fusion to *uidA*	This study
pAMG85	*M. acetivorans vht* promoter fusion to *uidA*	This study
pAMG89	*M. acetivorans hyp* promoter fusion to *uidA*	This study
pAMG90	*M. barkeri hyp* promoter fusion to *uidA*	This study
pAMG91	*M. acetivorans vhx* promoter fusion to *uidA*	This study
pAMG92	*M. acetivorans frh* promoter fusion to *uidA*	This study
pAMG93	*M. barkeri frh* promoter fusion to *uidA*	This study
pAMG94	*M. barkeri fre* promoter fusion to *uidA*	This study
pAMG99	*M. barkeri ech* promoter fusion to *uidA*	This study
pAMG101	*M. barkeri vhx* promoter fusion to *uidA*	This study

Growth of *Methanosarcina* strains. *Methanosarcina* strains were grown as single cells (46) in anaerobic tubes sealed with butyl rubber stoppers at 37°C on HS media (34). In liquid media, 50 mM TMA, 120 mM acetate, or 125 mM methanol was provided as a growth substrate under an N_2-CO_2 (80:20) headspace. H_2-CO_2 (80:20) was provided for growth at an overpressure of 140 kPa when it was combined with methanol and at an overpressure of 220 kPa when it was used as the sole growth substrate. Growth on agar-solidified media has been described previously (4, 46). Puromycin was added to a final concentration of 2 μg ml^{-1} to select for the presence of *pac* (40). 8-Aza-2,6-diaminopurine was added to a final concentration of 20 μg ml^{-1} to select against the presence of *hpt* (41).

Plasmids and strains. Standard techniques were used for plasmid manipulation and isolation in *E. coli* (1). Relevant plasmids and their functions are listed in Table 1. *E. coli* WM3118 was used as the host strain for plasmids containing *oriV*, so that copy number could be induced prior to plasmid purification (14). BW25141 was the host strain for Π-dependent plasmids (15). DH10B was the host strain for all other plasmids (Invitrogen, Carlsbad, CA). Promoter fusion plasmids were constructed by first PCR amplifying ca. 1 kb of DNA upstream of the translational start site of the first gene of each operon of interest. This PCR fragment was then cloned in frame into pAMG82, creating a translational fusion to *uidA* using ATG as the start site (14).

Construction of *Methanosarcina* strains. Promoter fusion plasmids were transformed into *M. acetivorans* WWM82 (Δ*hpt*::ϕC31*int-attP*) and *M. barkeri* WWM85 (Δ*hpt*::ϕC31*int-attP*) (14) via liposome-mediated transformation (4, 33). These nonreplicating plasmids integrated into the *M. acetivorans* and *M. barkeri* chromosomes via ϕC31 Int-mediated site-specific recombination. Promoter fusions were verified to be integrated into each strain as single copies using the four-primer PCR screen as described previously (14).

During the course of this study, we determined that the *M. acetivorans* transformation efficiency was higher when organisms were grown on 50 mM TMA or 50 mM methanol than when they were grown on 125 mM methanol (A. Bose and W. W. Metcalf, unpublished data). Therefore, while *M. barkeri* strains were constructed using either 125 mM or 50 mM methanol as the growth substrate on agar plates, some *M. acetivorans* strains were constructed using methanol and some *M. acetivorans* strains were constructed using TMA. No phenotypic differences were observed between strains isolated on 125 mM methanol, strains isolated on 50 mM methanol, and strains isolated on 50 mM TMA.

β-Glucuronidase activity determination. Strains were adapted to each growth substrate (without puromycin) for at least 15 generations prior to measurement. Triplicate cultures were measured on at least two separate days. β-Glucuronidase activity levels were determined as described previously (42). UV-visible absorbance spectra were recorded with a Hewlett Packard 8453 diode array spectrophotometer. β-Glucuronidase activity was calculated by following cleavage of *p*-nitrophenyl-β-D-glucuronide at 415 nm (12,402 mM^{-1} cm^{-1}). The protein concentration was determined by the Bradford method (5), using bovine serum albumin as the standard. The limit of detection is 0.4 mU mg $protein^{-1}$.

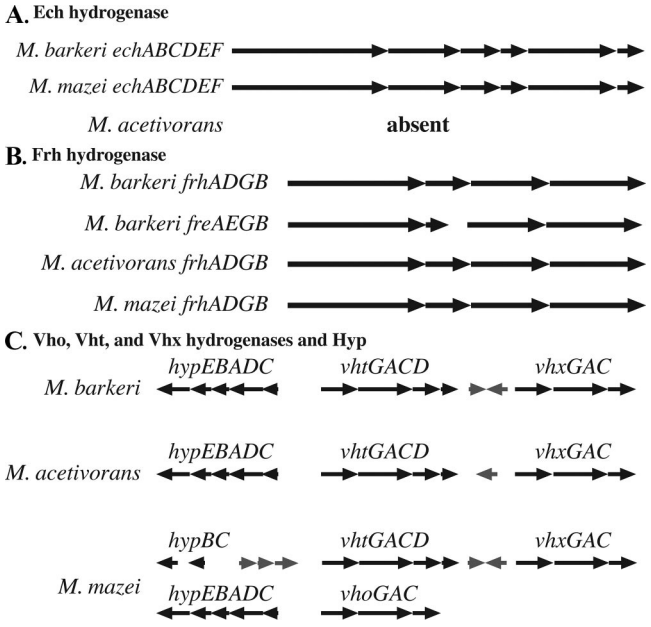

FIG. 1. Alignments of *Methanosarcina* genomic regions encoding hydrogenases. The black arrows represent hydrogenase and hydrogenase-related genes. All other genes are represented by gray arrows.

RESULTS

***M. acetivorans* lacks *ech* but contains *frh*, *vht*, and *vhx* hydrogenase genes.** Three types of hydrogenases are thought to be required for growth of *Methanosarcina* on H_2-CO_2: a ferredoxin-reducing Ech hydrogenase, an F_{420}-reducing hydrogenase, and a so-called F_{420}-nonreducing (methanophenazine-reducing) hydrogenase. *M. acetivorans* was first hypothesized to not grow on H_2-CO_2 due to the lack of *ech* (12). However, more recent studies indicate that *M. acetivorans* does not produce any functional hydrogenase when it is grown on methanol or methanol-H_2-CO_2 (13). To address the mechanism of inactivation of the *M. acetivorans* hydrogenases, genomic regions containing putative hydrogenase genes were aligned to compare the *M. acetivorans* sequences to those of *M. barkeri* and *M. mazei*, both of which grow on H_2-CO_2 (Fig. 1).

While *M. acetivorans* lacks genes encoding the Ech hydrogenase, which is present in both *M. barkeri* and *M. mazei* (Fig. 1A), each sequenced *Methanosarcina* isolate contains one full F_{420}-reducing hydrogenase operon (*frh*) (Fig. 1B). *M. barkeri* has a second copy of the F_{420}-reducing hydrogenase (*fre*) operon, which does not encode a homolog of the maturation peptidase FrhD (49). It seems possible, given the very high levels of amino acid identity, that the Fre hydrogenase could be processed by FrhD and therefore may represent a functional hydrogenase. However, the second copy may not be required for growth on H_2-CO_2, because *M. mazei* grows on H_2-CO_2 with only a single *frh* operon.

M. mazei was reported previously to contain two operons encoding the F_{420}-nonreducing hydrogenase, designated *vho* and *vht* (*v*iologen *h*ydrogenase *o*ne and *v*iologen *h*ydrogenase *t*wo), which are ca. 95% identical to each other (7). However, *Methanosarcina* genome sequences reveal a more complex scenario (Fig. 1C). *M. mazei* also contains a third, more divergent operon homologous to *vho* and *vht*. Here, we refer to this third operon as *vhx* (for *v*ho-like *h*ydrogenase with unknown [*x*] function). *M. barkeri* and *M. acetivorans* each encode two F_{420}-nonreducing hydrogenases: one hydrogenase most similar to the *vho*/*vht*-encoded hydrogenase and one hydrogenase most similar to the *vhx*-encoded hydrogenase. Phylogenetic analysis of the predicted protein sequences indicates that Vho and Vht group together to the exclusion of Vhx (Fig. 2) and that Vho likely arose from a recent duplication within the *M. mazei* lineage. Interestingly, the *M. mazei vho* and *vhx* operons each lack a homolog of *vhtD*, encoding the putative maturation

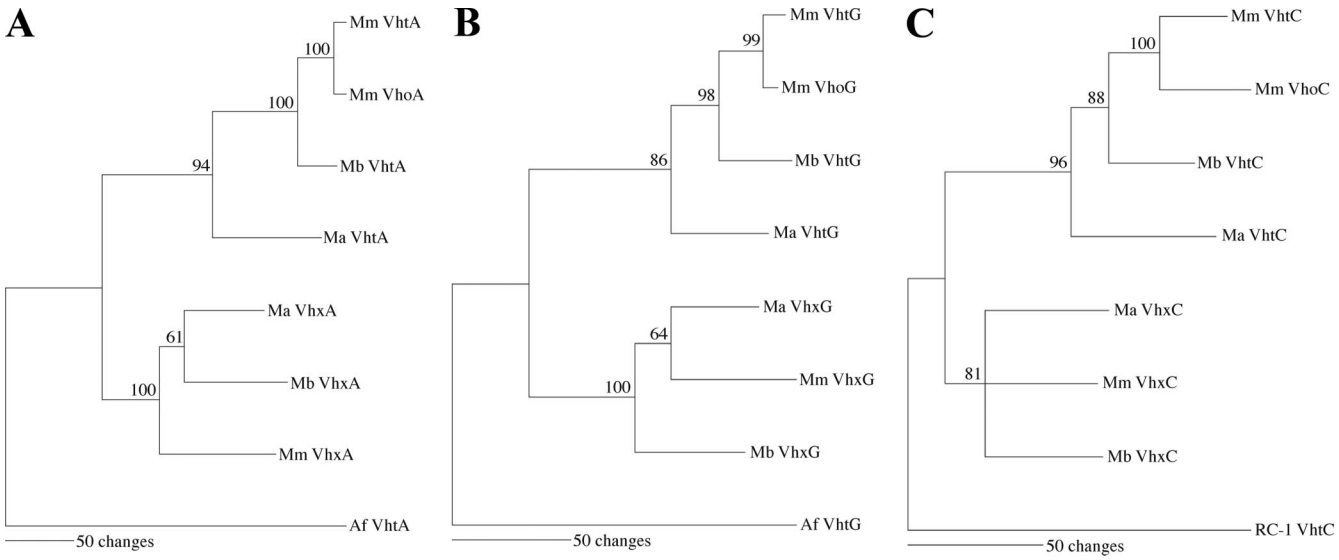

FIG. 2. Maximum parsimony phylogenetic trees for *Methanosarcina* Vht and Vhx subunits. Mm, *M. mazei*; Mb, *M. barkeri*; Ma, *M. acetivorans*; RC-I, rice cluster I methanogen; Af, *Archaeoglobus fulgidus*. Bootstrap scores greater than 50 are indicated at the nodes. (A) VhtA/VhxA subunits; (B) VhtC/VhxC subunits; (C) VhtG/VhxG subunits.

peptidase, which should be required for posttranslational maturation of the hydrogenase enzyme.

Just upstream of *vht*, *M. barkeri* contains a putative *hyp* operon, which is presumed to be required for posttranslational modification of the Ni-Fe hydrogenases (Fig. 1C). This location of *hyp* is conserved in *M. acetivorans*. In *M. mazei*, *hyp* is adjacent to *vho* instead of *vht-vhx*. An additional copy of *hypB* and *hypC* is located upstream of *M. mazei vht*.

Known active site residues are conserved in *M. acetivorans* hydrogenases. *M. acetivorans* produces no detectable hydrogenase enzyme activity under most growth conditions (13). Numerous types of changes could account for this inactivation, including disruption of transcription or translation of hydrogenase genes, mutation of residues essential for enzyme function, lack of posttranslational modification of the enzymes, or a combination of the these factors. To address the mechanism of inactivation, the inferred amino acid sequences of hydrogenase subunits (encoded by *ech*, *frh*, *vht*, and *vhx*) from *M. barkeri*, *M. acetivorans*, and *M. mazei* were first aligned to determine if important amino acid residues are conserved.

While the *M. acetivorans* genome does not encode an Ech hydrogenase, Ech hydrogenase subunits from *M. barkeri* and *M. mazei* show 82% to 97% identity. All known essential residues are present in each strain (see Fig. S1 in the supplemental material). EchA and EchB are predicted integral membrane proteins thought to be involved in ion translocation. EchC is the hydrogenase small subunit, which contains four Cys residues conserved in all hydrogenase small subunits. EchE is the hydrogenase large subunit and contains the conserved RXCGXCXXXH and DPCXXCXXH motifs that coordinate the hydrogenase Ni-Fe center. EchF is a polyferredoxin and contains two prototypical [4Fe-4S] cluster-binding sites. Conserved acidic amino acids in EchA, EchB, EchC, EchE, and EchF are thought to be involved in coupling electron transfer to ion translocation (16, 22; R. Hedderich, personal communication).

In Frh, all known essential active site residues are conserved in *M. acetivorans*, as well as in *M. barkeri* and *M. mazei* (see Fig. S2 in the supplemental material). FrhA is the hydrogenase large subunit and contains the conserved RXCGXCXXXH and DPCXXCXXH Ni-coordinating residues. FrhD is a putative maturation peptidase and contains the proposed catalytic Asp residue. FrhG is the hydrogenase small subunit and includes two conserved [4Fe-4S] motifs and six other conserved Cys residues thought to coordinate metal centers. FrhB is the F_{420}-binding subunit. Furthermore, pairwise comparisons of each subunit reveal that *M. acetivorans* Frh is not more divergent than the *M. barkeri* and *M. mazei* copies (see Table S1 in the supplemental material). Thus, there is no obvious reason that the *M. acetivorans frh* operon should not produce active hydrogenase. Nevertheless, it is still possible that other nonobvious mutations may inactivate the Frh hydrogenase.

Essential amino acid residues are also generally conserved in *M. acetivorans* Vht and Vhx. VhtG, the hydrogenase small subunit, contains 11 conserved Cys residues (see Fig. S3 in the supplemental material), 10 of which are presumed to coordinate Fe-S centers (7). The VhtG homologs of each organism contain most of the $CXXCX_nGXCXXXGX_mGCPP$ motif commonly found in hydrogenase small subunits but lack the final Pro residue. Interestingly, the VhxG homologs from *M. barkeri* and *M. mazei* also lack the first Cys residue in the motif, while it is present in *M. acetivorans* VhxG. The ability of *M. barkeri* and *M. mazei* Vhx to function as a hydrogenase in the absence of this Cys residue is not known. Another interesting feature, conserved in each sequence of subunit G, is the Tat signal peptide ($RRXFXKX_{18}V/AXA$), indicating that this hydrogenase is probably localized to the periplasmic side of the membrane (2, 7). VhtA/VhxA is the hydrogenase large subunit and contains the RXCGXCXXXH and DPCXXCXXH Ni-binding motifs. VhtC/VhxC is the cytochrome *b* subunit, which contains two His residues proposed to coordinate heme (7). However, the second His residue is not conserved in VhxC, while several other His residues are conserved. Therefore, one of the other His residues may coordinate heme instead. VhtD is predicted to be a maturation peptidase that contains a conserved, catalytic Asp residue. Like Frh/Fre discussed above, it seems possible that the VhtD peptidase may process each of the Vht, Vho, and Vhx hydrogenases.

The level of identity was calculated for each pairwise set of Vht and Vhx subunits (see Table S2 in the supplemental material). While the levels of identity between Frh subunits are approximately equal for all three organisms, *M. acetivorans* Vht is more divergent from *M. barkeri* and *M. mazei* Vht, and the levels of identity are 8 to 16% lower between *M. acetivorans* and either *M. barkeri* or *M. mazei* than between *M. barkeri* and *M. mazei*. Despite this divergence, *M. acetivorans* Vht and Vhx contain all of the proposed active site residues and *vht* and *vhx* may encode functional hydrogenases.

The *vht* and *ech* promoters are well conserved, while the *frh* and *vhx* promoters are not well conserved. The lack of obvious inactivating mutations in the important catalytic and structural residues in the hydrogenase-encoding regions suggests that the *M. acetivorans* hydrogenase operons have the potential to encode functional proteins. Therefore, the putative promoter region of each operon (ca. 1 kb upstream of each translational start site) was examined for conserved sequences involved in transcription and translation initiation. Although we have not mapped the transcription start sites of these operons, we are not aware of any archaeal transcriptional units whose start sites are not within 1 kbp of the coding sequence.

The *echA* genes from *M. barkeri* and *M. mazei* contain a conserved ATG start site and a putative ribosome binding site (RBS) (see Fig. S4 in the supplemental material). While the *ech* promoters have not been characterized, a putative TATA box and BRE element are present.

The *frh* promoters show very little conservation between the organisms (see Fig. S5 in the supplemental material). The TTG translational start site is the only portion that is absolutely conserved. The sequence for the putative RBS is conserved, but the spacing is different for the two *M. barkeri* promoters compared to the *M. mazei* and *M. acetivorans* promoters. Neither characterized *M. barkeri* transcriptional start site (49) is conserved in either *M. mazei* or *M. acetivorans*.

The *vht* promoters, on the other hand, are highly conserved in all three *Methanosarcina* strains, from the putative BRE element to the start site (see Fig. S6 in the supplemental material). Using the characterized *M. mazei* promoters as a reference, all four promoters share a conserved ATG start site, RBS, transcriptional start site, TATA box, and BRE element.

Unlike the *vht* promoters, the *vhx* promoters share only

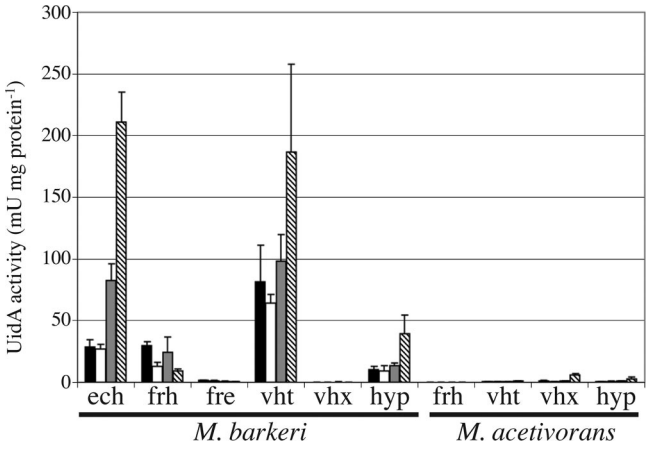

FIG. 3. Hydrogenase promoter activity in *M. barkeri*. β-Glucuronidase activity (UidA activity) was measured for *M. barkeri* strains carrying single-copy reporter gene fusions to each *M. barkeri* and *M. acetivorans* hydrogenase promoter. The growth substrates used were methanol (black bars), methanol plus H_2 (open bars), H_2-CO_2 (gray bars), and acetate (striped bars).

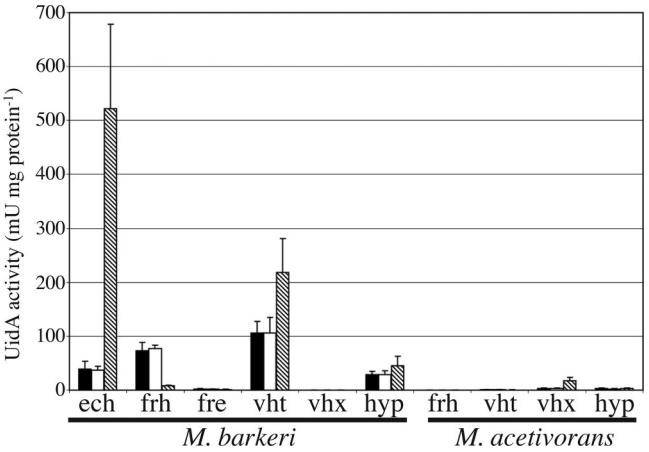

FIG. 4. Hydrogenase promoter activity in *M. acetivorans*. β-Glucuronidase activity (UidA activity) was measured for *M. acetivorans* strains carrying single-copy reporter gene fusions to each *M. barkeri* and *M. acetivorans* hydrogenase promoter. The growth substrates used were methanol (black bars), methanol plus H_2 (open bars), and acetate (striped bars).

limited identity near the putative translational start site (see Fig. S7 in the supplemental material). No putative RBS could be identified upstream of either potential translational start site, and the transcriptional start site has not been identified.

***M. barkeri* hydrogenase promoters are functional, while *M. acetivorans* hydrogenase promoters are nonfunctional.** The analysis described above shows that the lack of hydrogenase activity in *M. acetivorans* extracts is not due to obvious mutations in either the coding regions or the promoters. To experimentally test the functionality of the *Methanosarcina* hydrogenase promoters, translational fusions to *uidA* to the first genes in each operon from *M. barkeri* and *M. acetivorans* were constructed. For *frh* and *fre* the promoter fusions were constructed using an ATG start site rather than the native TTG translational start site. The 10 *uidA* fusions (*M. acetivorans* P*vht*, P*vhx*, P*frh*, and *hyp* and *M. barkeri* P*ech*, P*vht*, P*vhx*, P*frh*, P*fre*, and P*hyp*) were inserted into both the *M. barkeri* chromosome and the *M. acetivorans* chromosome via ϕC31 Int-mediated site-specific integration (14).

In *M. barkeri*, the *M. barkeri* P*hyp*, P*vht*, P*ech*, and P*frh* promoters are all expressed on all substrates (Fig. 3). P*hyp* and P*vht* are expressed at the same level on methanol or methanol-H_2-CO_2, but they are upregulated ca. twofold on acetate. P*ech* is also expressed at the same level on methanol and methanol-H_2-CO_2, but it is upregulated ca. 10-fold on acetate. P*frh*, on the other hand, is expressed at similar levels on H_2-CO_2 and methanol, but it is downregulated two- to threefold both on acetate and methanol-H_2-CO_2. *M. barkeri* P*fre* is expressed at only a very low level and is downregulated on acetate, like P*frh*. No expression was detected for *M. barkeri* P*vhx*. The *M. acetivorans* promoter fusions in *M. barkeri* were expressed at a level near or below the background level for nearly all fusions on each substrate. The exception is *M. acetivorans* P*vhx*, which was upregulated ca. fivefold on acetate.

The same pattern was generally seen when these promoter fusions were inserted into the *M. acetivorans* chromosome (Fig. 4). The exception to this pattern is the expression of *M. barkeri* P*frh* on methanol-H_2-CO_2. In *M. barkeri*, this promoter is downregulated on methanol-H_2-CO_2 compared to the expression on methanol alone. In *M. acetivorans*, the expression levels were the same on methanol with and without H_2-CO_2. Additionally, the regulation is more drastic in *M. acetivorans*: *M. barkeri frh* expression is 10-fold lower on acetate than on methanol. These data are fully consistent with previous proteomic and microarray experiments showing that the levels of expression of the *M. acetivorans* hydrogenases and maturation proteins are at or below the limit of detection (25–28).

DISCUSSION

The data presented here provide additional mechanistic evidence regarding the inability of *M. acetivorans* to metabolize hydrogen. The previously published genome sequence established that *ech* is not present, presumably due to deletion (12). However, genetic studies clearly showed that the lack of *ech* alone did not explain the dearth of hydrogen metabolism in this organism (13). Here we show that the remaining *M. acetivorans* hydrogenases have been inactivated at the level of transcription and/or translation.

Several mechanisms could be envisioned for this hydrogenase inactivation. For example, the *M. acetivorans* promoters could be inactivated by a *trans*-acting factor, such as a nonfunctional activator or a constitutive repressor. If this occurs, one would expect all 10 hydrogenase promoters to be inactive in *M. acetivorans* and active in *M. barkeri* (which clearly expresses its own hydrogenases). Alternatively, each *M. acetivorans* promoter could be inactivated via *cis*-acting mutations, resulting in genes that are not transcribed. If this model were correct, all the *M. acetivorans* promoters should be nonfunctional in both *M. acetivorans* and *M. barkeri*, while the *M. barkeri* promoters should be active in both *M. acetivorans* and *M. barkeri*. Because the *M. acetivorans* promoters showed similarly low levels of activity in both *M. acetivorans* and *M. barkeri*, while the *M. barkeri* promoters were expressed at similar

levels in both organisms, it seems that the latter mechanism is present; i.e., the *M. acetivorans* genes are nonfunctional due to *cis*-acting mutations in either transcriptional or translational sequence elements. Nevertheless, the mutations resulting in this inactivation are not always obvious.

The *M. acetivorans vht* promoter shares the conserved BRE element, TATA box, +1 transcriptional start site, RBS, and ATG translational start site that have been characterized in *M. mazei*. One explanation for the lack of *vht* expression in *M. acetivorans* could be that this promoter has lost either the binding site for a transcriptional activator or a required secondary structure in the mRNA somewhere in the nonconserved region. The *M. acetivorans frh* promoter, on the other hand, has little, if any, similarity to the *M. barkeri* and *M. mazei* promoters, which are also dissimilar to each other. Thus, the sequence motifs needed for *frh* expression cannot be determined by such a comparative approach. Because the *M. barkeri* and *M. mazei frh* promoters are so dissimilar, *M. mazei* may use a different regulatory mechanism for *frh* than *M. barkeri* uses.

Based upon operon structure and proximity to *vhx*, we refer to the operon encoding the first *M. barkeri* methanophenazine-reducing hydrogenase as *vht* rather than *vho*, but interestingly, the expression of *M. barkeri vht* more closely resembles the reported expression pattern of *M. mazei vho*. In *M. mazei*, *vho* is expressed on methanol, acetate, and H_2-CO_2, while *vht* is not expressed on acetate (6). The fact that the *M. mazei vho* and *vht* promoters share so many features but are expressed differently indicates that another level of regulation has yet to be elucidated. This regulation may be absent from *M. acetivorans*. Because *vhtD* encodes the putative maturation peptidase, it is unclear whether *M. mazei vho* can produce a functional hydrogenase during growth on acetate in the absence of *vhtD* expression.

The function of *vhx* is still cryptic. The Vhx gene is present in the genomes of all three sequenced *Methanosarcina* species, indicating it was in the genome since before the organisms diverged from each other. All known essential Vht residues are present in Vhx with the exception of the first conserved Cys in VhxG in *M. barkeri* and *M. mazei*. Thus, whether Vhx can function as a hydrogenase also remains unclear. However, *M. barkeri vhx* does not seem to be expressed. Therefore, either very low levels are required or Vhx no longer has a function in *M. barkeri* under laboratory conditions.

In contrast, *vhx* seems to be the only *M. acetivorans* hydrogenase operon expressed (albeit at a low level), and it is upregulated on acetate. Furthermore, *M. acetivorans* VhxG is the only VhxG that contains the first conserved Cys present in VhtG. Taken together, these findings may explain the very low levels of hydrogenase detected in *M. acetivorans* during growth on acetate (37) but not during growth on methanol or methanol-H_2-CO_2 (13). Presuming that Vhx requires proteolytic processing like other hydrogenases, it is also unclear what protein would perform this cleavage in the absence of *vht* expression. Therefore, the physiological role of Vhx in *M. acetivorans* remains unclear as well.

The differential regulation of *M. barkeri frh* suggests that *M. barkeri* and *M. acetivorans* can both sense a flux of carbon between CO_2 and methyl-H_4MPT. Accordingly, we observed that the expression of *M. barkeri* P*frh* is decreased on acetate in both organisms but is decreased on the combination of methanol, H_2, and CO_2 only in *M. barkeri.* A commonality between *M. barkeri* growth on acetate and *M. barkeri* growth on methanol-H_2-CO_2 is a decrease in this flux of carbon between CO_2 and methyl-H_4MPT. Methanogenesis from acetate or methanol-H_2-CO_2 does not require this portion of the pathway, and it may be relegated to a strictly biosynthetic role (13). Indeed, the levels of many of the enzymes in this portion of the pathway are reduced during growth on acetate (17). During methanogenesis from methanol, on the other hand, 25% of the methyl groups are oxidized to CO_2 via this pathway, while all carbon passes through this pathway during methanogenesis from H_2-CO_2. Therefore, a decrease in the concentration of one of these methanogenic intermediates during growth on acetate and on methanol-H_2-CO_2 may serve as the signal to downregulate expression of *frh*. In *M. acetivorans*, however, one would not expect to see this effect on methanol-H_2-CO_2 because the organism does not use H_2. Thus, even in the presence of H_2, methanol is still presumably oxidized via this pathway, and the level of *M. barkeri* P*frh* expression would be predicted to be high. Instead, flux through this pathway should decrease in *M. acetivorans* only during growth on acetate, resulting in decreased *M. barkeri* P*frh* expression.

Like the physiological role of *vhx*, the physiological role of *fre* is unknown. This work suggests that *M. barkeri fre* has minor importance in *M. barkeri* due to a low level of expression. Furthermore, *M. mazei* grows on H_2-CO_2 without *fre*, so *fre* must not be absolutely required. It is therefore not surprising that *fre* is expressed at a much lower level than *frh*, implying that it has decreased importance under laboratory conditions. Similar to Vhx, Fre lacks a homolog of the maturation peptidase encoded by *frhD*. Therefore, Fre functionality may be strictly dependent on the presence of the *frh* operon for post-translational processing.

Finally, this study demonstrates the importance of testing the hypotheses derived from genome sequences. Based solely on a comparison of genomic data, *M. acetivorans* was predicted to produce a hydrogenase, implying that H_2 could play a significant role in its physiology. While no deleterious mutations were found within the coding region of the *M. acetivorans* hydrogenase genes, biochemical, genetic, and now expression data suggest that these genes are inactive. In support of this idea, we have been unable to transform *M. acetivorans* with plasmids encoding the active Vht hydrogenase from *M. barkeri*, suggesting that *M. acetivorans* cannot tolerate Vht hydrogenase expression, at least under the conditions tested (data not shown). It should be noted, however, that it remains possible that we simply have not yet determined appropriate conditions needed to elicit expression of the *M. acetivorans* genes. If such conditions exist, then it is clear that the regulatory mechanism of the *M. acetivorans* genes is distinct from that of the orthologous *M. barkeri* genes. If they are never expressed, they should be considered pseudogenes despite the lack of obvious deleterious mutations in the coding regions.

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