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Methanogenesis by *Methanosarcina acetivorans* involves two structurally and functionally distinct classes of heterodisulfide reductase

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Summary

Biochemical studies have revealed two distinct classes of Coenzyme B-Coenzyme M heterodisulfide (CoB-S-S-CoM) reductase (Hdr), a key enzyme required for anaerobic respiration in methane-producing archaea. A cytoplasmic HdrABC enzyme complex is found in most methanogens, whereas a membrane-bound HdrED complex is found exclusively in members of the order *Methanosarcinales*. Unexpectedly, genomic data indicate that multiple copies of both Hdr classes are found in all sequenced *Methanosarcinales* genomes. The *Methanosarcina acetivorans hdrED1* operon is constitutively expressed and required for viability under all growth conditions examined, consistent with HdrED being the primary Hdr. HdrABC appears to be specifically involved in methylotrophic methanogenesis, based on reduced growth and methanogenesis rates of an *hdrA1C1B1* mutant on methylotrophic substrates and downregulation of the genes during growth on acetate. This conclusion is further supported by phylogenetic analysis showing that the presence of *hdrA1* in an organism is specifically correlated with the presence of genes for methylotrophic methanogenesis. Examination of mRNA abundance in methanol-grown *ΔhdrA1C1B1* strains relative to wild-type revealed upregulation of genes required for synthesis of (di)methylsulfide and for transport and biosynthesis of CoB-SH and CoM-SH, suggesting that the mutant has a defect in electron transfer from ferredoxin to CoB-S-S-CoM that causes cofactor limitation.

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Introduction

Methanogens are single-celled organisms of the domain Archaea that are responsible for biological methane production (Rouviere and Wolfe, 1988). They grow by anaerobically converting simple inorganic C sources thought to be abundant in early Earth environments (CO_2, CO, acetate, methylamines, methylsulfides, methanol) to methane gas. The metabolism of these organisms is therefore thought to represent an ancient strategy for conserving energy. Most methanogens are restricted to the use of $H_2 + CO_2$; however, members of the order *Methanosarcinales* are able to use CO, acetate, methylamines, methylsulfides and methanol in addition to $H_2 + CO_2$. Regardless of the substrate used, all methanogens produce a Coenzyme B-Coenzyme M (CoB-S-S-CoM) heterodisulfide molecule in the last step of methanogenesis (CH_3-S-CoM + CoB-SH→CH_4 + CoB-S-S-CoM) (Bobik *et al.*, 1987; Bobik and Wolfe, 1988). CoB-S-S-CoM subsequently must be reduced to regenerate the CoB-SH and CoM-SH thiols that are used as electron donors by methyl-CoM reductase and methyl-CoM methyltransferases. This regeneration reaction (CoB-S-S-CoM + $2e^-$ + $2H^+$→CoB-SH + CoM-SH) is catalysed by the enzyme heterodisulfide reductase (Hdr).

Two distinct classes of Hdr are known. Purification of Hdr activity from *Methanosarcina barkeri* resulted in identification of a cytochrome-containing, two-subunit Hdr complex that localizes to the membrane, HdrED (Heiden *et al.*, 1994) (Fig. 1A). HdrED operates much like a bacterial terminal oxidoreductase: it is an integral membrane complex and the electron donor for HdrED is reduced methanophenazine, a quinone-like electron carrier localized to the membrane. Reduction and oxidation of methanophenazine produces a proton-translocating oxidation-reduction loop (analogous to the bacterial Q-loop). CoB-S-S-CoM reduction by HdrED results in net consumption of cytoplasmic protons and therefore contributes to production of a chemiosmotic gradient across the cell membrane.

Purification of Hdr activity from *Methanothermobacter thermoautotrophicum* ΔH and *Methanothermobacter marburgensis* led to identification of a distinct class of

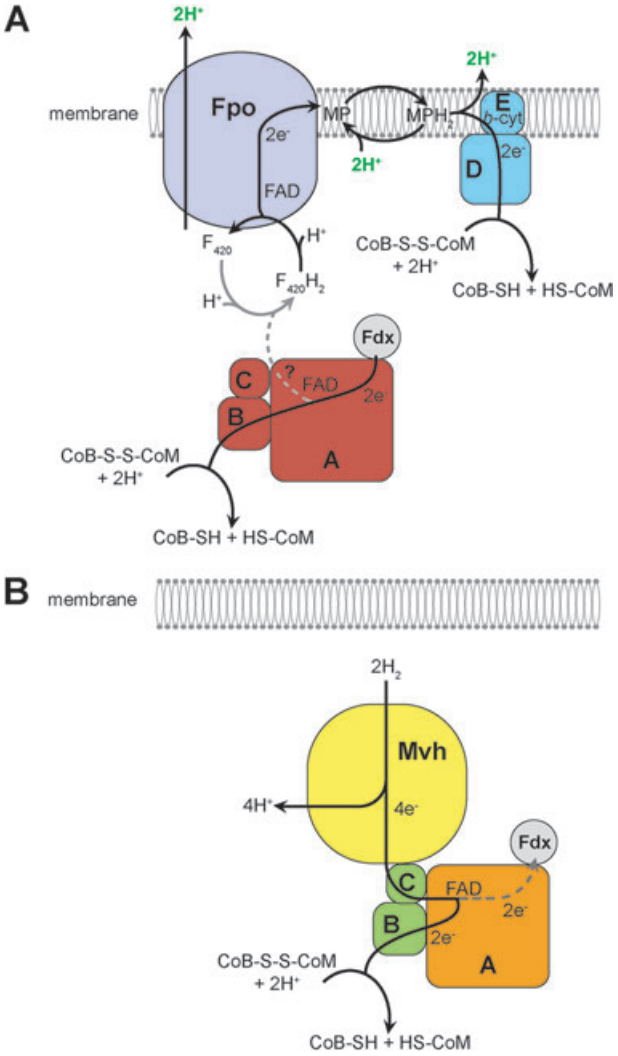

Fig. 1. Polypeptide composition of the soluble and membrane-bound heterodisulfide reductases.
A. *Methanosarcina acetivorans* HdrED (blue) accepts electrons from reduced methanophenazine (MPH_2) in the membrane. Methanophenazine (MP) is reduced by the proton-translocating F_{420}:methanophenazine oxidoreductase, Fpo (lavender). In *M. acetivorans*, HdrABC (red) oxidizes ferredoxin and reduces CoB-S-S-CoM. If electron bifurcation occurs, it likely results in reduction of F_{420}, which can then be used as substrate by Fpo (dashed lines).
B. HdrABC (orange, green) from *Methanobacterium* is localized in the cytoplasm and co-purifies with an associated hydrogenase, Mvh (yellow). Electrons are derived from H_2 via Mvh, and a proposed bifurcation mechanism in HdrABC may result in co-reduction of CoB-S-S-CoM heterodisulfide and ferredoxin (Fdx, grey). Protons translocated out of the cell are shown in green. CoB-SH and CoM-SH, reduced thiols of Coenzyme B and Coenzyme M; FAD, flavin adenine dinucleotide; *b*-cyt, *b*-cytochrome.

HdrABC enzyme, which has also been biochemically characterized (Setzke *et al.*, 1994). These methanogens are capable of growth on $H_2 + CO_2$ or formate and cannot synthesize cytochromes. HdrABC co-purifies with Mvh hydrogenase (methyl viologen-reducing hydrogenase) in a large soluble enzyme complex (Stojanowic *et al.*, 2003) (Fig. 1B). In these organisms, H_2 is the electron donor for reduction of CoB-S-S-CoM via Mvh (Deppenmeier, 2004). However, currently there is no *in vitro* assay for HdrABC that relies solely on physiological electron donors and acceptors, and how HdrABC contributes to energy-conservation is not well understood. A recent review by Thauer *et al.* (2008) postulates that in methanogens without cytochromes, flavin-mediated electron bifurcation by HdrABC results in co-reduction of ferredoxin. Reduced ferredoxin would then be used in the reduction of CO_2 to formyl-methanofuran (CHO-MFR) in the first step of methanogenesis from $H_2 + CO_2$. This model predicts that HdrABC does not directly contribute to energy conservation in these organisms.

Based on biochemical evidence it was hypothesized that $H_2 + CO_2$-utilizing methanogens like *M. thermoautotrophicum* use HdrABC, while the *Methanosarcinales*, which have acquired the ability to synthesize cytochromes, use HdrED. However, all five sequenced *Methanosarcinales* genomes (*Methanosarcina barkeri* Fusaro, *Methanosarcina acetivorans* C2A, *Methanosarcina mazei* Gö1, *Methanosaeta thermophila*, *Methanococcoides burtonii*) were found to contain *hdrED* as well as putative *hdrACB* genes. This was a surprise because HdrABC had never been identified by biochemical approaches in *Methanosarcinales*. The unexpected presence of genes for both Hdr classes in *Methanosarcina* suggests two possibilities: conditions exist whereby either HdrED or HdrABC is preferred as the dominant Hdr activity in the cell, or HdrED and HdrABC have specialized physiological functions. The aim of the work presented here was to determine the roles of putative *hdrABC* genes on the *M. acetivorans* chromosome in methanogenesis.

Results

Several hdr *alleles are present in the* M. acetivorans *C2A chromosome*

Methanosarcina acetivorans, M. barkeri and *M. mazei* chromosomes each encode one *hdrED1* operon and one *hdrA1C1B1* operon (Fig. 2). In addition, they each have an apparently monocistronic *hdrD2*, an *hdrA2* in an operon with a putative polyferredoxin, and an *hdrC2B2* operon. In *M. acetivorans* these *hdr* alleles encode proteins with relatively low amino acid similarity to each other (between 47% and 56%) (Table S1).

Phylogenetic analyses were performed to elucidate the evolutionary relationships among the Hdr proteins in methanogens. As expected, the only methanogen genomes with significant HdrE BLAST hits were members of the family *Methanosarcinales* (Fig. S1). HdrE is the

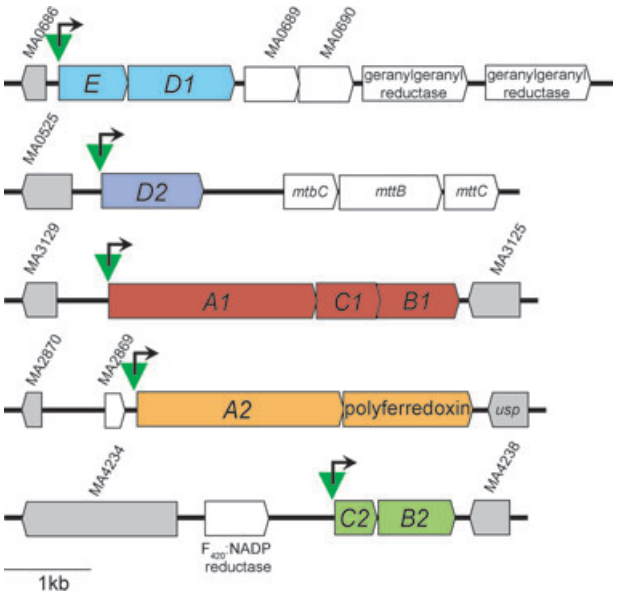

Fig. 2. *hdr* loci on the *Methanosarcina acetivorans* chromosome. Each putative *hdr* allele is shown in the context of nearby ORFs. The co-transcribed ORFs are indicated by corresponding colours. Inverted green triangles symbolize the position of the P_{tet} promoter used to drive controlled expression of the downstream genes in strains used for growth curve experiments. Genes with unknown functions are designated by MA#. Annotated functions are designated appropriately. ORFs that are transcribed in the opposite direction versus *hdr* genes are shaded in grey.

cytochrome-*b* subunit of HdrED (Heiden *et al*., 1994; Murakami *et al*., 2001), and only the *Methanosarcinales* synthesize cytochromes (Kamlage and Blaut, 1992). Each *Methanosarcinales* genome has only one *hdrE*, which is always the first gene in an operon with an *hdrD1* allele. HdrD contains the HdrED catalytic subunit where the CoB-S-S-CoM substrate is reduced. The *Methanosarcinales* HdrD1 proteins form a monophyletic clade (Fig. S1). HdrD2 is only found in *M. acetivorans, M. barkeri* and *M. mazei* and is specifically related to the HdrD1 clade, despite its absence in the other members of the *Methanosarcinales*.

Among methanogens, HdrA phylogeny generally mirrors 16s rRNA phylogeny; the *Methanobacteriales* and *Methanococcales* are closely related, while the *Methanosarcinales* lineage encodes a discrete HdrA clade. However, superimposed on the evolutionary ancestry are multiple duplication and recombination events (Fig. S2).

The deepest-branching HdrA proteins are found in the *Methanomicrobiales*. In these organisms, it appears that HdrA was duplicated. One copy of HdrA appears to be monocistronic, while another copy is combined in an operon with HdrBC proteins that are closely related to *Methanosarcina* HdrC2B2. An *hdrACB* operon is not found in the *Methanococcales*. However, the *Methanococcales* have two closely related copies of HdrA, one of which contains a selenocysteine residue in place of a cysteine. All sequenced *Methanobacteriales* genomes encode at least one HdrA. *Methanobrevibacter* and *Methanosphaera* also contain an *hdrACB* operon (Fricke *et al*., 2006; Samuel *et al*., 2007). In these two methanogens the proteins encoded by the *hdrACB* operon are specifically related to the HdrA1B1C1 proteins from *Methanosarcina*. Interestingly, *Methanobrevibacter* and *Methanosphaera* are the only methanogens outside of the *Methanosarcinales* to carry genes for the catabolism of methanol. These relationships suggest that this subfamily of *hdrA1B1C1* genes may be specifically involved in methylotrophic methanogenesis.

Of the five sequenced *Methanosarcinales* family members, only *Methanosarcina* species encode more than one copy of HdrA in their genome. *Methanococcoides burtonii* has only one *hdrA* operon and one *hdrCB* operon, similar to *Methanothermobacter*. *Methanosarcina* species have two HdrA copies. The *hdrA2* gene is in an operon with a polyferredoxin and the other is found in an *hdrA1C1B1* operon with a deeply branching HdrB and an HdrC protein that is most closely related to HdrC-like proteins of unknown function found in crenarchaea (*Sulfolobus*, *Metallosphaera*) and bacteria (*Hydrogenobaculum*, *Aquifex*) (Fig. S2). *Methanosaeta* has only one copy each of HdrA, HdrB and HdrC proteins (Smith and Ingram-Smith, 2007). In this organism, HdrA is most similar to *Methanosarcina* HdrA2 but is combined in an operon with bacterial HdrBC proteins. As *Methanosaeta* is an obligate aceticlastic methanogen, the phylogenetic relationship between the HdrA subunits suggests the possibility that HdrA2 in *Methanosarcina* may be involved in methanogenesis from acetate.

Transcription of M. acetivorans hdr *genes is regulated by methanogenic substrate*

Methanosarcina acetivorans strains carrying reporter gene fusions to all five putative *hdr* promoters were constructed to assess the transcriptional regulation of each. As shown in Table 1, the *hdrED1* fusion strain displayed the highest reporter activity on all media with approximately fourfold higher expression on methanol (MeOH) than on trimethylamine (TMA) or acetate. These data suggest that HdrED1 is used on all growth substrates. Transcriptional fusions to *hdrD2* showed significant activity only when acetate is the growth substrate, suggesting that HdrD2 may be preferentially used during aceticlastic methanogenesis.

The *hdrA1C1B1* transcriptional fusion strains had low but detectable activity on TMA, MeOH and MeOH + acetate, but activity was undetectable in cells growing on acetate as the sole energy source. HdrABC is therefore correlated with methanogenesis from methy-

Table 1. Reporter enzyme activity in *hdr* transcriptional fusion strains.

	β-glucuronidase activity[a]			
Genetic locus	TMA	MeOH	MeOH + acetate	acetate
hdrED1	106 (32)	401 (24)	172 (21)	83 (5.2)
hdrD2	3.9 (0.3)	4.8 (0.2)	4.5 (0.1)	24 (7.3)
hdrA1C1B1	18 (2.9)	49 (2.1)	32 (3.0)	< 1
hdrA2	2.7 (0.5)	22 (7.4)	12 (0.2)	24 (3.1)
hdrC2B2	35 (9.9)	116 (13)	23 (4.5)	65 (4.1)

Means were calculated from three independent cultures each measured in triplicate ($n = 9$). Values in parentheses indicate SEM.
a. Specific activity, nmoles min^{-1} mg^{-1}.

lotrophic substrates and is predicted to be dispensable for growth on acetate. Reporter activity from the *hdrA2* transcriptional fusion strains is very low on TMA but is fivefold to 10-fold higher when cells are grown on MeOH, MeOH + acetate and acetate. The *hdrC2B2* transcriptional fusion strains exhibited reporter activity under all conditions tested, with highest β-glucuronidase activity detected on MeOH.

HdrED is essential in M. acetivorans

The *hdr* promoters were individually replaced by a tetracycline-controllable $P_{mcrB(tet01)}$ promoter (hereafter abbreviated as P_{tet}) to determine the effect of repressing *hdr* expression (Fig. 2). Strains were constructed and adapted to each growth substrate in the presence of tetracycline, then inoculated into medium without tetracycline to examine the phenotype of cultures when the *hdrED1* locus is repressed.

Based on a variety of evidence, the *hdrED1* operon appears to be essential on all energy sources tested. In the absence of tetracycline induction, growth was observed in all but two TMA cultures and two acetate cultures by 2000 h (four cultures per condition, 16 cultures total). However, the lag phase before growth occurred in these cultures was much longer than in cultures where the tetracycline induction was maintained. Without exception, the growing strains were found to contain mutations in the TetR repressor binding site and, thus, were almost certainly expressing HdrED (Fig. S3). No mutations were ever observed in cultures maintained with tetracycline (five cultures per condition, 20 total). Numerous attempts were made to delete the ORFs encoding HdrED1 without affecting the upstream promoter or downstream ORFs. However, a *ΔhdrED1* mutant could not be obtained by either double-recombination or markerless deletion methods. The inability to construct a *ΔhdrED1* strain and the strong selection for tetracycline-independent mutants in a P_{tet}*hdrED1* strain supports the interpretation that *hdrED1*(MA0689) is essential.

No growth defect is observed when *hdrD2* expression is repressed. A *ΔhdrD2* mutant strain was constructed and had indistinguishable phenotype from wild-type when methanol (8.0 ± 0.8 h for the *ΔhdrD2* mutant strain versus 7.0 ± 0.5 h for wild-type) or acetate (32 ± 2.0 h for the *ΔhdrD2* mutant versus 31 ± 2.4 h for wild-type) were provided as growth substrates.

HdrABC is not essential, but contributes to growth and methane production from methylotrophic substrates

Repression of *hdrA1C1B1* results in a prolonged lag phase and increased doubling time on TMA (13 ± 2.3 h versus 9.0 ± 0.2 h for wild-type) and MeOH (18 ± 1.4 h versus 6.3 ± 0.7 h for wild-type) (Fig. 3). This growth lag is not observed when cells are adapted to MeOH + acetate, although the doubling time was much longer for the mutant (41 ± 6 h versus 7.5 ± 0.2 h for wild-type). The phenotype of the P_{tet}*hdrA1C1B1* strain is indistinguishable from wild-type on acetate; no growth lag is observed, no decrease in final culture OD_{600}, and equivalent doubling times (66 ± 11 h for wild-type and 69 ± 8.9 h for P_{tet}*hdrA1C1B1*) (Fig. 3D).

A *ΔhdrA1C1B1* deletion mutant was constructed to help understand the *in vivo* function of HdrA1B1C1. The mutant recapitulated the growth phenotype of the P_{tet}*hdrA1C1B1* strain; when methanol is the growth substrate, the generation time of *ΔhdrA1C1B1* is 15 (± 1.0) h versus 7.0 (± 0.5) h for wild-type. No significant difference was observed in the stationary phase culture densities. The rate of methanogenesis was determined using resting cell suspension assays. Methane production from methanol was twofold faster by wild-type cells versus *ΔhdrA1C1B1* mutant cells (372 ± 69 versus 143 ± 16 nmoles min^{-1} mg^{-1}). This suggests that a reduced growth rate by *ΔhdrA1C1B1* mutant cells is due to a reduced rate of methanogenesis and not due to a block in biosynthesis. When acetate is provided as the energy source, the generation times of wild-type and the *ΔhdrA1C1B1* mutant are 51 ± 6 h and 72 ± 18 h respec-

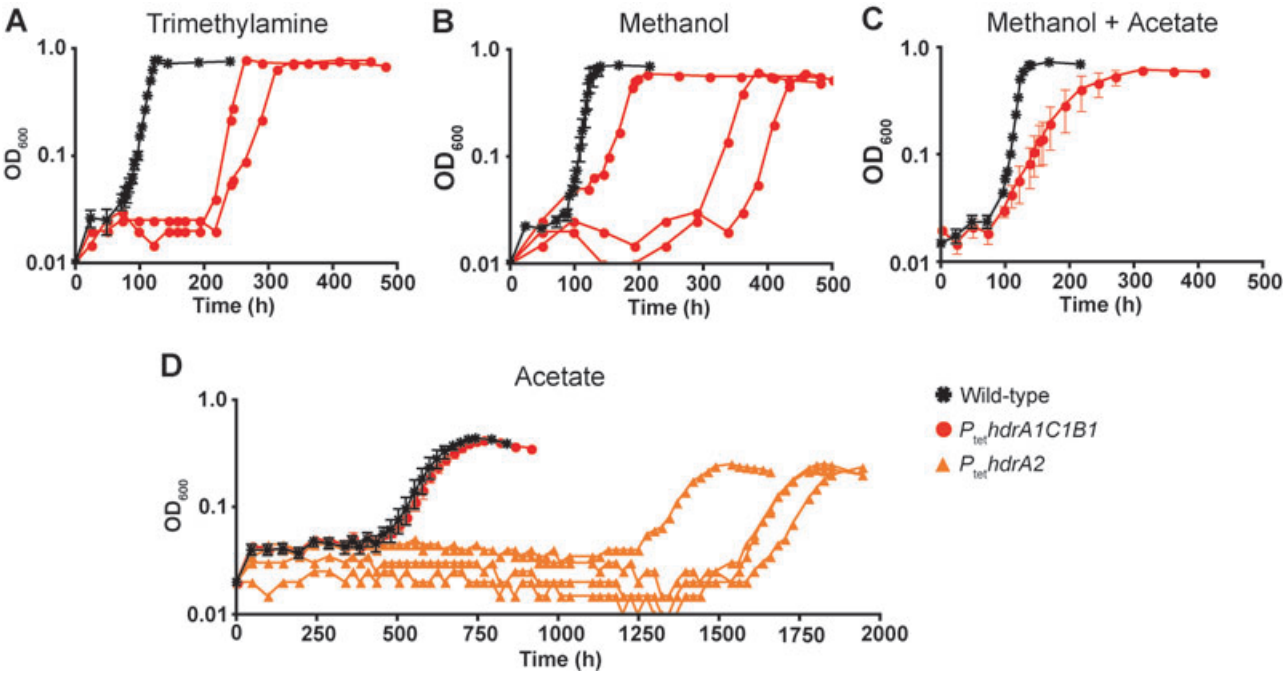

Fig. 3. Growth curves of P_{tet}*hdr* strains on trimethylamine (A), methanol (B), methanol + acetate (C) or acetate (D) as sole energy sources. Strains were adapted to each growth substrate in the presence of tetracycline. To observe the phenotypes of strains when transcription of *hdr* genes is repressed, adapted cells were inoculated at 10^{-4} dilution into medium without tetracycline. Error bars indicate cultures inoculated in triplicate. Individual data are plotted for P_{tet}*hdrA1C1B1* cultures (on trimethylamine, methanol and methanol + acetate) and P_{tet}*hdrA2* cultures (on acetate).

tively, and the rate of methanogenesis from acetate by wild-type and *ΔhdrA1C1B1* mutant cells is roughly equivalent (82 ± 31 nmoles min^{-1} mg^{-1} and 63 ± 18 nmoles min^{-1} mg^{-1} respectively). Therefore, HdrA1B1C1 plays a role in methylotrophic methanogenesis (when TMA, MeOH or MeOH + acetate are growth substrates), but not in aceticlastic methanogenesis, when acetate is the sole carbon and energy source.

Deletion of hdrA1C1B1 *results in upregulation of CoB-SH and CoM-SH synthesis, transport and methylsulfide methyltransferases*

A microarray mRNA abundance analysis was performed using total RNA isolated from methanol-grown cells of the *ΔhdrA1C1B1* mutant and from its isogenic parent. Only eight loci showed higher mRNA levels in the *ΔhdrA1C1B1* mutant (Table 2). These data were confirmed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) using total RNA isolated from four independent cultures. Deletion of *hdrA1C1B1* results in an eightfold increase in mRNA for genes that code for subunits (MA0063 and MA0065) of an ABC-family sulfonate transporter that has been shown to be involved in transport of Coenzyme M and the CoM-SH competitive inhibitor, bromoethanesulfonate (Zhang *et al.*, 2000). Transcripts for the sulfopyruvate decarboxylase (CoM-SH biosynthesis) and threonine synthase (CoB-SH biosynthesis) genes were found at levels sixfold higher than wild-type. The greatest increase in mRNA abundance is seen in the genes for three methylsulfide methyltransferase/corrinoid proteins know to be required for dimethylsulfide (DMS) production/consumption (Oelgeschlager and Rother, 2009). In particular, MA4558, a fused MT1/MT2 methyltransferase, is upregulated more than 1000-fold. Two other fused MT1/MT2s on the chromosome, MA4384 and MA0859, are upregulated 100–300-fold. Consistent with this observation, significant production of ^{13}C-dimethylsulfide from ^{13}C-methanol was observed by the *ΔhdrA1C1B1* strain, but not by wild-type cells (Fig. 4). These data suggest that a lack of HdrA1C1B1 during methylotrophic growth is overcome by increasing biosynthesis and uptake of CoB-SH and CoM-SH, and by synthesizing (di)methylsulfide. This response is predicted to increase the free CoB-SH and CoM-SH pools in the cell.

Notably, none of the other putative *hdr* genes, nor MA0658-0665 (encoding an acetate-expressed putative sodium-pumping ferredoxin : methanohenazine oxidoreductase, Rnf) (Li *et al.*, 2006) was significantly (more than twofold up- or down-) regulated in the *ΔhdrA1C1B1* mutant (Table S2). This suggests that either the methylotrophic HdrA1B1C1 has a physiological function that is not overcome by simply increasing the expression of the other *hdr* genes and MA0658-0665, or that activating

Table 2. Genes with increased relative mRNA abundance in *ΔhdrA1B1C1* versus wild-type cultures growing on MeOH as sole carbon and energy source.

Genome MA#	Putative function	Fold regulation *ΔhdrA1C1B1*/WT (range)[a]	
		Microarray	qRT-PCR
MA4558	Hypothetical protein. MtsH. Fused MT1/MT2?	95	1375 (1127–1678)
MA0859	Hypothetical protein. MtsD. Fused MT1/MT2?	30	116 (98.8–136)
MA4164	5-hydroxybenzimidazolyl-cobamide protein. MT1?	25	121 (104–140)
MA4165	Methyl-5-hydroxybenzimidazolyl-cobamide : CoM methyltransferase isozyme A. MT2?	23	ND
MA4384	Hypothetical protein. MtsF. Fused MT1/MT2? Next to MsrC, MtaCB2.	11	335 (297–377)
MA3298	Sulfopyruvate decarboxylase. CoM-SH synthesis.	6.0	ND
MA3297	Threonine synthase. CoB-SH synthesis.	5.3	5.7 (5.2–6.3)
MA4399	Acetyl-CoA decarbonylase/synthase complex subunit alpha CdhA2. CODH : Fdx activity, next to MsrE.	4.4	0.9 (0.8–1.0)
MA0065	Sulfonate ABC transporter, solute-binding protein. CoB-SH, CoM-SH scavenging.	2.8	8.3 (7.7–8.9)
MA0063	Sulfonate ABC transporter, ATP-binding protein. CoB-SH, CoM-SH scavenging.	2.6	ND
MA4615	2-isopropylmalate synthase. CoB-SH synthesis.	2.5	1.2 (1.1–1.2)

a. Calculated by ΔΔCt method versus *rpoA1* transcripts. *rpoA1* transcripts ng^{-1} total RNA in all samples was not statistically different by one-way ANOVA $P > 0.5$.
WT, wild-type WWM82.

such a bypass pathway would require multiple mutations and thus would have been too rare of an event to be observed.

Evidence for HdrA2 involvement in aceticlastic methanogenesis

Phylogenetic analysis of the HdrA2, HdrC2 and HdrB2 proteins from *M. acetivorans* shows that they form a clade with proteins found in other aceticlastic methanogens (Fig. S2). This association suggests that these proteins may be important for methanogenesis from acetate. The P_{tet}*hdrA2* strains showed no obvious phenotype on most media with, or without, induction by tetracycline; however, uninduced cultures grown on acetate displayed a long lag phase, a decreased growth rate and decreased final OD_{600} (Fig. 3). The decreased growth rate and stationary culture density on acetate indicates an involvement of HdrA2 in aceticlastic methanogenesis. However, the apparent lack of selection for repressor mutations in cultures grown without tetracycline suggests cells can overcome HdrA2 repression. No growth defects are observed when *hdrC2B2* expression is repressed.

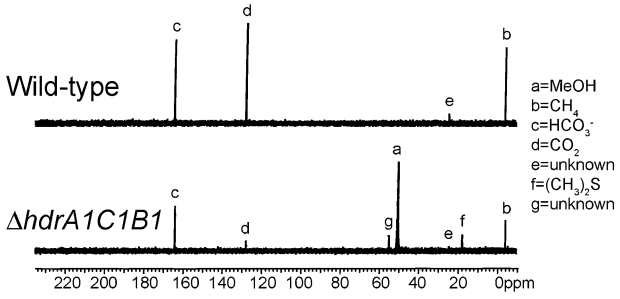

Fig. 4. ^{13}C-methanol metabolism by cell suspensions. ^{13}C NMR spectra are shown for wild-type and the *ΔhdrA1C1B1* mutant strain after 4 days' incubation at 37°C. A minor peak g was present in day 1 samples in both wild-type and the *ΔhdrA1C1B1* mutant. Peak g disappeared by day 2 in the wild-type suspension but accumulated in the *ΔhdrA1C1B1* mutant suspension. Expected chemical shifts are as follows: MeOH = 50.2 ppm, CH_4 = –2.3 ppm, CO_2 = 124.2 ppm, HCO_3^- = 165.7 ppm, $(CH_3)_2S$ = 19.6 ppm.

Discussion

The data presented here highlight a number of unanswered questions regarding the electron transport pathways used by *M. acetivorans* to deliver electrons to the CoB-S-S-CoM heterodisulfide, particularly during methylotrophic methanogenesis (Fig. 5A). *M. acetivorans* can grow on a variety of one-carbon (C-1) compounds, including methanol, methylsulfides and methylamines via the methylotrophic pathway for methane production (Keltjens and Vogels, 1993). The oxidative branch of the methylotrophic pathway produces two moles of reduced F_{420} and one mole of reduced ferredoxin, which is believed to carry two electrons using two [4Fe-4S] clusters (Meuer *et al.*, 1999; Meuer *et al.*, 2002). In the reductive branch of the pathway, electrons from reduced F_{420} and ferredoxin are used to reduce the CoB-S-S-CoM heterodisulfide in an energy conserving process. Current evidence suggests that energy is conserved via proton pumping at the $F_{420}H_2$:methanophenazine oxidoreductase (Fpo) step and via a redox loop at the methanophenazine : heterodisulfide oxidoreductase step (HdrED) (Deppenmeier, 2004). However, it is not clear how electrons from reduced ferredoxin are delivered to the CoB-S-S-CoM heterodisulfide (Guss *et al.*, 2005). This pathway must exist to maintain redox balance and to account for the observed 1 CO_2/3

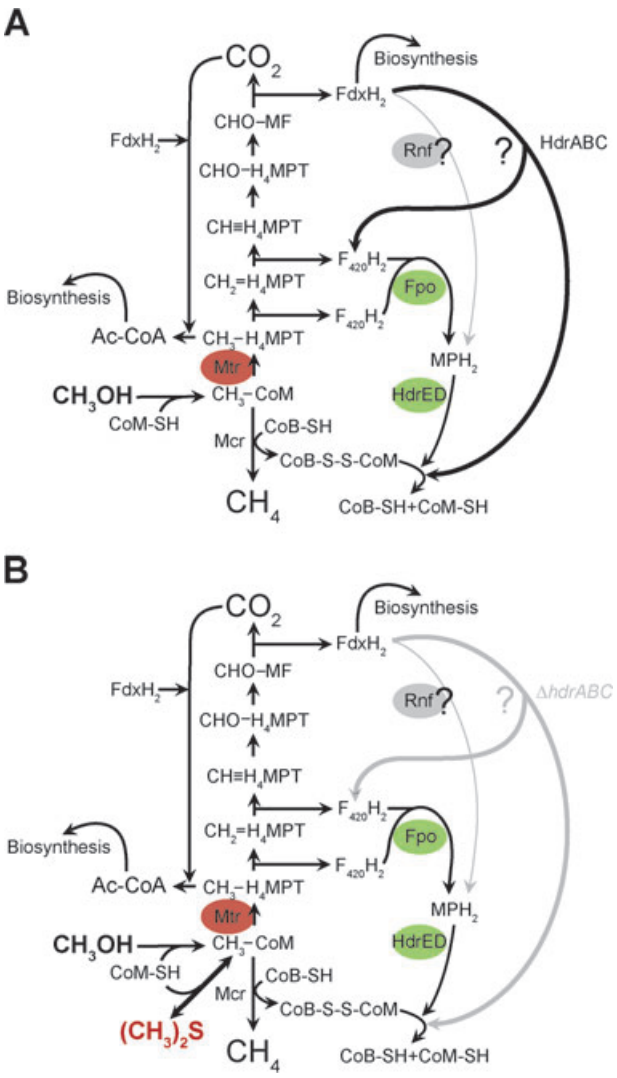

Fig. 5. Model for the *in vivo* physiological function of HdrA1C1B1 in *Methanosarcina acetivorans*. The pathway for methanogenesis from methanol in wild-type cells is shown in (A) and in the *ΔhdrA1C1B1* mutant in (B). Reactions that result in generation of a proton/sodium ion gradient are shown in green ovals, those reactions that require consumption of a proton/sodium ion gradient are shown with red ovals. The grey oval indicates a possible energy-conserving ion-pumping function for Rnf, the putative ferredoxin : methanophenazine oxidoreductase encoded by genes MA0658-0665. $FdxH_2$, reduced ferredoxin; MF, methanofuran; H_4MPT, tetrahydromethanopterin; $F_{420}H_2$, reduced F_{420}; Ac-CoA, acetyl Coenzyme A; CoB-SH, Coenzyme B thiol; CoM-SH, Coenzyme M thiol.

CH_4 product ratio. One possible route is via the MA0658-0665 (Rnf) complex, a putative proton-pumping ferredoxin : methanophenazine oxidoreductase that is highly upregulated in acetate-grown cells (Li *et al.*, 2006). We have shown that a *ΔMA0658-0665* deletion mutant is viable on methylotrophic substrates, although it fails to grow on acetate (A.M Guss and W.W. Metcalf, unpubl. results). Therefore, if *MA0658-0665* indeed encodes a ferredoxin : methanophenazine oxidoreductase, it cannot be the sole route for oxidation of ferredoxin during methylotrophic methanogenesis in *M. acetivorans.*

The presence of HdrABC in *M. acetivorans* suggests that reduction of the heterodisulfide need not include a methanophenazine-dependent step (as with HdrED). Based on the proposed function of HdrABC in *Methanothermobacter*, it seems likely that the *Methanosarcina* enzyme is a ferredoxin : CoB-S-S-CoM heterodisulfide oxidoreductase and, thus, may represent the missing ferredoxin-dependent electron transfer pathway linking the oxidative and reductive branches of methylotrophic methanogenesis. Standard redox potentials indicate that electron flow from reduced ferredoxin (–500 mV) to CoB-S-S-CoM (–140 mV) is highly exergonic, and therefore a stoichiometric (1:1) electron transfer mechanism is possible; however, based on the model suggested by Thauer *et al.* (2008), we believe that an HdrABC-mediated bifurcation mechanism is more likely to occur. Accordingly, *M. acetivorans* HdrABC would reduce both F_{420} (–360 mV) and CoB-S-S-CoM after two successive two-electron transfers from ferredoxin. This mechanism would allow additional energy conservation via Fpo (Fig. 1A). Regardless of whether an energy conserving bifurcation is catalysed by the enzyme, electron transport via HdrABC cannot conserve as much energy as is conserved through the HdrED pathway. Thus, no energy is conserved if HdrABC transfers electrons directly to CoB-S-S-CoM; whereas energy is conserved only in the putative F_{420}-reducing step if HdrABC catalyses a bifurcation reaction. This reduced level of energy conservation raises questions regarding the *in vivo* function of the HdrABC enzyme.

The evidence presented here suggests that function of HdrA1B1C1 is specific to methylotrophic methanogenesis. On these substrates, the *hdrA1C1B1* mutants have a pronounced growth defect and produce methane at much lower rates relative to the isogenic parental strain. Further, expression of the *hdrA1C1B1* locus is highly induced on methylotrophic substrates. These phenotypes are especially pronounced when methanol is the substrate. In contrast, expression of the *hdrA1C1B1* operon is undetectable during growth on acetate and the *ΔhdrA1C1B1* mutant has no obvious phenotype during growth on this substrate. Phylogenetic analysis also supports the role of HdrA1B1C1 in methylotrophic methanogenesis. Nevertheless, the precise function of HdrA1B1C1 remains enigmatic because the enzyme is not essential for growth on methylotrophic substrates.

Clues to the role of HdrABC can be found in the phenotype of the *ΔhdrA1C1B1* mutant during growth on methanol. The available evidence suggests that the mutant experiences CoM-SH and CoB-SH limitation during growth on this substrate. If HdrABC is truly a ferredoxin : CoB-S-S-CoM oxidoreductase, then CoB-S-

S-CoM reduction may be rate-limiting in the mutant. This would result in shortage of free CoB-SH and CoM-SH. Because CoB-SH is the electron donor for methyl-CoM reductase (Mcr), this activity would also be slowed, resulting in accumulation of CH_3-S-CoM. Consistent with this model, the genes in cofactor biosynthesis and transport are upregulated in the mutant, presumably to cope with the shortage of free cofactors. The *ΔhdrA1C1B1* mutant also induces expression of methylsulfide methyltransferases, which are thought to convert CH_3-S-CoM to (di)methylsulfide and free CoM-SH (Oelgeschlager and Rother, 2009). Synthesis of (di)methylsulfide has previously been proposed as a response to reduced methyl-CoM reductase activity (Moran *et al.*, 2008; Bose and Metcalf, 2009), which is fully consistent with our proposed role for HdrABC (Fig. 5).

The question of why the cell requires a non-energy-conserving pathway from ferredoxin to CoB-S-S-CoM also remains. It should be stressed that the role of MA0658-0665 as a ferredoxin : methanophenazine oxidoreductase has yet to be validated experimentally. Thus, a simple explanation is that HdrABC is required because the model for MA0658-0665 function is incorrect. It should also be stressed that our proposed role for HdrABC also lacks direct biochemical support. If both enzymes use ferredoxin as an electron donor as proposed, then it may be important for the cell to have a mechanism to bypass the methanophenazine step (via HdrABC) when methanophenazine pools are fully reduced. This bypass would be especially important during rapid growth (such as during exponential growth on methanol); it would be less important when growth rate is slower, for instance during stationary phase or during growth on acetate or trimethylamine. Growth phenotypes of the P_{tet}*hdrA1B1C1* and *ΔhdrA1B1C1* strains are consistent with this scenario. Cells grow faster on methanol than on trimethylamine and the *ΔhdrA1B1C1* phenotype is more severe in cells growing on methanol versus trimethylamine. Under conditions when HdrED is operating at maximum capacity, HdrABC may provide a selective advantage for cells by increasing the speed of CoB-S-S-CoM reduction while sacrificing translocation of protons across the cell membrane. Lastly, it should be noted that neither MA0658-0665 nor HdrA1B1C1 are absolutely required for growth on methylotrophic substrates. It is possible that either enzyme alone is sufficient for growth and that a double ΔMA0658-0665 *ΔhdrA1C1B1* mutant would be unable to grow on methylotrophic or aceticlastic substrates. Alternatively, a third pathway could exist to mediate electron transfer between ferredoxin and CoB-S-S-CoM. For example, a putative ferredoxin:F_{420} oxidoreductase could fill this role. Reduced F_{420} could then be used by Fpo to reduce methanophenazine, the substrate for HdrED. At least 19 genes proposed to encode flavoredoxins can be found in the *M. acetivorans* genome. One of these proteins has been shown *in vitro* to accept electrons from ferredoxin leading to the suggestion that it plays a role in delivery of electrons to CoB-S-S-CoM during aceticlastic methanogenesis (Suharti *et al.*, 2008); however, fate of the reduced flavoredoxin has yet to be shown. Clearly, additional biochemical and genetic studies, including verification of these proposed enzyme activities and construction of strains with mutations in multiple pathways, will be essential to further our understanding of the electron transport process in *Methanosarcina* species.

Our data indicate that HdrA1B1C1 is not required for growth on acetate. Aceticlastic methanogenesis is very different from methylotrophic methanogenesis (Ferry, 1992). The rate-limiting step of the aceticlastic pathway is thought to occur during activation of acetate to acetyl-CoA via acetate kinase and phosphotransacetylase, when one ATP is consumed (Peinemann *et al.*, 1988). The rest of the aceticlastic pathway involves reduction of the methyl moiety of acetate to methane with concomitant oxidation of the carbonyl moiety to CO_2 and reduction of ferredoxin. Reduced ferredoxin is then used to generate a proton gradient across the cell membrane and the electrons are used to reduce methanophenazine and ultimately CoB-S-S-CoM via HdrED1. Most of the enzymes responsible for the oxidative branch of the methylotrophic pathway are downregulated during growth on acetate (Li *et al.*, 2007). MA0658-0665, a putative proton-pumping oxidoreductase, is highly upregulated on acetate, and is hypothesized to use ferredoxin to reduce methanophenazine. Despite the high flux of electrons through ferredoxin and methanophenazine with respect to growth on methylotrophic substrates, the growth and methanogenesis rate on acetate is approximately fourfold slower than on methanol. Therefore we suspect that HdrA1B1C1 is not required to balance the ferredoxin pool during aceticlastic methanogenesis, and indeed, *hdrA1C1B1* transcription is shut off in cells growing on acetate. We suggest that CoB-S-S-CoM reduction by HdrED is not rate limiting on acetate and therefore the cell does not need to increase the rate of CoB-S-S-CoM reduction via HdrA1B1C1 at the expense of an opportunity to conserve energy.

Based on our phylogenetic analyses we expected that the other *hdr* genes on the chromosome (*hdrA2*, polyferredoxin and *hdrC2B2*) would encode an aceticlastic HdrA2C2B2 and its accompanying polyferredoxin. Indeed we showed that the P_{tet}*hdrA2* mutant strain shows an acetate-dependent phenotype similar to the *ΔhdrA1C1B1* deletion strain on methylotrophic substrates. However, repression of *hdrC2B2* transcription in the P_{tet}*hdrC2B2* mutant does not have an affect on growth rate when cells are grown on acetate and the HdrA2 subunit does not contain the predicted Hdr active site. If these proteins functioned together in a single complex, the phenotypes of

the P_{tet}*hdrA2* and P_{tet}*hdrC2B2* strains would be identical. Because their phenotypes are different, these data suggest that the *hdrA2* and *hdrC2B2* gene products are not operating in the same pathway. Therefore our data do not support the formation of an HdrA2C2B2 aceticlastic enzyme. Furthermore, neither *hdrA2* nor *hdrC2B2* appears to be essential when repressed in the respective P_{tet}*hdrA2* or P_{tet}*hdrC2B2* strains and therefore obtaining deletion strains at each locus should be possible. However, numerous attempts have been made to generate markerless *ΔhdrA2* or *ΔhdrC2B2* mutants, to no avail. Until these seemingly paradoxical observations can be resolved, we can make no conclusions about the physiological functions of the HdrA2, polyferredoxin or HdrC2B2 proteins.

Experimental procedures

Bioinformatics

The annotated *M. acetivorans* C2A sequences for *hdrED1*, *hdrD2*, *hdrA1C1B1*, *hdrA2*, *hdrC2B2* (Galagan *et al.*, 2002) were used to find orthologous sequences in all completed microbial genome sequences via BLAST with blastn or blastp at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi. Nucleotide sequences were aligned with ALIGN (Pearson and Lipman, 1988) to determine % identity and similarity. Predicted amino acid sequences were aligned in ClustalX2.0 (Thompson *et al.*, 1997). For phylogenetic analysis the Phylip3.67 package was used (http://www.evolution.genetics.washington.edu/phylip.html) (Felsenstein, 1989). Maximum-likelihood trees were assembled in proml using slower analysis, global rearrangements and randomized input order (one jumble). Bootstrap values were obtained using seqboot, proml (same conditions as for generating phylogenetic trees except 100 datasets were analysed and global rearrangements was turned off), and the consensus trees were assembled with consense.

Culture conditions

Escherichia coli strains were grown in Luria–Bertani medium (Uetake *et al.*, 1958) with the appropriate antibiotics or additions in the following concentrations: rhamnose 5 mM, ampicillin 100 μg ml^{-1}, chloramphenicol 35 or 5 μg ml^{-1}. *Methanosarcina* strains were grown under strictly anaerobic conditions in HS mineral salts medium (Metcalf *et al.*, 1996). For growth on solid medium, cells were plated on HS medium containing 1.4% agar (w/v) with the appropriate carbon source and additions as previously described (Metcalf *et al.*, 1998). The following anaerobic additions were added when appropriate: MeOH (125 mM), acetate (120 mM), TMA (50 mM), MeOH + acetate (125 mM MeOH + 40 mM acetate), Tetracycline (100 μg ml^{-1}), Puromycin (2 μg ml^{-1}), 8-ADP (8-aza-2,6-diaminopurine, 20 μg ml^{-1}).

Cloning and generation of Methanosarcina *strains*

P$_{tet}$ *plasmids.* Methods were as previously described (Guss *et al.*, 2008). Approximately 1 kb of upstream DNA and approximately 1 kb *hdr* coding sequence was amplified from the *M. acetivorans* genome. PCR products were digested and cloned into pGK050A. The resulting plasmids listed in Table S3 were transformed into WWM82 using standard techniques to create the strains listed in Table S3.

Each strain was verified by PCR to have the *hdr* coding sequence driven by the P_{tet} promoter. All cultures that grew after a lag phase were sequenced at the appropriate *hdr* locus and at the *hpt* locus to ensure that the P_{tet} promoter was intact and that neither of the *tetR* repressor cassettes had acquired mutations. The data presented in Fig. 3 are from cultures that retained the ability to respond to tetracycline, have intact P_{tet} promoters driving *hdr* gene expression and have two intact *tetR* repressor cassettes.

Point mutations in the P_{tet} promoters at the *hdrA1C1B1* locus were observed in eight cultures (out of 27 total) when cells were selected under repressed conditions (Fig. S3). No mutations were observed in P_{tet}*hdrA1C1B1* acetate cultures (8 total), and no mutations were ever observed in any cultures maintained with tetracycline (20 total).

ΔhdrA1C1B1 *deletion mutant.* Approximately 1 kb each upstream and downstream of the *hdrA1B1C1* locus was cloned into pMP44 as previously described (Pritchett *et al.*, 2004). Cells were transformed and selected for puromycin resistance. These merodiploid isolates were cultured for approximately five generations under the non-selective condition (without puromycin) and individuals whose chromosomes had resolved the merodiploid locus were selected by plating on the toxic purine analog 8-ADP. Colonies were screened by PCR and analysed by Southern blot to ensure deletion of *hdrA1C1B1*.

uidA fusion plasmids. Methods were as previously described (Pritchett *et al.*, 2004; Guss *et al.*, 2008). Approximately 1 kb of upstream DNA was amplified from the *M. acetivorans* genome. PCR products were digested and cloned into pAMG82. The resulting plasmids listed in Table S3 were transformed into WWM82 using standard techniques to create the strains listed in Table S3. Strains were verified using the PCR screening method described in Guss *et al.* (2008).

β-glucuronidase assays. Methods were followed as described previously (Rother *et al.*, 2005). Cultures were adapted on each substrate for at least 30 generations (or 15 generations on acetate). Ten millilitres of cells were harvested at ~OD_{600} = 0.3 and frozen at –80°C until ready for use. Triplicate cultures were measured three times each for a total of nine data points per condition. The limit of detection was determined to be 0.5 mU.

All plasmids were sequenced at the UIUC Core Sequencing Facility.

Growth curves

Methanosarcina P_{tet}*hdr* strains listed in Table S3 were maintained in medium containing tetracycline at all times. After adaptation for approximately 30 generations on the appropriate substrates, cultures were diluted to 10^{-4} into 25 ml Balch

tubes containing 10 ml HS medium. Cultures were incubated in quadruplicate at 37°C. Growth was measured every 4 h or 12 h by reading the optical density at 600 nm (OD_{600}) in a Spectronic 21 spectrophotometer (Milton Roy Company). Genomic DNA from each individual culture was alcohol precipitated and used for PCR and sequencing analysis.

qRT-PCR

Four millilitres of culture at OD_{600} 0.3–0.5 was directly lysed in 15 ml Qiagen RNEasy buffer RLT. The Qiagen RNEasy midiprep kit was then used to purify total RNA. *M. acetivorans rpoA1* was used as an internal reference. As an external reference, *rpoA1* RNA was transcribed with the MEGAscript T7 *in vitro* transcription kit (Ambion). DNA contamination was removed from all RNAs by digestion with TurboDNAfree DNAse (Ambion) and confirmed by lack of product after 40 cycles of PCR amplification. Quality of RNA was verified with an Agilent Bioanalyzer at the University of Illinois W.M. Keck Center for Functional Genomics.

The qRT-PCR oligos listed in Table S2 were designed using the program PrimerExpress. qRT-PCR reactions were performed in 384-well plates with 100 ng total RNA and 500 mM oligos per 20 ml reaction as per manufacturer instructions (Invitrogen SuperScript® III Platinum® SYBR® Green One-Step qPCR Kit w/ROX cat#11746). Data collection was obtained using the Agilent HT-7900 at the University of Illinois W.M. Keck Center for Functional Genomics.

Cell suspension assays

Rate of methane production. The method was previously described (Welander and Metcalf, 2008). For methylotrophic methanogenesis, MeOH-adapted cells were used and 250 mM MeOH was added as substrate. For aceticlastic methanogenesis assays, acetate-adapted cells were used and 20 mM acetate was added as substrate.

^{13}C NMR. Stationary-phase cultures of methanol-adapted wild-type (WWM82) and the *ΔhdrA1C1B1* deletion mutant (WWM287) were pelleted by centrifugation and resuspended in HS medium supplemented with 10% D_2O and 125 mM ^{13}C-methanol (Sigma). Cell suspensions were sealed in 5 mm Kontes NMR tubes (Fisher Scientific cat# K897635-800) fitted with septum caps and incubated at 37°C. At approximately 24 h intervals, ^{13}C NMR spectra (with and without proton decoupling) were obtained at the UIUC School of Chemical Sciences NMR Laboratory using a Varian Unity 500 NMR spectrometer (125MHz ^{13}C) for 832 acquisitions at acquisition times of 1.086 s. An external standard of tetramethylsilane [$(CH_3)_4Si$] was set to $\delta = 0$ ppm.

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References

Bobik, T.A., and Wolfe, R.S. (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.

Bobik, T.A., Olson, K.D., Noll, K.M., and Wolfe, R.S. (1987) Evidence that the heterodisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate is a product of the methylreductase reaction in *Methanobacterium*. *Biochem Biophys Res Commun* **149:** 455–460.

Bose, A., and Metcalf, W.W. (2009) Regulation of putative methyl-sulfide methyltransferases in *Methanosarcina acetivorans* C2A. *Mol Microbiol* **74:** 227–238.

Deppenmeier, U. (2004) The membrane-bound electron transport system of *Methanosarcina* species. *J Bioenerg Biomembr* **36:** 55–64.

Felsenstein, J. (1989) PHYLIP – Phylogeny Inference Package (Version 3.2). *Cladistics* **5:** 164–166.

Ferry, J.G. (1992) Methane from acetate. *J Bacteriol* **174:** 5489–5495.

Fricke, W.F., Seedorf, H., Henne, A., Kruer, M., Liesegang, H., Hedderich, R., *et al.* (2006) The genome sequence of *Methanosphaera stadtmanae* reveals why this human intestinal archaeon is restricted to methanol and H_2 for methane formation and ATP synthesis. *J Bacteriol* **188:** 642–658.

Galagan, J.E., Nusbaum, C., Roy, A., Endrizzi, M.G., Macdonald, P., FitzHugh, W., *et al.* (2002) The genome of *M. acetivorans* reveals extensive metabolic and physiological diversity. *Genome Res* **12:** 532–542.

Guss, A.M., Mukhopadhyay, B., Zhang, J.K., and Metcalf, W.W. (2005) Genetic analysis of *mch* mutants in two *Methanosarcina* species demonstrates multiple roles for the methanopterin-dependent C-1 oxidation/reduction pathway and differences in H_2 metabolism between closely related species. *Mol Microbiol* **55:** 1671–1680.

Guss, A.M., Rother, M., Zhang, J.K., Kulkarni, G., and Metcalf, W.W. (2008) New methods for tightly regulated gene expression and highly efficient chromosomal integration of cloned genes for *Methanosarcina* species. *Archaea* **2:** 193–203.

Heiden, S., Hedderich, R., Setzke, E., and Thauer, R.K. (1994) Purification of a two-subunit cytochrome-*b*-containing heterodisulfide reductase from methanol-grown *Methanosarcina barkeri*. *Eur J Biochem* **221:** 855–861.

Kamlage, B., and Blaut, M. (1992) Characterization of cytochromes from *Methanosarcina* strain Göl and their involvement in electron transport during growth on methanol. *J Bacteriol* **174:** 3921–3927.

Keltjens, J.T., and Vogels, G.D. (1993) Conversion of methanol and methylamines to methane and carbon dioxide. In *Methanogenesis*. Ferry, J.G. (ed.). New York, NY: Chapman & Hall.

Li, L., Li, Q., Rohlin, L., Kim, U., Salmon, K., Rejtar, T., *et al.* (2007) Quantitative proteomic and microarray analysis of the archaeon *Methanosarcina acetivorans* grown with acetate versus methanol. *J Proteome Res* **6:** 759–771.

Li, Q., Li, L., Rejtar, T., Lessner, D.J., Karger, B.L., and Ferry, J.G. (2006) Electron transport in the pathway of acetate

conversion to methane in the marine archaeon *Methanosarcina acetivorans*. *J Bacteriol* **188:** 702–710.
Metcalf, W.W., Zhang, J.K., Shi, X., and Wolfe, R.S. (1996) Molecular, genetic, and biochemical characterization of the *serC* gene of *Methanosarcina barkeri* Fusaro. *J Bacteriol* **178:** 5797–5802.
Metcalf, W.W., Zhang, J.K., and Wolfe, R.S. (1998) An anaerobic, intrachamber incubator for growth of *Methanosarcina* spp. on methanol-containing solid media. *Appl Environ Microbiol* **64:** 768–770.
Meuer, J., Bartoschek, S., Koch, J., Kunkel, A., and Hedderich, R. (1999) Purification and catalytic properties of Ech hydrogenase from *Methanosarcina barkeri*. *Eur J Biochem* **265:** 325–335.
Meuer, J., Kuettner, H.C., Zhang, J.K., Hedderich, R., and Metcalf, W.W. (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.
Moran, J.J., House, C.H., Vrentas, J.M., and Freeman, K.H. (2008) Methyl sulfide production by a novel carbon monoxide metabolism in *Methanosarcina acetivorans*. *Appl Environ Microbiol* **74:** 540–542.
Murakami, E., Deppenmeier, U., and Ragsdale, S.W. (2001) Characterization of the intramolecular electron transfer pathway from 2-hydroxyphenazine to the heterodisulfide reductase from *Methanosarcina thermophila*. *J Biol Chem* **276:** 2432–2439.
Oelgeschlager, E., and Rother, M. (2009) In vivo role of three fused corrinoid/methyl transfer proteins in *Methanosarcina acetivorans*. *Mol Microbiol* **72:** 1260–1272.
Pearson, W.R., and Lipman, D.J. (1988) Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* **85:** 2444–2448.
Peinemann, S., Muller, V., Blaut, M., and Gottschalk, G. (1988) Bioenergetics of methanogenesis from acetate by *Methanosarcina barkeri*. *J Bacteriol* **170:** 1369–1372.
Pritchett, M.A., Zhang, J.K., and Metcalf, W.W. (2004) Development of a markerless genetic exchange method for *Methanosarcina acetivorans* C2A and its use in construction of new genetic tools for methanogenic archaea. *Appl Environ Microbiol* **70:** 1425–1433.
Rother, M., Boccazzi, P., Bose, A., Pritchett, M.A., and Metcalf, W.W. (2005) Methanol-dependent gene expression demonstrates that methyl-coenzyme M reductase is essential in *Methanosarcina acetivorans* C2A and allows isolation of mutants with defects in regulation of the methanol utilization pathway. *J Bacteriol* **187:** 5552–5559.
Rouviere, P.E., and Wolfe, R.S. (1988) Novel biochemistry of methanogenesis. *J Biol Chem* **263:** 7913–7916.
Samuel, B.S., Hansen, E.E., Manchester, J.K., Coutinho, P.M., Henrissat, B., Fulton, R., *et al.* (2007) Genomic and metabolic adaptations of *Methanobrevibacter smithii* to the human gut. *Proc Natl Acad Sci USA* **104:** 10643–10648.
Setzke, E., Hedderich, R., Heiden, S., and Thauer, R.K. (1994) H_2:heterodisulfide oxidoreductase complex from *Methanobacterium thermoautotrophicum*. Composition and properties. *Eur J Biochem* **220:** 139–148.
Smith, K.S., and Ingram-Smith, C. (2007) *Methanosaeta*, the forgotten methanogen? *Trends Microbiol* **15:** 150–155.
Stojanowic, A., Mander, G.J., Duin, E.C., and Hedderich, R. (2003) Physiological role of the F_{420}-non-reducing hydrogenase (Mvh) from *Methanothermobacter marburgensis*. *Arch Microbiol* **180:** 194–203.
Suharti, S., Murakami, K.S., de Vries, S., and Ferry, J.G. (2008) Structural and biochemical characterization of flavoredoxin from the archaeon *Methanosarcina acetivorans*. *Biochem* **47:** 11528–11535.
Thauer, R.K., Kaster, A.K., Seedorf, H., Buckel, W., and Hedderich, R. (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat Rev Microbiol* **6:** 579–591.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25:** 4876–4882.
Uetake, H., Luria, S.E., and Burrous, J.W. (1958) Conversion of somatic antigens in *Salmonella* by phage infection leading to lysis or lysogeny. *Virology* **5:** 68–91.
Welander, P.V., and Metcalf, W.W. (2008) Mutagenesis of the C1 oxidation pathway in *Methanosarcina barkeri*: new insights into the Mtr/Mer bypass pathway. *J Bacteriol* **190:** 1928–1936.
Zhang, J.K., Pritchett, M.A., Lampe, D.J., Robertson, H.M., and Metcalf, W.W. (2000) In vivo transposon mutagenesis of the methanogenic archaeon *Methanosarcina acetivorans* C2A using a modified version of the insect mariner-family transposable element Himar1. *Proc Natl Acad Sci USA* **97:** 9665–9670.

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