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MINIREVIEW

Methanogenesis: Genes, Genomes, and Who's on First?

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INTRODUCTION

Recognition of the *Archaea* (*Archaebacteria*) in 1977 (127) and the subsequent widespread use of gene cloning and sequencing have led to molecular phylogenetics becoming a dominant theme in microbiology (73, 128). Regardless of the gene cloned, an almost mandatory component of the reporting publication is a sequence comparison and an inferred phylogeny for the gene. Reference is then made to the microorganism's position on the small-subunit rRNA (ssu-rRNA) tree (73), and inconsistencies are usually reconciled by evoking lateral gene transfer. As complete microbial genome sequences are becoming available (12, 26, 27, 44, 51, 59, 91), the phylogenetics of individual genes is being replaced by comparative genomics. Primary sequence comparisons remain the foundation, but operon organizations, local neighborhoods, and global patterns of gene arrangement and expression can now be compared. So far, there is little evidence of conservation above the level of homologous genes being clustered within similarly arranged operons and growing evidence that the ssu-rRNA tree may not reflect the phylogenies of all other subcellular components and metabolic pathways.

Of the six annotated genome sequences so far reported (12, 26, 27, 44, 51, 91), two are from methanogens, *Methanococcus jannaschii* (48) and *Methanobacterium thermoautotrophicum* ΔH (132), and methanogenesis is therefore one of the first complex metabolic pathways amenable to comparative genomics. Here we review the structure, organization, and expression of all sequenced methane genes that encode enzymes involved in the H_2-dependent pathway of CO_2 reduction to CH_4 (19, 82, 106, 115, 129). This is the only methanogenesis pathway available to *M. jannaschii* and *M. thermoautotrophicum*, and data from the two genome sequencing projects (12, 59, 91) provide a focus for comparative evaluations.

Methanogens are defined and unified as a group by methanogenesis, but otherwise they are extremely diverse with ssu-rRNA sequences that indicate very early divergences within the archaeal lineage (Fig. 2) (73). Most, but not all, members of the division *Euryarchaeota* are methanogens. Methanogens have genomic DNAs that range from 23 to 61 mol% G+C, they have coccal, spiral, and bacillary morphologies with a variety of cell envelope structures, and they inhabit ecological niches as varied as the human digestive system and submarine volcanic vents (3, 24, 49, 50, 133). Nevertheless, they all employ elements of the same biochemistry to synthesize methane (19, 24, 82, 106, 125, 129) and there are no facultative methanogens. Methanogenesis employs five unique cofactors (82, 106, 129), a complexity that argues against methanogenesis acquisition by lateral transfer and that the homologous enzymes essential for methanogenesis in all extant methanogens are encoded by genes that have evolved from one ancestral complement of methane genes (77). Comparison of the ssu-rRNA tree (Fig. 1) (3, 49, 73) with phylogenetic trees based on the sequences of essential methane gene products appears, therefore, to provide a test case for comparative genomics. Does the phylogeny of methanogenesis parallel the phylogeny of methanogens as defined by the ssu-rRNA tree?

AUTOTROPHY, METHANOGENESIS, AND GENOME SIZE

M. thermoautotrophicum and *M. jannaschii* gain energy, grow, and synthesize their biomass from only CO_2, H_2, N_2, or NH_4 and inorganic salts (48, 132) but have genomes that are only 1.75 and 1.66 Mbp, respectively (12, 91, 97). Despite being <40% of the size of the *Escherichia coli* genome, these small genomes must therefore encode all of the information needed for a fully autonomous, autotrophic lifestyle, and identifying a minimum complement of such genes is an important goal.

Based on the presence of homologous methane genes, *M. jannaschii* and *M. thermoautotrophicum* employ the same seven-step pathway for CH_4 biosynthesis from CO_2 plus H_2 (Fig. 2), although the relative locations of these genes are not conserved within the two genomes (Fig. 3). The biochemistry and enzymology of this pathway have been described in several recent reviews (19, 24, 82, 106, 125, 129), and the details of the methane genes and molecular biology involved at each step are described below.

STEP 1, CATALYZED BY FMD

The first step in methanogenesis, the synthesis of formylmethanofuran (formyl-MF) from CO_2 and methanofuran (MF), is catalyzed by formyl-MF dehydrogenases (FMDs), although the enzyme is named for the reverse reaction, the dehydrogenation of formyl-MF to CO_2. Methanogens synthesize isoenzymes or functionally equivalent but structurally unrelated enzymes to catalyze several steps in methanogenesis, and two FMDs are synthesized by *M. thermoautotrophicum*, *Methanobacterium wolfei*, and *Methanopyrus kandleri*. The *M. thermoautotrophicum* and *M. wolfei* FMDs differ in that one contains only molybdenum (Mo-FMD) whereas the second contains tungsten (W-FMD) (6). In *M. thermoautotrophicum*, Mo-FMD is encoded by an *fmdECB* operon that is transcribed only in the presence of Mo whereas the *fwdHFGDACB* operon that encodes W-FMD is transcribed in the presence of either W or Mo (45, 46). In *M. wolfei*, the regulation is reversed; W-FMD is synthesized only when W is available, and Mo-FMD is synthesized constitutively (84).

Both *M. kandleri* FMDs are W-FMDs, but one also contains selenium, incorporated into the active-site-containing subunit

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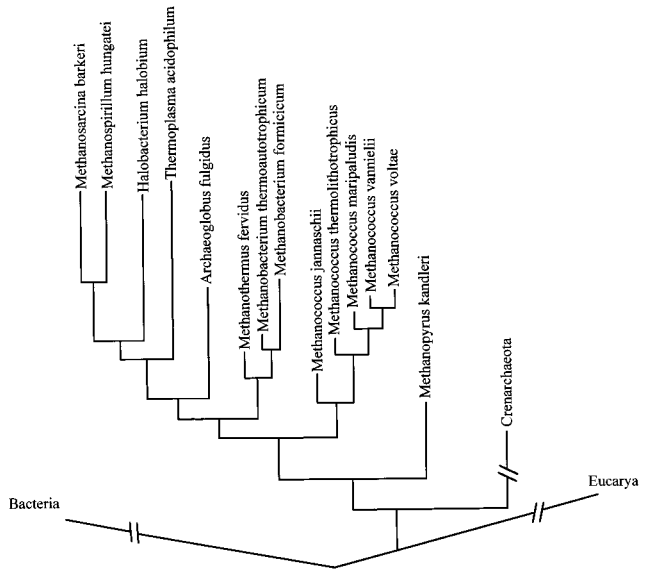

FIG. 1. Methanogen phylogenetic tree based on ssu-rRNA sequences. The members of the *Euryarchaeota* discussed in the text are identified on this phylogenetic tree abstracted from the full prokaryotic tree reported by Olsen et al. (73).

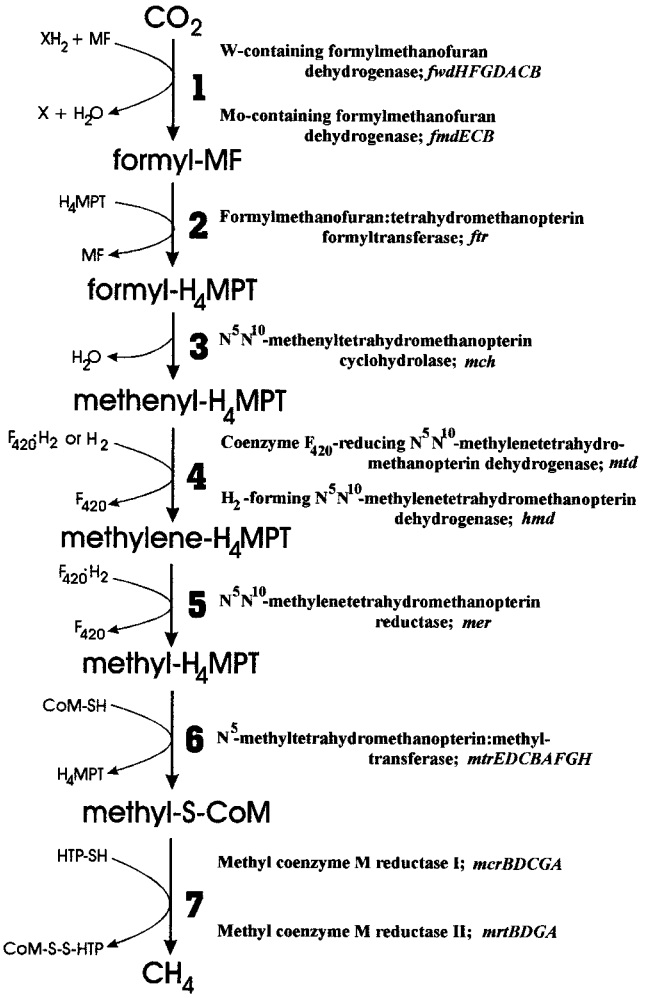

FIG. 2. Biochemical pathway of H_2-dependent reduction of CO_2 to CH_4. The C_1 moiety is transferred from CO_2 via MF, H_4MPT, and CoM (CoM-SH) into CH_4. The immediate sources of reductant (XH_2) used in step 1, and by HDR to reduce the heterodisulfide (CoM-S-S-HTP) generated at step 7, remain to be identified (19, 24, 106, 125, 129). The enzymes that catalyze each step and their encoding transcriptional units in *M. thermoautotrophicum* are listed.

(FWUB) as a selenocysteinyl residue (Se-cys) by translation of an in-frame UGA codon (120). The homologous subunit of the second W-FMD (FWCB) has a sequence that is 87% identical to that of FWUB, but the encoding gene (*fwcB*) has a UGU cysteine codon at the site of the UGA in *fwuB*. *fwuB* is transcribed in the presence and absence of Se, whereas *fwuC* transcription occurs only in the absence of Se. Se similarly negatively regulates transcription of two operons that encode cysteinyl-containing hydrogenases in *Methanococcus voltae*, whereas two operons that encode Se-cys-containing hydrogenases in *M. voltae* are transcribed constitutively (5, 32, 92, 93). In-frame UGA codons also direct Se-cys incorporation into the catalytic subunit of W-FMD and into five more methanogenesis-related gene products in *M. jannaschii* (see below) (126). *M. thermoautotrophicum* does not have the translation machinery needed for Se-cys incorporation (91).

FWDA is a subunit of both *M. thermoautotrophicum* FMDs. The W-FMD also contains the products of *fwdB*, *fwdC*, and *fwdD*, whereas the Mo-FMD contains FWDA plus the *fmdB* and *fmdC* gene products (45). FWDB and FMDB have amino acid sequences that are 47% identical and are members of a family of molybdopterin guanine dinucleotide cofactor-binding polypeptides that includes all FMD active-site subunits and the α subunits of formate dehydrogenases (FDHs). Consistent with this relationship, FMDs and FDHs both catalyze reactions that involve a C_1 moiety at the reduction level of formate and some FMDs have FDH activity (46). The α subunit of *E. coli* FDH contains a UGA-encoded Se-cys, located at the same site as in the FWDBs, and the Se atom has been shown to interact with the Mo atom of the molybdopterin cofactor (29). *fwdC* and *fwdD* of the W-FMD-encoding operon apparently fused to form *fmdC* in the Mo-FMD operon. Homologs of *fwdD* and *fwdG* from *M. kandleri* have also been sequenced (120), but their functions remain to be determined.

M. jannaschii and *Methanosarcina barkeri* appear to synthesize only one FMD. There is only one W-FMD-encoding *fwdHFGDAXC* operon in the *M. jannaschii* genome (12, 59), and *M. barkeri* has an *fmdEFACDB* operon that encodes Mo-FMD (85, 119). Consistent with this, hyperthermophiles, such as *M. jannaschii*, frequently have W-containing variants of enzymes that contain Mo in mesophiles, suggesting that W has better cofactor properties than Mo at high temperatures (1). *M. thermoautotrophicum* and *M. wolfei* retain both a W-FMD and a Mo-FMD, possibly to facilitate their growth at temperatures that range from 40 to 70°C, but presumably this is also advantageous in W- or Mo-limited environments. The *M. jannaschii fwdHFGDAXC* operon is similar in organization and sequence to the *M. thermoautotrophicum fwd* operon but has an additional short *fwdX* (MJ1170) and lacks *fwdB*, which is located ~18 kbp away within a second cluster of methane genes, arranged *hdrA-vhuDGAUB-fwdB* (Fig. 3). In this cluster, *hdrA* encodes the large subunit of a coenzyme M (CoM)-S-S–7-mercaptoheptanoylthreonine phosphate (HTP) heterodisulfide reductase (HDR) and *vhuDGAUB* encodes a coenzyme F_{420}-nonreactive hydrogenase (methyl viologen-reducing hydrogenase [MVH]) and a polyferredoxin. There are two in-frame UGA codons in *vhuD* and one in *hdrA*, *vhuU*, and *fwdB*, and Se-cys is incorporated into HDR, MVH, and FMD of *M. jannaschii* (12, 126). The locations of *hdrA* and *fwdB*,

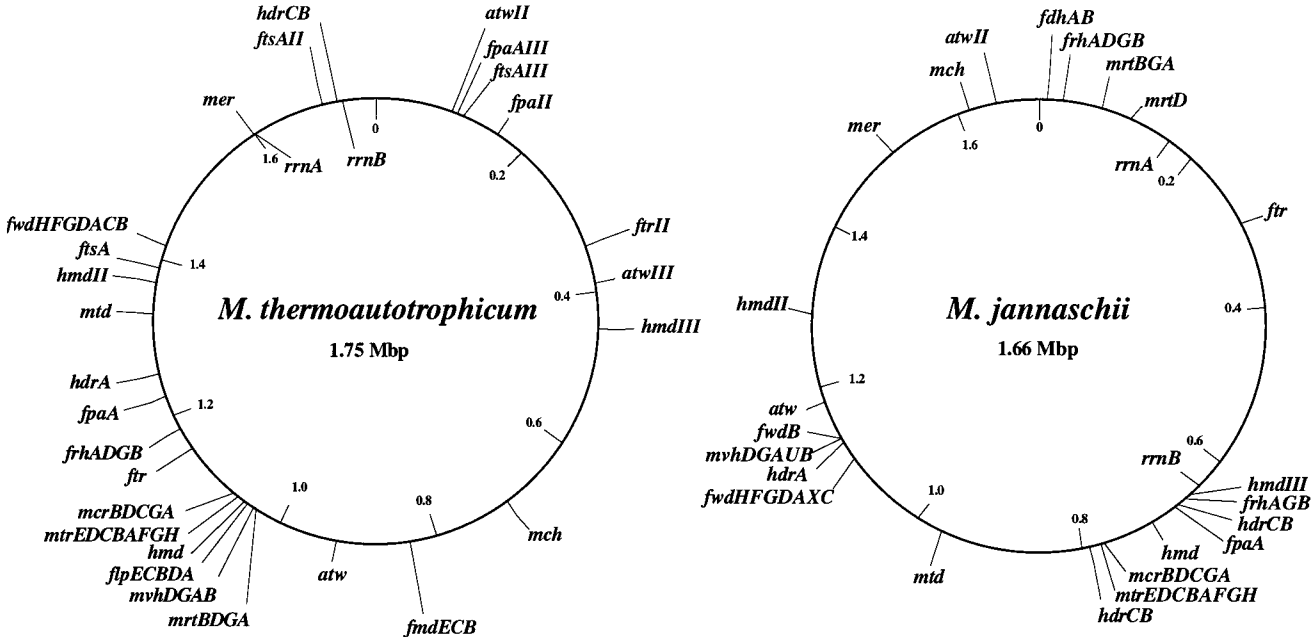

FIG. 3. Maps of the *M. jannaschii* and *M. thermoautotrophicum* genomes with methane gene locations identified. Both genomes also contain two rRNA operons, identified as *rrnA* and *rrnB*, and *M. jannaschii* has a 58-kbp plasmid and a 16-kbp plasmid, but neither plasmid contains methane genes.

flanking the *vhuDGAUB* operon, are intriguing, as the immediate sources of reductant used by HDRs and FMDs remain unknown. They probably oxidize reduced electron carriers, such as ferredoxins, polyferredoxins, flavodoxins, or cytochromes (18, 19, 41, 42, 57, 69, 106, 119), which must then be rereduced directly or indirectly by hydrogenase(s) at the expense of H_2, and the *vhu* operon encodes both a hydrogenase and a polyferredoxin. This arrangement could also just be coincidental, as there are eight more ferredoxin-encoding and three polyferredoxin-encoding genes dispersed around the *M. jannaschii* genome, assuming that a ferredoxin is defined by the presence of [4Fe-4S]-center motifs in polypeptides that contain <100 amino acid residues (12, 59). Based on this definition, the *M. thermoautotrophicum* genome encodes nine ferredoxins and five polyferredoxins (91).

STEP 2, CATALYZED BY FTR

Formyl-MF:tetrahydromethanopterin (H_4MPT) formyltransferases (FTRs) catalyze the second step in methanogenesis, and FTR-encoding *ftr* genes have been cloned from *M. thermoautotrophicum*, *M. kandleri*, *M. barkeri*, and *Methanothermus fervidus*. Except for *M. fervidus ftr*, all of these genes have been shown to direct the synthesis of functional FTRs in *E. coli* (20, 58, 62, 90). *M. kandleri* FTR synthesized in *E. coli* has been crystallized, and a structure has been established at a resolution of 1.73Å (21). FTR monomers have molecular masses of ~32 kDa and amino acid sequences that, in pairwise comparisons, are 60 to 70% identical (Table 1). Including the FTR encoded in the *M. jannaschii* genome, 42% of the positions in the five FTR sequences contain the same amino acid residues. The *M. thermoautotrophicum* genome also contains *ftrII* (Fig. 3), an open reading frame (ORF) that encodes a sequence which is 36 to 38% identical to the amino acid sequences of the established FTRs. With the FTRII sequence included, 32% of the positions in the six sequences contain the same amino acid residues. There is no biochemical evidence of a second FTR in *M. thermoautotrophicum*, and *ftrII* transcripts were not detected by Northern blotting in RNA preparations isolated from cells grown under a variety of different conditions (64). The presence of *ftrII* does, nevertheless, predict that *M. thermoautotrophicum* has metabolic flexibility at step 2. Two *ftr* homologs are also present in the *Archaeoglobus fulgidus* genome (http://www.ncbi.nlm.nih.gov/BLAST/tigr_db.html).

FTR from *M. fervidus* binds ATP (61), and a motif has been identified in the FTR sequences that is similar to the phosphate-binding P-loop motif ([A/V]XXGG [A/S] KVXX) conserved in phosphoglycerate kinases (61, 90). However, the *M. jannaschii* FTR is predicted to have an additional residue within this motif (12) and the motif is not encoded by *ftrII* (91). The structure determined for *M. kandleri* FTR indicates that the native enzyme is a homotetramer and that the active site is formed at the interface of two monomers (21).

STEP 3, CATALYZED BY MCH

Relatively few studies have focussed on the N^5,N^{10}-methenyl-H_4MPT cyclohydrolases (MCHs) that catalyze step 3, and MCH-encoding *mch* genes have been cloned from only *M. thermoautotrophicum* Marburg, *M. barkeri*, and *M. kandleri* (53, 103, 110, 111, 113). Their sequences, together with the two *mch* sequences from the genome projects, predict that MCH subunits have molecular masses of ~34 kDa and amino acid sequences that are 98% identical in the two *M. thermoautotrophicum* strains and 45 to 66% identical in different methanogens (Table 1) and that 36% of the positions in all five MCHs contain the same amino acid residues. There is no evidence of a second *mch* gene or a functionally equivalent but structurally different enzyme that can substitute for MCH.

In both *M. thermoautotrophicum* ΔH and Marburg, the 5′ region of *mch* overlaps the last three codons of the upstream gene *tysY*, which encodes thymidylate synthase (91, 111). However, *mch* and *tysY* are not cotranscribed and the *tysY-mch* overlap is not conserved in *M. jannaschii* or *M. kandleri*.

TABLE 1. Percent identities in amino acid sequences of methane gene products from *M. thermoautotrophicum*, *M. jannaschii*, *M. kandleri*, and *M. barkeri*[a]

Gene (step)	% Identity						
	Mt vs Mj	Mt vs Mk	Mt vs Mb	Mj vs Mk	Mj vs Mb	Mk vs Mb	All
fwdB (1)	68	61[c]	41[d]/63[e]	66[c]	41[d]	41[c,d]	52[f]
ftr (2)	70	66	65	64	66	60	38
mch (3)	66	58	45	66	48	52	35
mtd (4)[b]	66	55	NA	61	NA	NA	48
hmd (4)[b]	64	62	NA	64	NA	NA	48
mer (5)[b]	64	56	NA	59	NA	NA	46
mtrE (6)[b]	68/61[h]	62[g]	NA	67[g]	NA	NA	56[g]
mcrA (7)	71	72	65	76	67	63	51
16S RNA[i]	83	84	79	78	77	76	68

[a] Identities in pairwise comparisons of methane gene products are listed. The biochemical steps in the methanogenesis pathway (Fig. 2) catalyzed by the gene products are identified by the numbers in parentheses. Mt, *M. thermoautotrophicum*; Mj, *M. jannaschii*; Mk, *M. kandleri*; Mb, *M. barkeri.*
[b] *M. barkeri* sequences not available.
[c] Comparisons with the *fwuB* gene product of *M. kandleri* (120).
[d] Comparisons with the *fmdB* gene product of *M. barkeri* (119).
[e] Comparison of the *fmdB* gene products of *M. barkeri* and *M. thermoautotrophicum* (45, 119).
[f] Does not include *fmdB*-encoded sequences.
[g] Comparison limited to the 88 available N-terminal amino acids from *M. kandleri* (67).
[h] Comparison of the full-length sequences of the *mtrE* gene products from *M. thermoautotrophicum* and *M. jannaschii.*
[i] Identities in a Clustal alignment of 1,329 unambiguous nucleotide positions within the methanogens' 16S rRNA-encoding genes.

STEP 4, CATALYZED BY MTD AND HMD

Coenzyme F_{420}-dependent N^5,N^{10}-methylene-H_4MPT dehydrogenase (MTD) and H_2-forming N^5,N^{10}-methylene-H_4MPT dehydrogenase (HMD) reduce N^5,N^{10}-methenyl-H_4MPT to N^5,N^{10}-methylene-H_4MPT at step 4 by using reduced coenzyme F_{420} or molecular H_2 as the source of reductant, respectively (66, 102, 117). MTH has also been used as the abbreviation for the H_2-dependent enzyme, and *mth* has been used for the encoding gene (65, 71), but HMD and *hmd* have precedent (117) and are used here. Both MTD and HMD are oligomers of a single polypeptide, but *mtd*- and *hmd*-encoded amino acid sequences have no discernible features in common, consistent with MTD and HMD being genetically unrelated enzymes (117, 134).

Based on *mtd* sequences, the MTDs of *M. thermoautotrophicum* ΔH and Marburg (66, 91), *M. kandleri* (54), and *M. jannaschii* (12) have molecular masses of ~30 kDa and amino acids sequences that are 98% identical in the two *M. thermoautotrophicum* strains and 55 to 66% identical in different species and 48% of positions in all four MTDs contain the same residue (Table 1). Expression of *M. thermoautotrophicum* and *M. kandleri mtd*s in *E. coli* results in the synthesis of functional enzymes (54, 66).

Different genes, unrelated to methanogenesis, flank *mtd* in the genomes of *M. thermoautotrophicum* and *M. jannaschii*, but only 604 bp, that contain one ORF, separate *mtd* from *hmd* in *M. kandleri* (54). *M. kandleri mtd* and *hmd* are not, however, cotranscribed, and the ORFs that flank and separate these genes are homologs of *orf4*, *orf5*, *orf7*, and *orf6*, which are adjacent to *hmd* in *M. thermoautotrophicum* (71). Homologs of these ORFs are also present in *M. jannaschii*, MJ1251, MJ693, MJ927, and MJ489, respectively (12), but they are not clustered or adjacent to *hmd* or *mtd.*

Based on *hmd* sequences from *M. thermoautotrophicum* ΔH, Marburg, and Winter, *Methanobacterium thermoformicicum*, *M. kandleri*, *M. jannaschii*, *M. voltae*, and *Methanococcus thermolithotrophicus* (12, 36, 37, 71, 109, 117, 134), HMDs in the *M. thermoautotrophicum* strains and in *M. thermoformicicum* have amino acid sequences that are >95% identical whereas the methanococcal HMDs have sequences that are 76 to 84% identical. All other pairs of HMD sequences are 57 to 65% identical (Table 1), and 41% of positions contain the same amino acid residue in the eight HMDs. Immediately upstream, but transcribed divergently from *hmd* in the *M. thermoautotrophicum* genome, are the *flpECBDA*, *mvhDGAB*, and *mrtBDGA* operons that encode FDH-like proteins, an MVH, and methyl-CoM reductase II (MRII), respectively (Fig. 3) (71, 75). This cluster of three methanogenesis-related operons plus *hmd* is not conserved in the *M. jannaschii* genome or in *M. kandleri* (12, 54).

Completion of the genome sequences revealed the presence of *hmdII* and *hmdIII* in both *M. jannaschii* (MJ1338 and MJ1715) and *M. thermoautotrophicum*. These genes encode amino acid sequences that are almost the same length and 24 to 32% identical to the sequences of the established HMDs. HMDII and HMDIII are 66% identical in *M. jannaschii* and 79% identical in *M. thermoautotrophicum*, and the two HMDII and two HMDIII sequences are 59 and 53% identical, respectively. Related ORFs are located adjacent to *hmdIII* in both genomes. Northern blots indicate that H_2 availability differentially regulates *hmdII* and *hmdIII* transcription in *M. thermoautotrophicum* (see below and Fig. 4) (64), and determination of the functions of HMDII and HMDIII is therefore an important goal.

STEP 5, CATALYZED BY MER

Based on *mer* sequences from *M. thermoautotrophicum* Marburg and ΔH, *M. kandleri*, and *M. jannaschii* (12, 70, 112), coenzyme F_{420}-dependent N^5,N^{10}-methylene-H_4MPT reductases (MERs) that catalyze step 5 (63, 102, 104) are homotetramers of monomers with molecular masses of ~33 kDa. The two *M. thermoautotrophicum* MERs have sequences that are 98% identical, whereas MERs from different species have amino acid sequences which, in pairwise comparisons, are ~60% identical, and 46% of positions in the four MERs contain the same amino acids (Table 1). Unrelated genes flank *mer* in the *M. thermoautotrophicum* and *M. jannaschii* genomes.

In *M. thermoautotrophicum*, *mer* is transcribed into a mono-

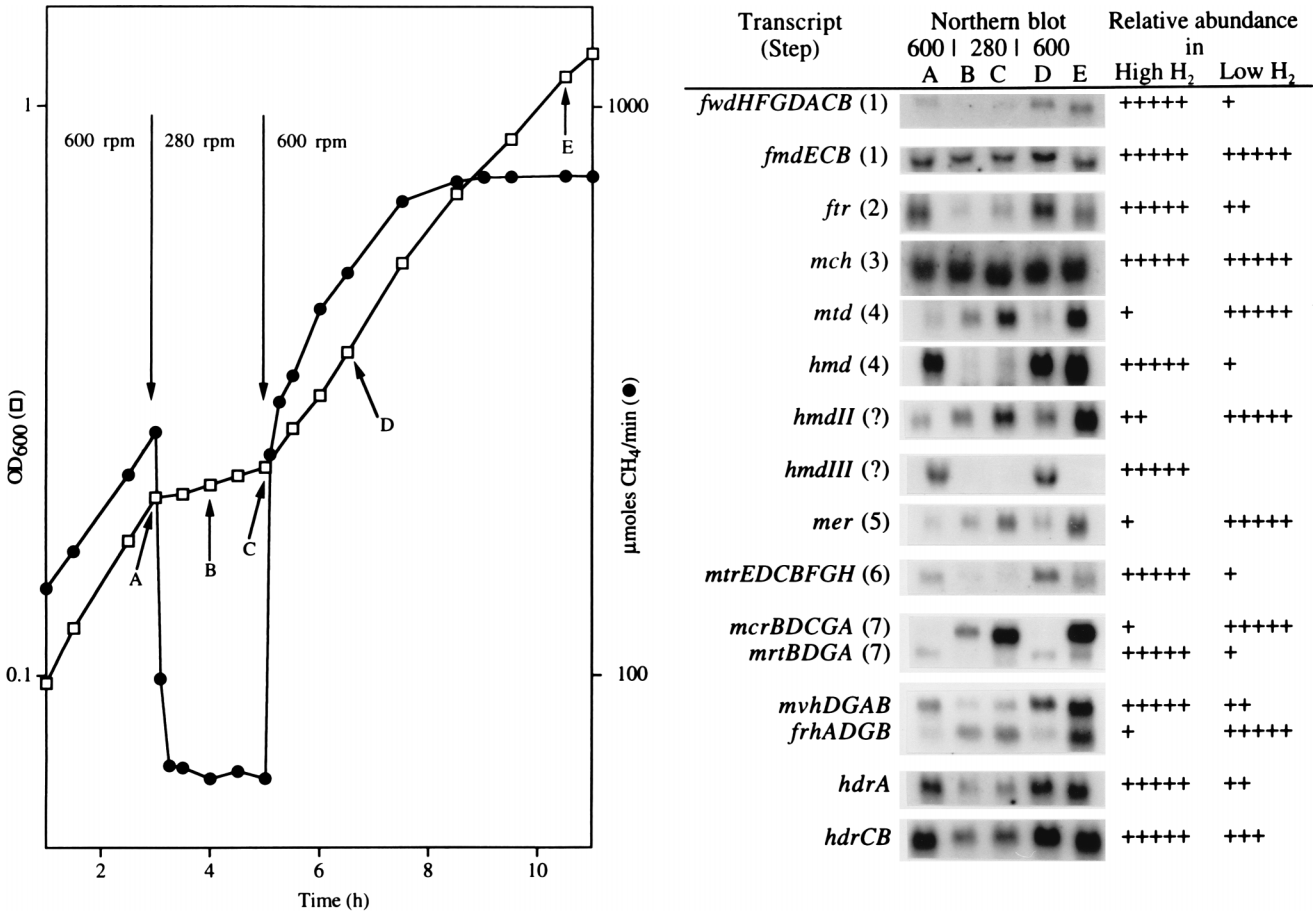

FIG. 4. Hydrogen-dependent regulation of methane gene transcription in *M. thermoautotrophicum*. The graph shows the growth at 55°C (□) and the rate of methane production (●) of a 1.5-liter culture supplied with 89% H_2–11% CO_2 at a flow rate of 200 ml/min (65). As indicated, the impeller speed was reduced from 600 to 280 rpm and then returned to 600 rpm. RNA preparations were isolated (75) at time points A, B, C, D, and E and hybridized with probes specific for the methane gene transcripts listed to the left of the Northern blots. The step in the methanogenesis pathway (Fig. 2) catalyzed by the enzyme encoded by each transcript is identified by the numbers 1 to 7 in parentheses, and a question mark indicates uncertainty. Based on the intensities of the hybridization signals, the relative abundance of each transcript under conditions of high H_2 availability (mixing at 600 rpm) and low H_2 availability (mixing at 280 rpm) is reported by the following symbols: +, detectable; ++, low; +++, medium; +++++, high. When mixing is reduced to 120 rpm, which imposes an even more severe H_2 limitation, only *mcr* transcription is stimulated (65, 72). OD_{600}, optical density at 600 nm.

cistronic transcript; however, the regulatory region upstream of *mer* contains sequences that are also present upstream of *mtd* (66, 70, 112). As predicted by this conservation, *mer* transcription parallels *mtd* transcription. Both genes are transcribed preferentially when the H_2 supply is growth rate limiting, and both MER and MTD are cofactor F_{420}-dependent enzymes (Fig. 2 and 4). In this regard, it is puzzling that there is no evidence of a second step 5-catalyzing enzyme that is H_2 dependent and synthesized preferentially when excess H_2 is available. *mer* appears to group with *mch* as a single-copy methane gene that encodes an enzyme essential for methanogenesis for which there is no substitute.

STEP 6, CATALYZED BY MTR

N^5Methyl-H_4MPT:CoM methyltransferases (MTRs) are complex, membrane-bound, corrinoid-containing enzymes that catalyze step 6 (4, 25). MTR purified from *M. thermoautotrophicum* contains eight different subunits with sizes of 12 to 34 kDa (28, 35), whereas MTR purified from *Methanosarcina mazei* Gö1 contains six subunits with sizes ranging from 9 to 34 kDa (19). The *mtrEDCBAFGH* operon encodes all eight *M. thermoautotrophicum* MTR subunits (35, 100), and an identically organized *mtr* operon is present in *M. jannaschii* (12). In both genomes, the *mtrEDCBAFGH* operon is positioned immediately downstream from the *mcrBDCGA* operon (Fig. 3) that encodes methyl-CoM reductase I (MRI), the enzyme that catalyzes the next and final step in methanogenesis (Fig. 2). *mtrE* sequences have also been identified downstream of *mcr* operons in *M. fervidus*, *M. kandleri*, and *Methanococcus vannielii* (16, 67, 123). Some transcripts initiated at the *mcr* promoter in *M. thermoautotrophicum* extend through the *mtr* operon, resulting in ~10-kb transcripts that encode both MRI and MTR (75). The adjacent positioning of these two methanogenesis-related operons does not, therefore, appear to be coincidental but apparently has a functional role consistent with the widespread conservation of this arrangement.

Most of the MTR subunits have sequences consistent with membrane-located proteins; however, MTRA, which binds the corrinoid prosthetic group (33, 100), is the only subunit whose specific function is known. Overall, the MTR subunit sequences are ~60% identical in *M. thermoautotrophicum* and *M. jannaschii* although codons 23 through 47 of *mtrE* encode the same sequence, AEDLESDVGSQSNPNSQVQLAPQM, in both methanogens. This sequence is also highly conserved in

other MTREs, indicating functional importance and a target for mutagenesis (Table 1) (67).

M. barkeri is more metabolically versatile than *M. jannaschii* and *M. thermoautotrophicum*. In addition to using H_2 to reduce CO_2 to CH_4, *M. barkeri* can also conserve energy by reducing CH_3 moieties from methylamines, methylthiols, methanol, and acetate to CH_4 (19, 23, 24). A methyl group is transferred from the substrate to the cobalt atom of a corrinoid cofactor by a substrate-specific methyltransferase, and a methylcobamide: CoM methyltransferase then catalyzes the transfer of the methyl group to CoM in a reaction that is, in essence, the same as the sixth step in the pathway of CO_2 reduction to CH_4 catalyzed by MTR (Fig. 2). Three methylcobamide:CoM methyltransferases from *M. barkeri* have been characterized (13, 22, 23, 34, 60, 101). Based on the sequences of their encoding genes, they have molecular masses of ~37 kDa and amino acid sequences that are ~30% identical and related to uroporphyrinogen III decarboxylases, but they have no detectable homology to any of the *mtrEDCBAFGH* gene products. Three substrate-specific methyltransferases have also been characterized (74, 83). These enzymes are similarly related to each other, with a large transferase subunit and a smaller, corrinoid-containing subunit that has sequence motifs in common with cobalamin-dependent methionine synthetases, but neither subunit exhibits any detectable relationship to the *mtr* gene products.

STEP 7, CATALYZED BY MRI AND MRII

MRs catalyze the last step of methanogenesis, the reduction of a methyl group bound to CoM, releasing CH_4. The presence of MR defines a cell as a methanogen, and historically MRs have been the most studied of all methanogenesis-related enzymes. All of the MRs that have been characterized, from a range of different methanogens (9, 11, 19, 24, 30, 38, 47, 81, 106, 129), contain α, β, and γ subunits encoded by *mcrA*, *mcrB*, and *mcrG* genes, respectively, located within *mcrBDCGA* operons. The *M. jannaschii* genome contains an *mcrBDCGA* operon, and *mcrBDCGA* operons have been cloned from *M. thermoautotrophicum* Marburg and ΔH, *M. fervidus*, *M. kandleri*, *M. barkeri*, *M. vannielii*, and *M. voltae* and sequenced (7, 8, 16, 52, 67, 75, 123). Despite their conservation and investigation (89, 98, 99), the functions of MCRC and MCRD have not been determined.

M. thermoautotrophicum strains contain two MR isoenzymes (7–9, 75, 81), and Southern blots indicate that a second MR-encoding region is likely to be present in the genomes of all members of the *Methanobacteriales* (Fig. 1) (95). The *mrtBDGA* operons that encode the α, β, and γ subunits of MRII in *M. thermoautotrophicum* and *M. fervidus* have been cloned and sequenced (62, 75). They lack a homolog of *mcrC* and encode α, β, and γ subunits with sequences that are only 61 to 72% identical to the sequences of their MRI homologs. These are values only marginally higher than the sequence identities of pairs of α, β, and γ subunits of MRIs from different methanogens (Table 1) (52, 67, 123, 124). The MRII subunit sequences are most similar to their methanococcal MRI homologs, indicating that the *mrt* operon originated in the methanococcal lineage (62, 67). The second MR-encoding operon in the *M. jannaschii* genome is arranged *mrtBGA* and therefore encodes only α, β, and γ subunits of MRII. The amino acid sequences of these subunits are 85, 73, and 79% identical, respectively, to the sequences of the *M. jannaschii mcrBGA* gene products, relatively high levels of identity that are consistent with the MR operon duplication having occurred in the methanococcal lineage. *M. jannaschii* has a *mrtD*, but this gene is separated from the *mrtBGA* operon by ~37 kbp (Fig. 3). Despite this novel location, *M. jannaschii mrtD* contains 11 contiguous codons that are also present in *mrtD* in *M. thermoautotrophicum* and *M. fervidus* and in *mcrD* in *M. jannaschii*, *M. vannielii*, *M. voltae*, *M. barkeri*, and *M. kandleri* but which appear to have been deleted from *mcrD* in *M. thermoautotrophicum* and *M. fervidus.*

By definition, methanogens must retain MR, suggesting that MR sequences might be used for methanogen phylogenetics (123), and MR-based trees have been reported (67, 94). However, in view of the operon duplication and the possibility of lateral transfer, it now seems less likely that an MR tree would parallel the phylogeny of all other methane genes or reflect the phylogeny of the remainder of the methanogen genome. Southern blots indicated that methanococci had only one MR-encoding region (43, 95), so the presence of the second MR operon in the *M. jannaschii* genome was unexpected. For both phylogenetic analyses and physiology studies, it is now important to determine if a second MR operon is also present in members of the *Methanopyrales* and *Methanomicrobiales* (Fig. 1).

MR purified from *M. thermoautotrophicum* ΔH requires the addition of two proteins, designated A2 and A3a, for activation in vitro (30, 129). The sequence of *atw*, the A2-encoding gene, predicts that A2 is an ~59-kDa ATP-binding protein related to the ABC family of proteins that participate in energy-dependent uptake and secretion processes (56). Several more genes encoding members of this family are present in the *M. thermoautotrophicum* and *M. jannaschii* genomes (12, 91), two of which in *M. thermoautotrophicum* encode sequences sufficiently similar to A2 to be designated *atwII* and *atwIII*. There are also two *atw* homologs (MJ1242 and MJ1662) in the *M. jannaschii* genome.

As described earlier, the only clustering of methane genes that is widely conserved is the adjacent positioning of the *mcrBDCGA* and *mtrEDCBAFGH* operons (67). The *mrtBDGA* operon in *M. thermoautotrophicum* is, however, located immediately downstream from the *mvhDGAB* operon (75, 95), and the *flpECBDA* and *hmd* genes are located immediately upstream from *mvhDGAB* (71). The *mcrBDCGA-mtrEDCB AFGH* cluster is also relatively close, separated by only 7.4 kbp from the *hmd-flp-mvh-mrt* cluster (Fig. 3), but this methanogenesis-related neighborhood is not conserved in the *M. jannaschii* genome or in *M. kandleri* (54).

MORE THAN ONE ENZYME FOR THE SAME REACTION

Before completion of the genome sequence, *M. thermoautotrophicum* was known to synthesize two enzymes that could catalyze steps 1, 4, and 7 of methanogenesis (Fig. 2) (106, 125, 129) and to have two [Ni,Fe]-containing uptake hydrogenases, a coenzyme F_{420}-reducing hydrogenase (FRH) (2) and a coenzyme F_{420}-nonreactive hydrogenase (MVH) (78). Now it appears that there are more players available to participate in methanogenesis. The presence of *ftrII* suggests that *M. thermoautotrophicum* has a second step 2-catalyzing FTR, and both *M. thermoautotrophicum* and *M. jannaschii* have *hmdII* and *hmdIII* genes, raising the possibility that both methanogens can synthesize four different enzymes, three HMDs and MTD, to catalyze step 4. HMD reduces N^5,N^{10}-methenyl-H_4MPT to N^5,N^{10}-methylene-H_4MPT by using H_2 as the source of reductant (Fig. 2) (134), and having three HMDs could optimize H_2 uptake under different H_2 supply conditions. Alternatively, HMDII and/or HMDIII might catalyze different reactions, combining H_2 uptake with a reduction elsewhere in the meth-

anogens' metabolism. One possibility would be at step 5 in methanogenesis (see below).

Retention of the genetic information needed to synthesize isoenzymes and functionally equivalent enzymes argues that this must be advantageous in environments encountered by the methanogens. The alternative FMDs that catalyze step 1 in *M. thermoautotrophicum*, *M. wolfei*, and *M. kandleri* differ in their W, Mo, and Se contents, and this presumably facilitates the growth of the methanogens when they are faced with a W, Mo, or Se limitation (6, 84, 85). Overcoming a metal limitation may also be one advantage of having both MTD and HMD to catalyze step 4. MTD requires reduced cofactor F_{420}, which contains Ni and must be reduced by a [Ni,Fe]-containing FRH, whereas HMD has no metal content or cofactor requirements. HMD, HMDII, and/or HMDIII may catalyze H_2 uptake when the Ni and/or Fe supply is limited, synthesizing N^5,N^{10}-methylene-H_4MPT, both as a step in methanogenesis and for oxidation by MTD to generate reduced cofactor F_{420} at the expense of H_2 without FRH participation (105).

Transcription studies indicate that MTD and HMD catalyze step 4 under different conditions of H_2 availability (see below; Fig. 4) (65, 71). HMD is synthesized when the dissolved H_2 level is very high, which must occur in the environments that surround thermal vents that are inhabited by both *M. thermoautotrophicum* and *M. jannaschii* (48, 131). Locally high H_2 concentrations may also occur within syntrophic and symbiotic associations of *M. thermoautotrophicum* with H_2-generating bacteria and protozoa (15, 31, 108). MTD, on the other hand, is synthesized when the H_2 supply is growth rate limiting, which appears to be the situation in most other anaerobic environments inhabited by *M. thermoautotrophicum* (55, 80, 133).

MRI and MRII are similarly synthesized preferentially under low- and high-H_2 conditions, respectively (10, 11, 65, 75, 81), but these are isoenzymes that employ the same cofactors to catalyze step 7. The advantage of having two MRs must therefore be explained in terms of different substrate affinities, catalytic rates, intracellular locations, and/or interactions with other cellular components. Consistent with this, MRII has a higher K_m for both CH_3-S-CoM and HTP (Fig. 2) and an approximately threefold higher V_{max} that MRI (9).

REGULATION OF METHANE GENE EXPRESSION: WHO'S ON FIRST?

MRII is synthesized only during the very early growth stages of fed-batch cultures of *M. thermoautotrophicum* sparged with H_2 plus CO_2 and is then replaced by MRI (10, 11, 81). This regulation occurs at the level of transcription initiation (75). Transcription of the MRII-encoding *mrtBDGA* operon is on first and is then replaced by transcription of the MRI-encoding *mcrBDCGA* operon. *hmd* transcription and *mtd* transcription follow parallel patterns; *hmd* transcription is on first during early exponential growth and is then replaced at later growth stages by *mtd* transcription (71). The growth medium used in these experiments contained bicarbonate and an adequate supply of trace metals, so the switch from *hmd* and *mrt* transcription to *mtd* and *mcr* transcription was predicted to result from a change in the availability of H_2 to the cells, despite there being no change in the H_2 supplied to the culture vessel (71, 75). Consistent with this, when the H_2 content of the gas mixture sparged into the culture was reduced, there was an immediate switch from *hmd* and *mrt* to *mtd* and *mcr* transcription (65, 72).

This growth phase-dependent regulation, or "who's on first" in a laboratory culture, must reflect a regulation that has an advantage in nature. *M. thermoautotrophicum* grows only on H_2 plus CO_2, and it is therefore not surprising that this methanogen senses and responds to changes in H_2 availability. But, in view of the small genome, how many alternative enzyme systems does *M. thermoautotrophicum* retain for use only under high or low H_2 supply conditions? This global-regulation question can be addressed by combining the genome sequence with Northern blot analyses, two-dimensional gel electrophoresis, and proteome mapping (76). The impeller mixing speed determines the rate of gas dissolution in a fermentor, so changing the mixing speed provides a simple, instantaneous, noninvasive, and reversible way to manipulate H_2 availability (65), and Fig. 4 illustrates how changing the H_2 supply affects the levels of all methane gene transcripts in *M. thermoautotrophicum*. Reducing the mixing speed, and therefore H_2 availability, has little effect on the *fwd* and *mch* transcripts and decreases the abundance of *fmd*, *ftr*, *mtr*, *frh*, and *hdr* transcripts, and *hmd*, *hmdIII*, and *mrt* transcripts become undetectable. In contrast, the *mtd*, *mer*, *mcr*, *hmdII*, and *mvh* transcripts increase in abundance when the H_2 supply is reduced. Returning the impeller speed to a high mixing rate reverses all of the transcript changes, and growth and methanogenesis also decrease and increase in parallel with the changes in H_2 availability.

As expected, the *hmd* and *mrt* transcripts that are on first in batch cultures (71, 75) disappear when the impeller speed, and therefore the H_2 supply, is reduced and are replaced by the late *mtd* and *mcr* transcripts. The *hmdIII* transcript also disappears when the impeller speed is reduced, consistent with the idea that HMDIII is synthesized under high-H_2 conditions. As the *mer* transcript, in contrast, increases when the impeller speed is reduced, MER is apparently synthesized preferentially when the H_2 supply is growth rate limiting and *M. thermoautotrophicum* should therefore synthesize a second step 5-catalyzing enzyme to replace MER when excess H_2 is available (71). There is no biochemical evidence of this enzyme, but the *hmdIII* transcript pattern (Fig. 4) suggests that HMDIII could be a candidate.

GENOME HINTS FOR METHANOGENESIS PUZZLES

Genome sequences allow positive conclusions to be drawn from negative observations. The absence of an FDH-encoding *fdhCAB* operon, for example, readily explains why *M. thermoautotrophicum*, unlike many of its close relatives (68, 72, 86), does not grow on formate. Unfortunately, this simple explanation does not extend to *M. jannaschii*, which also does not grow on formate but which does have *fdhBA* genes that in the presence of formate direct the synthesis of an active FDH with a Se-cys residue in FDHA (48, 126).

The hydrogenase(s) and electron carrier(s) that couple H_2 oxidation to the reductions at steps 1 and 7 of methanogenesis remain to be identified (Fig. 2) (19, 106). In *M. thermoautotrophicum*, *frhDBGA* encodes FRH (2), *mvhDGAB* encodes MVH and a polyferredoxin (MVHB) (39, 78, 96), and HMD, HMDII, and HMDIII probably also function as uptake hydrogenases (106, 117). *M. thermoautotrophicum* ΔH was reported to have MVHII (130), but this appears to have been MVH complexed with a flavoprotein (FPA). The FPA-encoding *fpaA* gene is not associated with the *mvh* operon but is clustered in what appears to be a redox-related operon, with genes predicted to encode a rubredoxin and a diiron-containing protein (69, 122). *fpaAII* and *fpaAIII* are also present at other sites in the *M. thermoautotrophicum* genome, and there is one *fpaA* homolog in *M. jannaschii* (Fig. 3). Based on the genes available, an attractive hypothesis is that MVH obtains the reductant from H_2 and supplies this into steps 1 and/or 7, via a polyferredoxin, FPA, and/or rubredoxin. MVHB contains 48

Fe atoms and requires a substantial Fe supply for synthesis (39, 96), whereas FPA is synthesized preferentially under Fe-limited conditions (69, 122). These proteins might therefore serve as alternative electron acceptors from MVH under different conditions of Fe availability.

MVH and the HDR that catalyzes the reduction of the CoM-S-S-HTP heterodisulfide generated at step 7 form a tight complex in vivo in *M. thermoautotrophicum* (40, 88). This supports the argument that MVH participates in step 7, but this complex does not contain a polyferredoxin or a flavoprotein, and *M. thermoautotrophicum* MVH does not reduce either MVHB or FPA effectively in vitro (39, 96).

Specific roles for different hydrogenases may be even more difficult to identify in other methanogens. *M. jannaschii* is predicted to have an MVH and two FRHs, *M. voltae* has two MVHs and two FRHs, and *Methanosarcina mazei* Gö1 has one FRH and two MVHs (5, 12, 18, 32, 92, 93). As in *M. thermoautotrophicum*, the MVH operons in *M. jannaschii* and *M. voltae* also encode polyferredoxins whereas the *M. mazei* MVH operons encode cytochromes (17, 18). Consistent with this, HDRs purified from *M. mazei* and *M. barkeri* contain cytochrome *b* (19, 41, 42, 57), indicating that cytochrome *b* supplies reductant to the HDR-catalyzed reaction these *Methanosarcinae* (Fig. 1). The presence of different methylated substrates determines which MVH is synthesized in *M. mazei* (17).

HDR purified from *M. thermoautotrophicum* contains HDRA, HDRB, and HDRC subunits, encoded in two separate transcriptional units, *hdrA* and *hdrCB*, and a flavin adenine dinucleotide (FAD) molecule bound to HDRA was, until recently, thought to be positioned at the active site (40, 85). The *M. jannaschii* genome contains one *hdrA* but has two *hdrCB* operons (12), suggesting the synthesis of two HDRs with the same large subunit (HDRA) but with different small subunits with sequences that are 65% (HDRB) and 53% (HDRC) identical. HDR purified from methanol-grown *M. barkeri* contains two subunits, designated HDRD and HDRE, neither of which is an HDRA homolog, and this enzyme has no flavin cofactor (41, 57). The encoding genes are organized in an *hdrED* operon, in which translation of *hdrE* initiates unusually at an ATC codon. *hdrD* is a homolog of a fusion of the *M. thermoautotrophicum* or *M. jannaschii hdrCB* genes, and *hdrE* encodes a cytochrome *b*. Based on the *M. barkeri* results, it appears that the active site of *M. thermoautotrophicum* HDR is within HDRB or HDRC and that FAD is not essential for catalysis (57).

M. thermoautotrophicum titrates the availability of exogenous H_2 and communicates this information into methane gene promoter functions, but the molecular details of this regulatory cascade are unknown. There are several two-component sensor kinase-response regulator systems encoded in the *M. thermoautotrophicum* genome (91), one or more of which could measure H_2 availability and activate or inactivate the appropriate methane gene promoters. Alternatively, and possibly more likely as there are no two-component systems encoded in the *M. jannaschii* genome, FRH, MVH, or one of the *hmd*-encoded hydrogenases might also function as an H_2 sensor. The intracellular level of a substrate or product of the hydrogenase might then signal the extracellular availability of H_2 to the methane gene regulatory systems. The level of an adenylated derivative of coenzyme F_{420}, known as coenzyme F_{390}, has been proposed to play this role, and *M. thermoautotrophicum* has enzymes that interconvert coenzymes F_{420} and F_{390} (71, 114–116). *ftsA* transcripts that encode cofactor F_{390} synthetase in *M. thermoautotrophicum* increase under conditions of high H_2 availability, consistent with an increase in cofactor F_{390} synthesis (65, 115). However, the presence of *ftsAII* and *ftsAIII*, genes that encode sequences that are 32 and 30% identical, respectively, to the *ftsA*-encoded cofactor F_{390} synthetase, suggests that cofactor F_{390} synthesis may be more complex in *M. thermoautotrophicum* than anticipated. The absence of an *ftsA* homolog in *M. jannaschii* also argues against any conserved signaling role for coenzyme F_{390} in methanogens. *ftsA* homologs are, however, intriguingly present in the *A. fulgidus* genome (http://www.ncbi.nlm.nih.gov/BLAST/tigr_db.html).

The participants in the final step of the H_2-dependent methane gene regulon, namely, promoter activation, are either known or predictable. Archaeal transcription initiation follows the eucaryal paradigm (79, 107). A preinitiation complex is therefore first assembled at the promoter that contains a TATA box binding protein, transcription factor TFIIB, and RNA polymerase, and the genes that encode all of these general transcription factors have been identified in both methanogen genomes (12, 59, 91). H_2 regulon-specific transcription factors must interact with this complex to initiate transcription from the appropriate methane gene promoters in response to an increase or decrease in the availability of H_2. The genes that encode these specific factors have also been sequenced in the *M. thermoautotrophicum* and *M. jannaschii* genomes, but they have yet to be identified.

METHANOGENESIS, COMPARATIVE GENOMICS, AND QUESTIONS

The ssu-rRNA tree predicts that *M. thermoautotrophicum* and *M. jannaschii* have diverged extensively (Fig. 1) (73), and this is substantiated by the genome sequences. Less than 1% of the ORFs conserved in both genomes encode amino acid sequences that are >70% identical, ~20% of the *M. thermoautotrophicum* genes have no recognizable homologs in the *M. jannaschii* genome, and ~15% of the *M. jannaschii* genes have no homologs in the *M. thermoautotrophicum* genome (91). Homologous methane genes encode sequences that are 60 to 70% identical in *M. thermoautotrophicum* and *M. jannaschii*, values that are generally higher than those obtained in pairwise comparisons of these *M. thermoautotrophicum* and *M. jannaschii* gene products with their homologs in *M. kandleri* and *M. barkeri*. As predicted by the ssu-rRNA tree, *M. barkeri* methane gene products consistently have the most divergent sequences (Table 1). *M. barkeri* can, however, also grow on methyl-containing substrates and is therefore not absolutely dependent on CH_4 synthesis from CO_2 plus H_2 (19). Possibly, this has provided more freedom for evolutionary change within the CO_2-to-CH_4 pathway in *M. barkeri* and raises the question of whether the sequences of obligatory- versus facultative-pathway enzymes can be directly compared without compensation for this difference.

The genome sequences have established that methane genes are not clustered in extended methanogenesis neighborhoods and that the relative locations of homologous methane genes are not conserved in two methanogen genomes (Fig. 3). Lateral transfer of methanogenesis in toto would therefore not now be readily accomplished, and the coordination of methane gene transcription must involve soluble *trans*-acting factors. The presence of duplicated genes and functionally equivalent genes and the possibility of lateral gene transfer raise the question of which gene sequences can be used to generate a methanogenesis phylogenetic tree valid for comparison with the ssu-rRNA tree. The step 1-catalyzing W-FMDs and Mo-FMDs share a common ancestry but have presumably evolved under different selective pressures and in the presence and absence of homologs. *M. thermoautotrophicum* contains both a

FIG. 5. Phylogenetic tree based on an alignment of MCH sequences. MCH sequences from *M. barkeri* (110), *M. jannaschii* (12), *M. kandleri* (53), *M. thermoautotrophicum* Marburg (111), and *A. fulgidus* (http://www.ncbi.nlm.nih.gov/BLAST/tigr_db.html) were aligned, and the tree was generated by Clustal V with the scale bar indicating substitution events (Dnastar Inc., Madison, Wis.). A parsimony analysis of the Clustal alignment using Paup 3.1.1 generated a tree with the same topology.

W-FMD and a Mo-FMD (45, 46), *M. kandleri* has two W-FMDs (120), *M. jannaschii* has only one W-FMD (12), and *M. barkeri* has only one Mo-FMD (85, 119). It seems unlikely that the rates and flexibility of primary sequence changes would be the same for the *fwd* and *fmd* genes in these different methanogen lineages. Similarly, the presence of two FTRs, three HMDs, and two MRs raises concerns that some of these step 2-, 4-, and 7-catalyzing enzymes, respectively, may no longer be essential for methanogenesis or may have diverged sufficiently to catalyze different reactions. Having duplicated copies of a gene also provides opportunities for gene conversion and therefore for unpredictable and possibly uneven sequence stabilization.

To avoid the potential complications inherent in isoenzymes and functionally equivalent enzymes, comparisons of MCH and MER sequences remain as viable options for establishing a methanogenesis phylogeny. *mch* and *mer* are not duplicated (Fig. 3), there are no substitutes for MCH and MER activities, and both enzymes fulfill the phylogenetic requirement of catalyzing the same essential reactions, steps 3 and 5, respectively, of methanogenesis, in all methanogens growing on CO_2 plus H_2 (Fig. 2) (106). MER sequences are not available from the *Methanomicrobiales* lineage, but MCH sequences have been determined from representatives of all four methanogen branches as defined by the ssu-rRNA tree (Fig. 1). An *mch* gene is also present in the *A. fulgidus* genome (http://www.ncbi.nlm.nih.gov/BLAST/tigr_db.html) that encodes a sequence that is 58, 55, 52, and 51% identical to the *M. jannaschii*, *M. kandleri*, *M. barkeri*, and *M. thermoautotrophicum* MCH sequences, respectively. These values are within the range of identities of pairs of methanogen MCH sequences (Table 1), and a phylogenetic tree based on MCH sequences is shown in Fig. 5. *A. fulgidus* is not a methanogen but is positioned on the ssu-rRNA tree within the lineage leading to the family *Methanomicrobiales* (Fig. 1) and synthesizes the enzymes that catalyze steps 1 through 5 of methanogenesis (Fig. 2) (53, 87, 118). These enzymes are, however, used to catalyze this portion of the pathway in the reverse direction, oxidizing methyl groups to CO_2 and thereby generating reduced cofactor F_{420} (87). The MCH tree is not inconsistent with the ssu-rRNA tree, but more MCH sequences are needed for a valid comparison of the two trees. Additional *Archaeoglobus* species are available (118), and an *mch* homolog has also been sequenced from *Methylobacterium extorquens* AM1, a bacterial methylotroph (14). The MCH tree can therefore be extended beyond methanogens, but this raises a question that is not inherent in the ssu-rRNA tree. Can the sequences of homologous enzymes that have evolved to catalyze the same reaction but in opposite directions or to catalyze similar but not homologous reactions be compared directly without compensation for these differences?

Initially, a simple question was posed. Does the phylogeny of methanogenesis parallel the phylogeny of the ssu-rRNA? The data reveal that this is not a simple question to answer and that although increasing the database will help, this may not be the limiting issue. The ssu-rRNA tree is based on the premise that this molecule has the same conserved and essential function in all cells under all physiological conditions and is subjected to the same selective pressures and constraints in all cells. Most species contain more than one ssu-rRNA-encoding gene, which in some species do have different sequences (121), but all ssu-rRNAs are nevertheless assumed to have the same in vivo function. It is clearly evident that these criteria cannot be met for all of the enzymes needed to catalyze methanogenesis, but they still may be met by MCH and MER, which do appear to be essential, not duplicated, and not subject to substitution by functionally equivalent enzymes. With sufficient sequences, MCH and MER trees may well accurately reflect the divergence of the methanogenesis pathway, although compensating for sequence differences that result from species-specific physiological demands may still be difficult. For example, *M. kandleri* grows optimally at ~100°C and contains ~3 M K^+ which, through salt interactions with surface-located, charged amino acid residues, helps stabilize the native configuration of *M. kandleri* proteins (21). *M. kandleri* proteins must therefore maintain a relatively large number of charged residues, many of which presumably do not contribute to enzyme activity but are nevertheless essential in *M. kandleri*. Methanogens now have such different nonmethanogenesis physiologies and lifestyles that constructing an MCH tree, or any other enzyme sequence-based tree that reflects the phylogeny of methanogenesis, without bias inappropriately introduced by other physiological demands is a challenge.

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