Function and Regulation of the Formate Dehydrogenase Genes of the Methanogenic Archaeon *Methanococcus maripaludis*

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Methanococcus maripaludis is a mesophilic species of *Archaea* capable of producing methane from two substrates: hydrogen plus carbon dioxide and formate. To study the latter, we identified the formate dehydrogenase genes of *M. maripaludis* and found that the genome contains two gene clusters important for formate utilization. Phylogenetic analysis suggested that the two formate dehydrogenase gene sets arose from duplication events within the methanococcal lineage. The first gene cluster encodes homologs of formate dehydrogenase α (FdhA) and β (FdhB) subunits and a putative formate transporter (FdhC) as well as a carbonic anhydrase analog. The second gene cluster encodes only FdhA and FdhB homologs. Mutants lacking either *fdhA* gene exhibited a partial growth defect on formate, whereas a double mutant was completely unable to grow on formate as a sole methanogenic substrate. Investigation of *fdh* gene expression revealed that transcription of both gene clusters is controlled by the presence of H_2 and not by the presence of formate.

Hydrogen and carbon dioxide are common substrates for methanogenic *Archaea*. Many species of hydrogenotrophic methanogens can use formate in place of H_2, while others cannot. The ability to use formate can be attributed to the enzyme formate dehydrogenase, which in methanogens catalyzes the reaction that produces reduced coenzyme F_{420} from the oxidation of formate (9, 10, 18). The enzyme consists of two protein subunits (17), α and β, encoded by *fdhA* and *fdhB*, respectively (15, 19).

The presence or absence of formate dehydrogenase genes does not predict the ability to grow on formate. Species known to contain contiguously encoded homologs of *fdhA* and *fdhB* include *Methanococcus maripaludis*, *Methanococcus voltae*, *Methanobacterium formicicum*, *Methanococcus jannaschii*, and *Methanopyrus kandleri*. Of these species, only the first three can use formate (4). In the species *Methanothermobacter thermautotrophicus*, strain Z-245 can use formate but strain ΔH cannot. This difference is attributed to the presence in the former strain of *fdh* genes that are absent from the same genomic context in the latter (15). Nevertheless, *fdhA* and *fdhB* homologs are present in the genome of strain ΔH, albeit not closely related to those known in strain Z-245. Furthermore, at least one species that is unable to grow on formate, *M. jannaschii*, possesses formate dehydrogenase enzyme activity (11).

The organization and regulation of formate dehydrogenase genes have been studied in *M. formicicum* (23) and *M. thermoautotrophicus* strain Z-245 (15), both of which use formate. In each species a gene designated *fdhC* precedes *fdhA* and *fdhB*. FdhC contains several potential membrane-spanning regions (23) and may encode a formate transporter. Northern blots in both species indicated the presence of three mRNA types, one containing *fdhC*, *fdhA*, and *fdhB*, another containing *fdhC* alone, and the third containing *fdhA* and *fdhB*. An apparent transcription start site was identified upstream of *fdhC* in both species. The smaller mRNAs could be formed by termination or processing between *fdhC* and *fdhA*, although a separate initiation of transcription in this region could not be ruled out. In *M. thermoautotrophicus* Z-245 the levels of *fdh* mRNA increased when the availability of hydrogen was decreased (15).

We have studied the formate dehydrogenase genes of *M. maripaludis*. Examination of the genome sequence of *M. maripaludis* indicated the presence of genes encoding two distinct formate dehydrogenases. Biochemical studies had shown that two distinct formate dehydrogenases exist in the related species *Methanococcus vannielii* (9, 10), but genetic studies were not carried out. Due to the development of genetic methods for *M. maripaludis* (21), we have been able to produce mutants in both sets of *fdh* genes and to determine their roles in growth on formate. In addition, we used *lacZ* fusions to determine factors that regulated the expression of both gene sets.

MATERIALS AND METHODS

Strains and growth conditions. Strict anaerobic growth conditions were used when culturing *M. maripaludis* as previously described (2). The wild-type strain LL is also designated strain S2 (13, 24). LL and its derivatives were grown in McC (24) or in nitrogen-free liquid medium (3) supplemented with 10 mM ammonium chloride. Tubes contained 5 ml of medium and 20 ml of headspace pressurized to 40 lb/in² with either H_2:CO_2 (80%:20%) or N_2:CO_2 (80%:20%); 20% CO_2 was always necessary, since a CO_2–bicarbonate equilibrium buffers the pH of the medium. Cultures were supplemented with 100 mM sodium formate where appropriate, and Tris-HCl (100 mM, pH 7) was added to buffer the pH during formic acid utilization. Antibiotics were used at the following concentrations (in micrograms per milliliter) where appropriate: neomycin sulfate, 500 (solid medium) or 1,000 (liquid medium); puromycin, 2.5; kanamycin sulfate, 25; ampicillin, 100.

Construction of mutants. The *fdhA1::neo* mutant, MM707, was constructed as follows. A 2,064-bp PCR product containing the *fdhA1* gene was amplified from LL chromosomal DNA by using primers FdhA1 and FdhA2 (Table 1). The PCR product was TA-cloned into pCR2.1Topo (Invitrogen), producing pCRfdhA303. A promoterless, terminatorless neomycin resistance cassette was amplified from pRCN230 (pRCN115 [5] with the Pur cassette replaced by a Neo cassette [1]) by

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TABLE 1. Primers used in this study

Target	Plasmid	Primer name and sequence (5′-3′)
fdhA1	pCRfdhA303	FdhA1: CCTTTCAAAAGACACCCTG
		FdhA2: ATGCAGTAACTGCCCCACCAC
neo	pCRfdhA303neo	NeoForCla: CCATCGATGGATCTGATCAAGAGACAGG
		NeoRevRINde: GAATTCCATATGCGAACCCCAGAGTCCCGC
fdhA2	pBSfdh336-11	Fdh/dhySXba: GGTCTAGAATCTGGGCTGACGCAACG
		FdhB62Xho: GGCTCGAGGATCGTGAATTCTTGGAACTTTCAAT
Pmcr-pac-Tmcr	pBSfdh336-11pac	Pmcr2MluI: GGACGCGTTCTAGAGGATGATTAATTTTAAG
		Tmcr2PpuRI: GAATTCAGGTCCCTAAAAATATATAAAAAAAGGAAACC
fdhA2 upstream region	pFL10	Fdh336Nsi: CCCATGCATTATTCCACCTTTTAATTTTATACTTATTTTTAACC
		Fdh336Spe4: GGACTAGTCATTCAAATTTTTGAGACTAGG
fdhA2 upstream region	pFL11	Fdh336Nsi: CCCATGCATTATTCCACCTTTTAATTTTATACTTATTTTTAACC
		Fdh336Spe3: GGACTAGTCGATATAGGTATTATTTAACAGG
fdhA2 upstream region	pFL12	Fdh336Nsi: CCCATGCATTATTCCACCTTTTAATTTTATACTTATTTTTAACC
		Fdh336Spe2: CCACTAGTGAAAAGATATTTCTTGATATCTCTC
fdhA2 upstream region	pFL13	Fdh336Nsi: CCCATGCATTATTCCACCTTTTAATTTTATACTTATTTTTAACC
		Fdh336Spe1: GGACTAGTCGATTGTATTTCCAATGCTCG
fdhA1 upstream region	pFL20	Fdh303Nsi: GGGATGCATATTTCGATCACCATTTTATAA
		Fdh303Spe3: GGACTAGTGCACAAATTTGAATGGTGTG
fdhA1 upstream region	pFL21	Fdh303Nsi: GGGATGCATATTTCGATCACCATTTTATAA
		Fdh303Spe2: CCACTAGTGCCTACAATAATATACTTAACTATATC
fdhA1 upstream region	pFL22	Fdh303Nsi: GGGATGCATATTTCGATCACCATTTTATAA
		Fdh303Spe1: GGACTAGTGCCTAAAAAAGGGTAATGTGG
fdhC1 upstream region	pFL30	FdhCNsi: GGGATGCATGATATTACCTCCATGTGAATATTG
		Fdh303Spe4: GGACTAGTCCACTTGATGTTAATTACTAC
fdhC1 upstream region	pFL31	FdhCNsi: GGGATGCATGATATTACCTCCATGTGAATATTG
		Fdh303Spe5: GGACTAGTGTAGCGTATCGTTGAAGC
fdhC1 upstream region	pFL32	FdhCNsi: GGGATGCATGATATTACCTCCATGTGAATATTG
		Fdh303Spe6: GGACTAGTGGGGCGCACATATATAAGAGG
fdhC1 upstream region	pFL33	FdhCNsi: GGGATGCATGATATTACCTCCATGTGAATATTG
		Fdh303Spe7: GGACTAGTAAAGGAAAAGTGACATTTTACAC

using primers NeoForCla and NeoRevNde and then were cloned into the *Cla*I and *Nde*I sites of pCRfdhA303. The resulting plasmid, pCRfdhA303neo, was used to transform LL as described below.

To make the *fdhA2*::*pac* mutant, MM708, *fdhA2* and surrounding DNA was amplified from LL chromosomal DNA by using primers Fdh/dhySXba and FdhB62Xho. The resulting 3,327-bp product was cloned into the *Xho*I and *Xba*I sites of pBluescript KS(+) (Stratagene), forming pBSfdh336-11. A puromycin resistance cassette, in which *pac* expression is controlled by the promoter (*Pmcr*) and terminator (*Tmcr*) of the *M. voltae mcr* operon (8), was amplified from pJK3 (14) by using primers Pmcr2MluI and Tmcr2PpuRI. This PCR product was then cloned into the *Eco*RI and *Mlu*I sites of pBSfdh336-11 to form pBSfdh336-11pac.

Allelic exchange was accomplished by transforming (20) LL with pCRfdh303neo or pBSfdh336-11pac and selecting on McC agar plates containing neomycin or puromycin, respectively, under 16 lb of H_2:CO_2/in^2. PCR and Southern blots confirmed that the wild-type allele had been replaced by the disrupted allele in these mutants and that no vector sequences remained (data not shown). MM708 was then transformed with pBSfdh336-11pac to produce the double mutant MM709.

Growth curves. Overnight McC–H_2:CO_2-grown cultures were diluted 1:50 into duplicate tubes containing 5 ml of the appropriate fresh medium. Tubes were pressurized with H_2:CO_2 or N_2:CO_2 to 40 lb/in^2. The optical density at 600 nm was determined immediately and at intervals throughout the growth curve. Readings were taken from duplicate cultures and were averaged.

Construction of *lacZ* reporter plasmids. Various regions upstream of *fdhA1*, *fdhC1*, and *fdhA2* (see Fig. 4) were PCR amplified and then were cloned into the *Spe*I and *Nsi*I sites of pWLG40+lacZ (6) such that the ATG was fused in frame with the ATG of *lacZ*. Each construct used the native ribosome binding site present upstream of the respective gene. The primers used to construct each plasmid are shown in Table 1. Each plasmid was sequenced to confirm that no errors had been introduced by PCR and then were used to transform LL. Plasmid pWLG40+lacZ was used as a positive control because it contains the *hmv* promoter, which is constitutively expressed (6). Plasmid pWLGΔhmv is identical to pWLG40+lacZ except that the DNA between the *Spe*I and *Nsi*I sites was deleted, thus removing the *hmv* promoter; this plasmid served as a promoterless negative control.

β-Galactosidase assays. Overnight McC–H_2:CO_2-grown cultures were diluted 1:25 in fresh medium as appropriate and then were pressurized to 40 lb/in^2 with H_2:CO_2 or N_2:CO_2. The optical density at 600 nm of the cultures was determined immediately (0 h), an aliquot (0.3 ml) was removed to determine β-galactosidase activity, and the cultures were placed in a 37°C shaker. Samples for the 3-, 6-, and 9-h time points were collected similarly. β-Galactosidase activities were determined by using standard procedures in duplicate, and the means and standard deviations were determined.

Sequence analysis and nucleotide sequence accession numbers. The genome sequence of *M. maripaludis* strain LL is an ongoing collaboration of J. A. Leigh with M. Olson (University of Washington Genome Center). *M. maripaludis fdh* sequences are deposited in GenBank (accession numbers AY236515 and AY236516).

RESULTS

***M. maripaludis* strain LL uses formate as a substrate for methanogenesis.** It has previously been reported that *M. maripaludis* strain JJ can grow on formate as a methanogenic substrate (12). To determine if this is also true for strain LL we compared its growth with either H_2:CO_2 or sodium formate (in the presence of N_2:CO_2) as methanogenic substrates. As shown in Fig. 1, LL grew on both H_2:CO_2 and formate, with maximal growth on H_2:CO_2. A higher final culture density was achieved on H_2:CO_2, presumably because 40 lb of H_2:CO_2/in^2 provides approximately five times more growth substrate than 100 mM formate, assuming that all available substrate is consumed. Cultures were inhibited somewhat by the Tris buffer that was necessary to stabilize the pH of cultures utilizing formic acid. Strain LL did not grow when both H_2 and formate
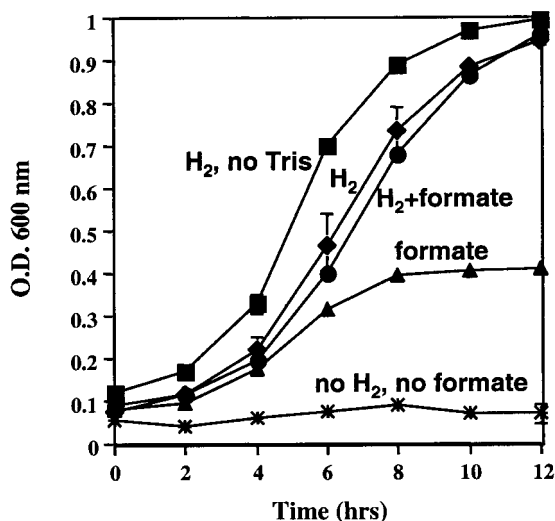
FIG. 1. Growth of *M. maripaludis* LL on $H_2:CO_2$ or formate. Squares, $H_2:CO_2$; circles, $H_2:CO_2$ plus Tris plus formate; triangles, $N_2:CO_2$ plus Tris plus formate; diamonds, $H_2:CO_2$ plus Tris; asterisks, N_2CO_2 plus Tris. The medium used was McC. O.D. 600 nm, optical density at 600 nm.

FIG. 3. Growth of *M. maripaludis fdh* mutants on $H_2:CO_2$ (upper curves) or $N_2:CO_2$ plus formate (lower curves). Solid lines, LL; dashed lines, *fdhA1* mutant; dashed and dotted lines, *fdhA2* mutant; dotted lines, double mutant. Lines join readings that were taken every 1 to 2 h. Readings from duplicate cultures were averaged. The medium used was McC plus Tris. O.D. 600 nm, optical density at 600 nm.

were absent, confirming the lack of growth-supporting substrate.

M. maripaludis LL contains two sets of formate dehydrogenase genes. We searched the genome sequence of *M. maripaludis* strain LL for formate dehydrogenase α subunit genes and found two separate gene clusters, which we named *fdh1* and *fdh2* (Fig. 2). We found no other formate dehydrogenases in the genome; distant homologs to FdhA appeared to be molybdopterin-containing proteins with different functions. The *fdhA1* gene lay immediately upstream of an *fdhB* homolog, *fdhB1*. An *fdhC* homolog, encoding a putative formate transporter, was found upstream of *fdhA1* separated by an open
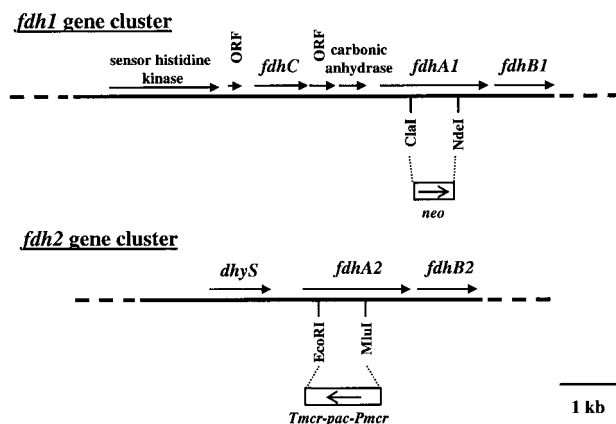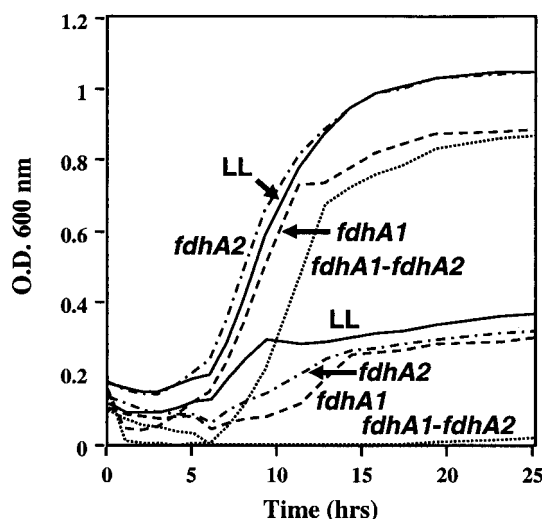

FIG. 2. The *fdh* gene clusters of *M. maripaludis* LL. Intergenic distances are as follows (in base pairs, between genes): sensor histidine kinase-213-ORF-247-*fdhC1*-40-ORF-23-carbonic anhydrase-304-*fdhA1*-38-*fdhB1*; *dhyS*-639-*fdhA2*-23-*fdhB2*. Mutations constructed in *fdhA1* and *fdhA2* are shown. *neo*, neomycin resistance gene; *pac*, puromycin resistance gene; *Pmcr* and *Tmcr*, promoter and terminator derived from the *M. voltae* methylreductase gene (7).

reading frame (ORF) of unknown function and a carbonic anhydrase homolog. In contrast, in *M. formicicum* (23) and *M. thermoautotrophicum* strain Z-245 (15) *fdhC* is immediately upstream of *fdhA*, and genes corresponding to the ORF of unknown function and the carbonic anhydrase homolog are not present. In addition, a small ORF of unknown function lay upstream of *fdhC* in *M. maripaludis*. The entire *fdh1* cluster was preceded by a sensor histidine kinase homolog. The *fdhA2* gene lay immediately upstream of an *fdhB* homolog (*fdhB2*); no *fdhC* homolog was found near *fdhA2*, nor was a carbonic anhydrase gene or a homolog of either unknown ORF present. The *fdh2* cluster was preceded by *dhyS* encoding a homolog of deoxyhypusine synthase. The two *fdh* clusters of *M. maripaludis* encoded closely related proteins; FdhA1 and FdhA2 were 65% identical and FdhB1 and FdhB2 were 62% identical. Each gene was preceded by a sequence similar to that of a ribosome binding site (data not shown). Both *fdhA* alleles contained a TGA codon at amino acid 132 (*fdhA1*) or 133 (*fdhA2*) which presumably encodes a selenocysteine residue. The presence of selenocysteine is not uncommon in formate dehydrogenases (8, 10).

In an effort to determine the origin of the two formate dehydrogenases of *M. maripaludis*, we carried out a phylogenetic analysis of FdhA and FdhB in methanogens. The results (not shown) indicated that the duplications leading to the presence of two *fdhA-fdhB* gene clusters in *M. maripaludis* occurred within the methanococcal lineage.

Both *fdhA* genes contribute to growth on formate. The relative importance of the two *fdh* gene clusters in growth on formate was assessed by constructing mutants in either or both *fdhA* genes. Internal sections of each gene were deleted and replaced by neomycin or puromycin resistance markers (Fig. 2). The mutants were then analyzed for growth on formate, and their growth was compared to that of wild-type strain LL
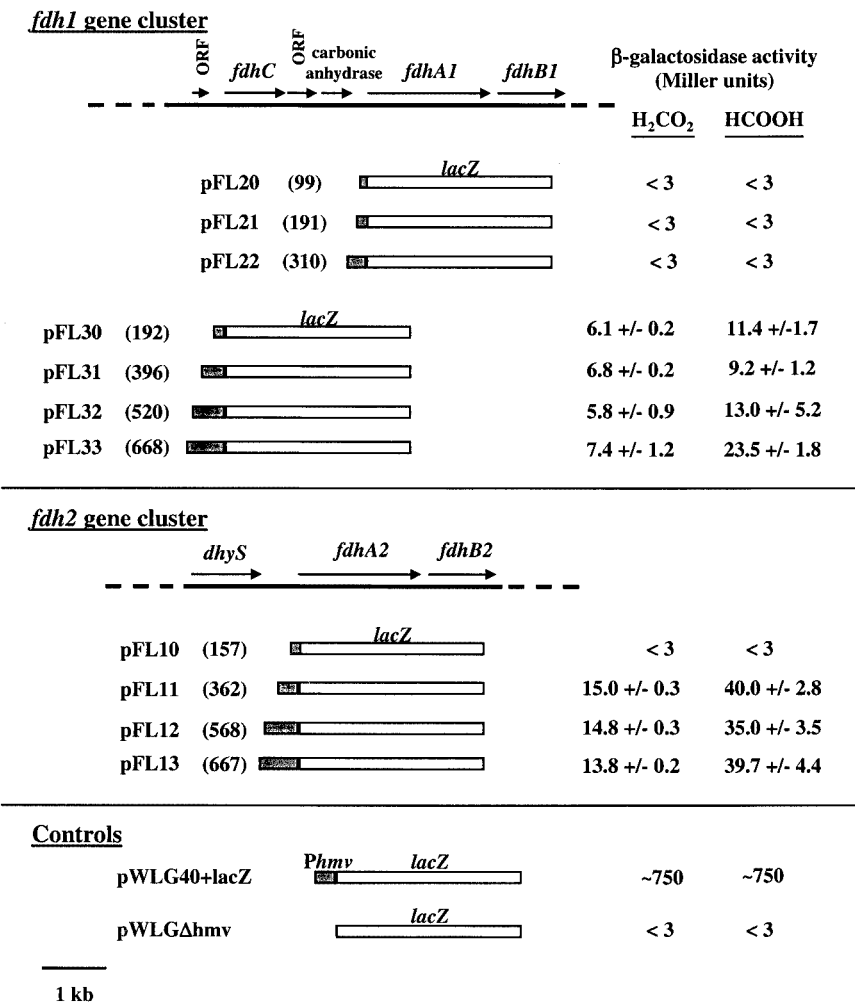
FIG. 4. Regulation mediated by regions upstream of *fdh* genes. Strains containing plasmids with *lacZ* fused to the indicated upstream regions were grown on $H_2:CO_2$ or on formate. Numbers in parentheses indicate the number of base pairs upstream of the putative start codon. β-Galactosidase activities are means ± standard deviations of duplicate measurements from a single culture after 9 h of growth on the indicated substrate. The medium used was McC plus Tris.

(Fig. 3). Mutants lacking either *fdhA1* or *fdhA2* showed a significant growth defect on formate. When both *fdhA* alleles were inactivated, *M. maripaludis* was completely unable to grow with formate as sole methanogenic substrate. All strains grew well on $H_2:CO_2$. The entire experiment was repeated, with similar results. These results suggest that both *fdhA* genes contribute to growth on formate as the methanogenic substrate.

White (22) found that free formate, not formyl-tetrahydromethanopterin, is the substrate for the in vitro synthesis of purines in *M. thermoautotrophicus* and *Sulfolobus solfataricus*. We hypothesized that in addition to functioning in growth on formate, the *fdh* genes may be required for the synthesis of formate and hence of purines during growth on $H_2:CO_2$ in *M. maripaludis*. To test this hypothesis we performed growth experiments in chemically defined medium that lacked exogenous purines (nitrogen-free medium supplemented with ammonium chloride). Both single mutants and the double mutant grew as well as LL, suggesting that neither *fdhA* allele is re-

quired to produce formate for purine biosynthesis (data not shown).

Regulation of *fdh* expression. We studied the expression of each *fdh* gene cluster by constructing a series of plasmid-encoded *lacZ* fusions to possible promoter region DNAs. Intergenic regions upstream of *fdhC1*, *fdhA1*, and *fdhA2* were judged to be of sufficient length to contain possible promoter regions (see Fig. 2). Therefore, regions extending various distances upstream of *fdhA1*, *fdhC1*, and *fdhA2* were PCR amplified and fused in frame with the ATG of *lacZ* on a low-copy replicative plasmid. Thus, each construct contained the native ribosome binding site and start codon fused to *lacZ*. The extent of each upstream region is shown in Fig. 4. *M. maripaludis* LL was transformed with these plasmids, and β-galactosidase assays were done with McC medium with either $H_2:CO_2$ or formate (with $N_2:CO_2$) as methanogenic substrate. Controls included a plasmid (pWLG40+lacZ) in which *lacZ* is constitutively expressed (6) and a plasmid (pWLGΔhmv) which lacks

a promoter upstream of *lacZ*. As shown in Fig. 4, fusions to DNA immediately upstream of *fdhA1* (pFL20 to pFL22) were not expressed on either substrate, suggesting that no promoter lies in this region. Fusions made to DNA upstream of *fdhC1* (pFL30 to pFL33) had β-galactosidase activity, suggesting that a promoter lies in this region. In all cases *fdhC-lacZ* expression was higher when cultures were grown on formate in the presence of $N_2:CO_2$ than when grown on $H_2:CO_2$. Plasmids pFL30 to pFL32, collectively containing DNA ranging up to 520 bp upstream of *fdhC*, gave similar results. In contrast, higher expression and greater regulation appeared to occur when DNA between 520 and 668 bp upstream of *fdhC* was included in the region fused to *lacZ* (pFL33). This result suggests that the regions between the start codon of *fdhC* and 520 bp and between 520 and 668 bp both contain separate sequences involved in the activation of *fdhC* expression with formate plus $N_2:CO_2$. Notably, 520 bp upstream of *fdhC* is only 30 bp upstream of a small ORF of unknown function (see Fig. 2). Therefore, activation involving the region upstream of 520 bp might be due not only to DNA that acts *cis* to *fdhC* but also to the expression of this ORF.

β-Galactosidase fusions were also made by using DNA upstream of *fdhA2* (pFL10 to pFL13, Fig. 4). No β-galactosidase was expressed from plasmid pFL10, suggesting that no promoter is present in the 157 bp immediately upstream of the start codon of *fdhA2*. Fusions containing ≥362 bp of DNA were all expressed, indicating the presence of a promoter in this region. Likewise, all three fusions were upregulated two- to threefold in formate-containing medium with $N_2:CO_2$, suggesting the presence of a regulatory site.

A time course experiment was performed to extend the above observations. In all cases, enhancement of *fdhC1* (Fig. 5A) and *fdhA2* (Fig. 5B) expression in the presence of formate and $N_2:CO_2$ was observed starting 3 h after inoculation. As before, the highest expression of *fdhC1* occurred only with pFL33, which contains 668 bp of upstream DNA. An increase in expression of both *fdhC1* and *fdhA2* was consistently seen after 9 h of growth on $H_2:CO_2$. One possible explanation is that $H_2:CO_2$ pressure was decreasing and that, as shown below, *fdh* expression is controlled by the presence of H_2.

To test the hypothesis that *fdh* expression is regulated by the presence of H_2 we repeated the time course experiment, this time including cultures containing formate with an $H_2:CO_2$ atmosphere. Thus, three conditions were compared: H_2 alone, H_2 plus formate, and formate alone (N_2 replacing H_2). Comparison of these three conditions allowed us to separately assess the effects of hydrogen and of formate (Fig. 6). Constructs containing the maximum upstream regions of *fdhC1* (Fig. 6A) and *fdhA2* (Fig. 6B) were used. When both $H_2:CO_2$ and formate were present (growth condition B), *fdhC1-lacZ* and *fdhA2-lacZ* expression were both low compared to that of formate with an $N_2:CO_2$ atmosphere (growth condition C). These results suggested that the presence of H_2 resulted in decreased gene expression. To further test the effect of H_2, cultures were grown for 6 h in tubes containing both $H_2:CO_2$ and formate, β-galactosidase activity was measured, and the $H_2:CO_2$ was flushed away and replaced with 40 lb of $N_2:CO_2/in^2$ (growth condition E). Control cultures were flushed with $N_2:CO_2$ and were repressurized with 40 lb of $H_2:CO_2/in^2$ (growth condition D). β-Galactosidase activities increased at
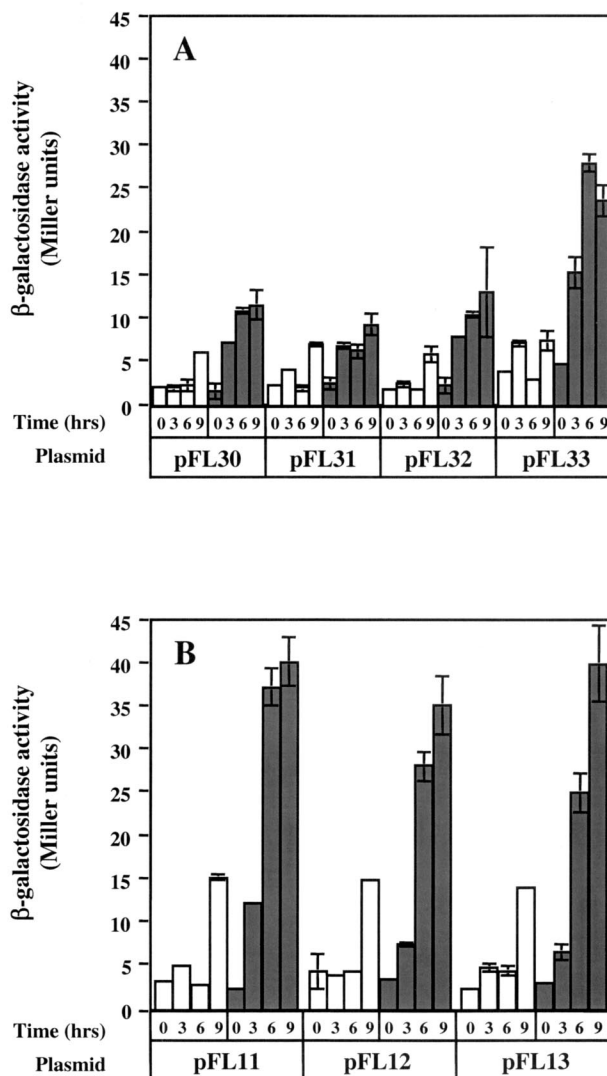

FIG. 5. Time course of *fdh* regulation. Strains containing plasmids as shown in Fig. 4 were grown with $H_2:CO_2$ and then were used to inoculate medium with $H_2:CO_2$ (open bars) or formate plus $N_2:CO_2$ (filled bars). The medium used was McC plus Tris.

9 h due to the removal of H_2. Furthermore, repeat experiments with each construct revealed the same pattern. These results confirm that both *fdhC1* expression and *fdhA2* expression are controlled by the presence or absence of H_2. In contrast, formate had no marked effect.

DISCUSSION

Apparent gene duplication within the methanococcal lineage has resulted in *M. maripaludis* containing two sets of formate dehydrogenase genes. We considered two alternative hypotheses for the function of these genes. One hypothesis was that duplication of function has occurred: both formate dehydrogenases convert formic acid to carbon dioxide and reduce F_{420} for the purpose of methanogenesis. The other hypothesis was that only one formate dehydrogenase has this catabolic function while the other functions biosynthetically, producing
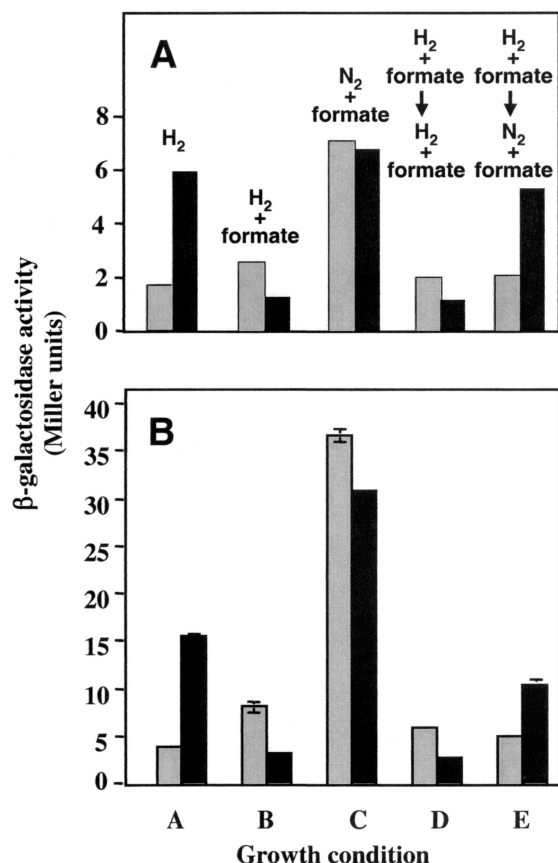
FIG. 6. Effects of H_2 and formate on *fdhC1* and *fdhA2* regulation. LL containing pFL33 (A) or pFL13 (B) was grown under the following conditions: lanes A, H_2:CO_2; lanes B, H_2:CO_2 plus formate; lanes C, N_2:CO_2 plus formate; lanes D, H_2:CO_2 plus formate flushed with H_2:CO_2 at 6 h; lanes E, H_2:CO_2 plus formate flushed with N_2:CO_2 at 6 h. Grey bars, 6 h; black bars, 9 h. The medium used was McC plus Tris.

from H_2 and CO_2 the formate that has been suggested as a substrate for purine biosynthesis in other methanogens (22). Our results support the first hypothesis: both FdhA proteins appeared to contribute to the ability to grow on formate as the catabolic substrate, and together the two formate dehydrogenases accounted fully for this ability. Our findings are in concurrence with those of a recent study in which the *selB* gene encoding the archaeal translation factor specialized for selenocysteine insertion was inactivated in *M. maripaludis* strain JJ (16). The resulting mutant was unable to grow on formate, presumably because both *fdhA* genes encode selenocysteine-containing proteins. We found no evidence that either formate dehydrogenase is required for biosynthesis, since the double mutant grew well in minimal medium on H_2 and CO_2.

Maximum expression of both *fdh* clusters depended on the absence of H_2 and not the presence of formate. This observation parallels that of Nolling and Reeve (15), who noted that in *M. thermoformicicum* Z-245, *fdh* expression was regulated by the availability of H_2. Whether formate had any regulatory effect was not addressed in that study. In *M. maripaludis*, growth on H_2 was superior to growth on formate (Fig. 1), and

downregulation of *fdh* by H_2 would be in accordance with the organism's tendency to grow on formate only when H_2 is absent. However, why significant *fdh* expression still occurs when hydrogen is present is unknown; a biosynthetic function would be one explanation, but as stated above we were unable to find evidence to support this idea. Besides regulation by H_2, *fdh* expression in *M. formicicum* is stimulated by molybdate (23), a phenomenon we did not investigate with *M. maripaludis*.

By fusing different segments of *fdh* promoter region DNA to *lacZ* we were able to localize the sites necessary for expression and regulation. In the case of *fdh2* both expression and regulation were mediated by DNA between 157 and 362 bp upstream of the *fdhA* coding sequence. In the case of *fdh1* transcription was initiated within 192 bp upstream of *fdhC*, and no transcription was initiated upstream of *fdhA*. These results are consistent with those of studies with *M. formicicum* (23) and *M. thermoautorophicus* strain Z-245 (15) which suggested that transcription occurred from a promoter upstream of *fdhC* with processing of some transcripts between *fdhC* and *fdhA*. Regulation of *fdh1* appeared to be more complex than that of *fdh2*, being mediated partially by DNA within 192 bp of *fdhC* but with additional activation depending on DNA between 520 and 668 bp upstream of *fdhC*. Future experiments will characterize the regulatory roles of these upstream regions in more detail, including the possible function of the small ORF upstream of *fdhC*.

REFERENCES

1. **Argyle, J. L., D. L. Tumbula, and J. A. Leigh.** 1996. Neomycin resistance as a selectable marker in *Methanococcus maripaludis*. Appl. Environ. Microbiol. **62:**4233–4237.
2. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43:**260–296.
3. **Blank, C. E., P. S. Kessler, and J. A. Leigh.** 1995. Genetics in methanogens: transposon insertion mutagenesis of a *Methanococcus maripaludis nifH* gene. J. Bacteriol. **177:**5773–5777.
4. **Boone, D. R., and R. W. Castenholz (ed.).** 2001. The Archaea and the deeply branching phototrophic bacteria, 2nd ed., vol. 1. Springer-Verlag, New York, N.Y.
5. **Cohen-Kupiec, R., C. Blank, and J. A. Leigh.** 1997. Transcriptional regulation in Archaea: in vivo demonstration of a repressor binding site in a methanogen. Proc. Natl. Acad. Sci. USA **94:**1316–1320.
6. **Gardner, W. L., and W. B. Whitman.** 1999. Expression vectors for Methanococcus maripaludis: overexpression of acetohydroxyacid synthase and beta-galactosidase. Genetics **152:**1439–1447.
7. **Gernhardt, P., O. Possot, M. Foglino, L. Sibold, and A. Klein.** 1990. Construction of an integration vector for use in the archaebacterium Methanococcus voltae and expression of a eubacterial resistance gene. Mol. Gen. Genet. **221:**273–279.
8. **Jones, J. B., G. L. Dilworth, and T. C. Stadtman.** 1979. Occurrence of selenocysteine in the selenium-dependent formate dehydrogenase of *Methanococcus vannielii*. Arch. Biochem. Biophys. **195:**255–260.
9. **Jones, J. B., and T. C. Stadtman.** 1980. Reconstitution of a formate-NADP+ oxidoreductase from formate dehydrogenase and a 5-deazaflavin-linked NADP+ reductase isolated from Methanococcus vannielii. J. Biol. Chem. **255:**1049–1053.
10. **Jones, J. B., and T. C. Stadtman.** 1981. Selenium-dependent and selenium-independent formate dehydrogenases of Methanococcus vannielii. Separation of the two forms and characterization of the purified selenium-independent form. J. Biol. Chem. **256:**656–663.
11. **Jones, W. J., J. A. Leigh, F. Mayer, C. R. Woese, and R. S. Wolfe.** 1983. *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. Arch. Microbiol. **136:**254–261.
12. **Jones, W. J., M. J. B. Paynter, and R. Gupta.** 1983. Characterization of

Methanococcus maripaludis sp. nov., a new methanogen isolated from salt marsh sediment. Arch. Microbiol. **135:**91–97.

13. **Lie, T. J., and J. A. Leigh.** 2002. Regulatory response of *Methanococcus maripaludis* to alanine, an intermediate nitrogen source. J. Bacteriol. **184:** 5301–5306.

14. **Metcalf, W. W., J. K. Zhang, E. Apolinario, K. R. Sowers, and R. S. Wolfe.** 1997. A genetic system for Archaea of the genus Methanosarcina: liposome-mediated transformation and construction of shuttle vectors. Proc. Natl. Acad. Sci. USA **94:**2626–2631.

15. **Nolling, J., and J. N. Reeve.** 1997. Growth- and substrate-dependent transcription of the formate dehydrogenase (*fdhCAB*) operon in *Methanobacterium thermoformicicum* Z-245. J. Bacteriol. **179:**899–908.

16. **Rother, M., I. Mathes, F. Lottspeich, and A. Bock.** 2003. Inactivation of the *selB* gene in *Methanococcus maripaludis*: effect on synthesis of selenoproteins and their sulfur-containing homologs. J. Bacteriol. **185:**107–114.

17. **Schauer, N. L., and J. G. Ferry.** 1986. Composition of the coenzyme F_{420}-dependent formate dehydrogenase from *Methanobacterium formicicum*. J. Bacteriol. **165:**405–411.

18. **Schauer, N. L., and J. G. Ferry.** 1982. Properties of formate dehydrogenase in *Methanobacterium formicicum*. J. Bacteriol. **150:**1–7.

19. **Shuber, A. P., E. C. Orr, M. A. Recny, P. F. Schendel, H. D. May, N. L. Schauer, and J. G. Ferry.** 1986. Cloning, expression, and nucleotide sequence of the formate dehydrogenase genes from Methanobacterium formicicum. J. Biol. Chem. **261:**12942–12947.

20. **Tumbula, D. L., T. L. Bowen, and W. B. Whitman.** 1994. Transformation of Methanococcus maripaludis and identification of a PstI-like restriction system. FEMS Microbiol. Lett. **121:**309–314.

21. **Tumbula, D. L., and W. B. Whitman.** 1999. Genetics of Methanococcus: possibilities for functional genomics in Archaea. Mol. Microbiol. **33:**1–7.

22. **White, R. H.** 1887. Purine biosynthesis in the domain Archaea without folates or modified folates. J. Bacteriol. **179:**3374–3377.

23. **White, W. B., and J. G. Ferry.** 1992. Identification of formate dehydrogenase-specific mRNA species and nucleotide sequence of the *fdhC* gene of *Methanobacterium formicicum*. J. Bacteriol. **174:**4997–5004.

24. **Whitman, W. B., J. Shieh, S. Sohn, D. S. Caras, and U. Premachandran.** 1986. Isolation and characterization of 22 mesophilic methanococci. Syst. Appl. Microbiol. **7:**235–240.