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Organization and Growth Phase-Dependent Transcription of Methane Genes in Two Regions of the *Methanobacterium thermoautotrophicum* Genome

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Two regions of the *Methanobacterium thermoautotrophicum* genome containing genes that encode enzymes involved in methanogenesis (methane genes) have been cloned and sequenced to determine the extent of methane gene clustering and conservation. One region from the *M. thermoautotrophicum* strains ΔH and Winter, extending ~13.5 kb upstream from the adjacent *mvhDGAB* and *mrtBDGA* operons that encode the methyl-viologen-reducing hydrogenase (MVH) and the methyl coenzyme M reductase II (MRII), respectively, was sequenced, and 76% sequence identity and very similar gene organizations were demonstrated. Five closely linked open reading frames were located immediately upstream of the *mvh* operon and were designated *flpECBDA*. The *flpCBD* genes encode amino acid sequences that are 31, 47, and 65% identical to the primary sequences of the α and β subunits of formate dehydrogenase and the δ subunit of MVH, respectively. Located immediately upstream of the *flp* genes was the *mth* gene, which encodes the H_2-dependent methylene-tetrahydromethanopterin dehydrogenase (MTH). In contrast to this *mth-flp-mvh-mrt* cluster of methane genes, a separate ~5.4-kb genomic fragment cloned from *M. thermoautotrophicum* ΔH contained only one methane gene, the *mtd* gene, which encodes the 8-hydroxy-5-deazaflavin (H_2F_{420})-dependent methylene-tetrahydromethanopterin dehydrogenase (MTD). Northern (RNA) blot experiments demonstrated that *mth* was transcribed only at early growth stages in fermentor-grown cultures of *M. thermoautotrophicum* ΔH, whereas *mtd* was transcribed at later growth stages and in the stationary phase. Very similar transcription patterns have been observed by T. D. Pihl, S. Sharma, and J. N. Reeve (J. Bacteriol. 176:6384–6391, 1994) for the MRI- and MRII-encoding operons, *mrtBDGA* and *mcrBDCGA*, in *M. thermoautotrophicum* ΔH, suggesting coordinated regulation of methane gene expression. In contrast to the growth phase-dependent transcription of the *mth*/*mrt* and *mtd*/*mcr* genes, transcription of the *mvhDGAB* and *frhADGB* operons, which encode the two (NiFe) hydrogenases in *M. thermoautotrophicum* ΔH, was found to occur at all growth stages.

Seven enzymatic steps in the H_2-dependent reduction of CO_2 to CH_4 by strains of the thermophilic archaeon *Methanobacterium thermoautotrophicum* have been characterized. During this process, CO_2 is progressively reduced through the formyl, methenyl, methylene, and methyl levels and transferred from methanofuran to tetrahydromethanopterin (H_4MPT) to coenzyme M (CoM). In the final reaction, methyl-CoM is reduced and methane is released. Characterization of the enzymes involved in this pathway has led to the discovery of isoenzymes and of functionally equivalent enzymes (for a review see reference 35). The first step in methanogenesis is catalyzed by either a tungsten- or molybdenum-containing formyl-methanofuran dehydrogenase (29). The fourth reaction, the reduction of N^5,N^{10}-methenyl-H_4MPT to N^5,N^{10}-methylene-H_4MPT, can be catalyzed by either an H_2-dependent (cofactor F_{420}-independent) or F_{420}-dependent methylene-H_4MPT dehydrogenase, designated MTH and MTD, respectively (44). Finally, two methyl-CoM reductases, designated methyl-CoM reductase I (MRI) and MRII, have been demonstrated to catalyze the methane-releasing step (26). By using antisera specific for MRI and MRII, it was shown that MRII is the predominant isoenzyme in cells in the early exponential growth phase and is then replaced by MRI at later growth stages and in the stationary phase (4). Recently, we demonstrated that the regulation of MRI and MRII syntheses occurs at the level of transcription initiation (24).

To continue these investigations into the regulation of methane gene expression, additional methane genes had to be located, cloned, and sequenced. The physical map of the *M. thermoautotrophicum* Marburg genome, generated by Southern hybridizations, indicated that methane genes were clustered in an ~200-kb region of this 1.6-Mb genome (33) and suggested that most, if not all, of these genes might be very closely linked. Consistent with this, we found that, in the genomes of *M. thermoautotrophicum* ΔH and *Methanothermus fervidus*, the genes encoding the methyl-viologen-reducing hydrogenase (MVH), *mvhDGAB*, were located immediately upstream of the MRII-encoding genes, *mrtBDGA*, and that the genes encoding the methyl-H_4MPT:CoM-methyltransferase, *mtrEDCBA*, were located immediately downstream of the MRI-encoding genes, *mcrBDCGA* (24, 32). The same adjacent organization of the *mcr* and *mtr* operons has also been demonstrated to occur in the *Methanococcus vannielii* genome (18).

To extend this analysis of methane gene clustering, we have sequenced an additional ~13.5 kb of genomic DNA directly upstream from the *mvh* operons in the *M. thermoautotrophicum* strains ΔH and Winter. This region contains at least one additional bona fide methane gene, the MTH-encoding *mth* gene, plus five closely linked genes, designated *flpECBDA*, that appear likely to be methane genes. We have also cloned and sequenced an ~5.4-kb region from *M. thermoautotrophicum* ΔH that contains the MTD-encoding *mtd* gene. As both MTH and MTD catalyze the fourth step in methanogenesis and can

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be considered effectively equivalent enzymes, it seemed unlikely that *M. thermoautotrophicum* cells would synthesize both enzymes under the same conditions. Therefore, the possibility of growth phase-dependent regulation of *mth* and *mtd* transcription has been investigated. In addition, as MTH can also be considered a metal-free hydrogenase (44, 45), the effects of the growth phase on transcription of the *frhADGB* and *mvhDGAB* operons which encode the F_{420}-dependent hydrogenase (FRH) and MVH, the two previously well-characterized (NiFe) hydrogenases in *M. thermoautotrophicum* ΔH (for a review see reference 6), were also investigated in this study.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. thermoautotrophicum* strains ΔH (DSM 1053) and Winter and *Methanobacterium thermoformicicum* Z-245 (DSM 3720) were cultivated in a minimal salts medium (19) in 20- or 200-ml batch cultures, or in a 20-liter fermentor as previously described (24). *Escherichia coli* DH5α (11) was used as the host strain to grow the λ-Charon35 recombinant phages λH1 and λα32, which were isolated previously (25, 32), and for the propagation of pUC18- and pUC19-based recombinant plasmids (41). The λH1 and λα32 phages contain DNA located immediately upstream of the *mvhDGAB* genes in the genomes of *M. thermoautotrophicum* strains ΔH and Winter, respectively (25, 32). LambdaGEM-11 recombinant phages were propagated in *E. coli* KW251 (Promega Corp., Madison, Wis.). *E. coli* cultures were grown at 37°C in Luria-Bertani medium (27) that, when appropriate, also contained 50 μg of ampicillin per ml or 5 μg of tetracycline per ml.

Enzymes and chemicals. Enzymes used for the manipulation of nucleic acids were purchased from Gibco/BRL (Gaithersburg, Md.) or Boehringer Mannheim Biochemicals Co. (Indianapolis, Ind.). Radioactively labeled nucleotides ([α-^{32}P]dATP [110 TBq/mmol], [γ-^{32}P]ATP [259 TBq/mmol], and ^{35}S-dATP [37 TBq/mmol]) were purchased from ICN Biomedicals, Inc. (Costa Mesa, Calif.) and from DuPont Co. (Hoffman Estates, Ill.). All other chemicals were obtained either from Sigma Chemical Co. (St. Louis, Mo.) or Jenneile Enterprises (Cincinnati, Ohio).

Genomic DNA library construction. An *M. thermoautotrophicum* ΔH genomic library was constructed in LambdaGEM-11 by using the recommended protocol (Promega Corp.). In brief, genomic DNA was isolated as previously described (38) and partially digested with *Sau*3AI, and the single-stranded ends generated were partially filled in by using the Klenow fragment of DNA polymerase I supplemented only with dGTP and dATP. This mixture of DNA molecules was ligated to LambdaGEM-11 arms that had been digested with *Xho*I and partially filled in by using only dCTP and dTTP. The ligation mixture was packaged in vitro with a commercially obtained λ packaging kit (Promega Corp.), which resulted in a total of 3×10^5 PFU with an average insert size of ~15 kb. There is a 99.9% probability that this represented a fully comprehensive library of the 1.6-Mb genome of *M. thermoautotrophicum* ΔH.

DNA manipulations and DNA sequencing. Routine DNA manipulations were performed as described by Sambrook et al. (27). Recombinant λ DNA was isolated from phage particles by the procedure described by Meese et al. (15). To subclone the insert DNA from λ phages into pUC vectors, both the linearized plasmid DNA and the insert DNA were purified from agarose gels by the GeneClean procedure (Bio 101, La Jolla, Calif.). PCR was performed to amplify DNA regions from the *M. thermoautotrophicum* Winter and *M. thermoformicicum* Z-245 genomes under the conditions described earlier (20) except that the oligonucleotide primers were annealed at 40°C. The amplified DNA fragments were manipulated as previously described (21) and cloned into *Hin*cII-digested pUC vectors. Double-stranded DNA templates were prepared for DNA sequencing according to the method of Yie et al. (42). Vector- and insert-specific oligonucleotides were used to prime dideoxy chain-terminated reactions (28) with the 7-deaza-dGTP version of the Sequenase 2.0 kit (U.S. Biochemical, Cleveland, Ohio). The sequences obtained were analyzed by using the University of Wisconsin Genetics Computer Group package version 6.0 (7) and the BLAST program (2).

RNA isolation, Northern (RNA) blots, and primer extension reactions. The protocol used to isolate RNA from fermentor-grown *M. thermoautotrophicum* ΔH cells has been described in detail previously (24). Northern blots were obtained by a modified version of the procedure described by Hennigan and Reeve (13). After electrophoresis of the RNA samples and capillary transfer to Zeta-probe membranes (Bio-Rad, Hercules, Calif.), the membranes were incubated for 20 min at 65°C in hybridization buffer (0.5 M Na_2HPO_4 [pH 7.2], 7% [wt/vol] sodium dodecyl sulfate [SDS], 1 mM EDTA, 1% [wt/vol] crystalline-grade bovine serum albumin), after which the ^{32}P-end-labeled oligonucleotide probes (12 pmol each) were added. Hybridization was allowed to occur for ~16 h at 45°C. The membranes were then washed twice for 20 min in 2× SSC (0.3 M NaCl plus 0.03 M sodium citrate) that contained 0.1% SDS at a temperature that was 10°C below the lowest dissociation temperature calculated for the probes. The blots were visualized by autoradiography and quantitated by using a Packard Instant Imager 2024. RNA size standards were purchased from Boehringer Mannheim Biochemicals Co. The primer extension reactions were performed as described by Montzka and Steitz (16). The same oligonucleotides were used as probes in the Northern blot hybridizations and as primers in the primer extension reactions. Oligonucleotides with the following sequences that are complementary to regions near the 5′ termini of the *mth*, *mtd*, *frh*, and *mvh* transcripts were synthesized: 5′-GGGTCCTGTAACATCCTGCACCTAG (*mth*), 5′-GTCGGC CCTCTCATCTAGTAACAG (*mtd*), 5′-CGCCCCTTTGTCACGATTCCCTC AT (*frh*), and 5′-CCCGCAGTGTCGGCTCCACCGTAGG (*mvh*). The *mcr* and *mrt* transcripts were detected by using the same oligonucleotide, with the sequence 5′-TTACTGTGCCTTGTAACGA, which is complementary to a sequence conserved near the 3′ ends of both the *mcrB* and *mrtB* transcripts (24).

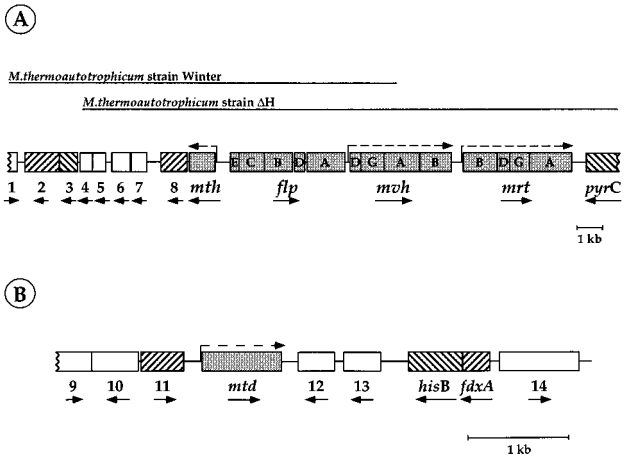


FIG. 1. Maps of the genes in the *mth* regions of *M. thermoautotrophicum* strains ΔH and Winter (A) and in the *mtd* region of *M. thermoautotrophicum* ΔH (B). Each gene or ORF listed in Table 1 or 3 is shown as a box, with the solid arrows indicating the gene orientations. The numbers below the boxes are ORF designations. Methane genes are indicated by shaded boxes, whereas ORFs encoding amino acid sequences that are similar to the sequences of enzymes not involved in methanogenesis are shown by hatched boxes. ORFs for which functions could not be predicted are shown by open boxes. The transcripts identified in this study and by Pihl et al. (24) are indicated above the genes by dashed lines, which also indicate the approximate sites of transcription initiation and termination. The overlapping regions of the genome of *M. thermoautotrophicum* ΔH that was cloned into the recombinant phages λH1 and λA6 (25, 32) and of the Winter strain genome cloned into λα32 (25), sequenced in this and previous studies (24, 25), are indicated by the horizontal lines above the *mth* region in panel A. Note the difference in the scales of panels A and B.

Nucleotide sequence accession numbers. The sequences obtained from the regions upstream of the *mvh* operon in the *M. thermoautotrophicum* strains ΔH and Winter have been assigned the GenBank accession numbers U19363 and U19364, respectively, and the sequence obtained from the *mtd* region from *M. thermoautotrophicum* ΔH has been assigned the GenBank accession number U19362.

RESULTS

Analysis of the sequences in the *mth* region in the genomes of *M. thermoautotrophicum* strains ΔH and Winter. In *M. thermoautotrophicum* ΔH, the *mrtBDGA* genes are immediately downstream from the *mvhDGAB* genes and upstream from the *pyrC* gene that encodes dihydroorotase (24). As *pyrC* is not a methane gene, we sequenced regions further upstream from the *mvh* operon, in both *M. thermoautotrophicum* ΔH and Winter, to determine if there were additional methane genes adjacent to the *mvh* and *mrt* operons. On the basis of their 16S rRNA sequences, *M. thermoautotrophicum* strains ΔH and Winter are representatives of the Z-245 and CB12 groups, respectively, and could be considered different species (20).

Sequences of 10,852 and 14,039 bp, starting from within the *mvhD* genes, were obtained from the genomic DNAs of *M. thermoautotrophicum* strains ΔH and Winter that were cloned in λH1 and λα32, respectively (Fig. 1A). When optimally aligned, these sequences have an overall identity of 76%, with

TABLE 1. Gene locations upstream of the *mvh* operon in *M. thermoautotrophicum* strains ΔH and Winter and features predicted for the encoded gene products

Gene locations and features of gene products for *M. thermoautotrophicum* ΔH						Gene locations and features of gene products for *M. thermoautotrophicum* Winter						Identity (%)[c]
Gene	Size[a]	M_r (kDa)	pI	Start codon	Position[b]	Gene	Size[a]	M_r (kDa)	pI	Start codon	Position[b]	
						orf1[d]	122				1>369	
						orf2	409	45.1	4.8	ATG	855<2087	
						orf3	251	27.7	4.6	ND[e]	2069<2824	
orf4[d]	96			ATG	2<289	*orf4*	162	18.1	5.2	ATG	2884<3372	76
orf5	180	19.9	4.7	ATG	286<828	*orf5*	180	19.9	5.4	ATG	3369<3911	69
orf6	222	24.2	4.2	ATG	978<1646	*orf6*	221	23.9	4.0	ATG	4072<4737	65
orf7	258	27.9	4.3	ATG	1669<2445	*orf7*	257	28.6	4.4	ATG	4788<5561	65
orf8	343	37.5	6.0	TTG	3009<4040	*orf8*	343	37.9	4.7	TTG	6175<7206	80
mth	344	37.6	4.5	ATG	4145<5179	*mth*	344	37.5	4.5	ATG	7308<8342	97
flpE	119	13.7	4.6	ATG	5804>6163	*flpE*	119	13.8	4.4	ATG	8941>9300	71
flpC	343	37.8	4.6	ATG	6167>7198	*flpC*[f]				ATG	9305>10347	65
flpB	380	43.7	4.5	ATG	7200>8342	*flpB*	380	43.6	4.7	ATG	10353>11495	81
flpD	136	15.5	8.7	ATG	8394>8804	*flpD*	141	16.2	10.0	ATG	11505>11930	92
flpA	505	55.1	4.7	ATG	8921>10435	*flpA*	505	54.8	4.7	ATG	12044>13561	86

[a] Number of amino acid residues.
[b] Position 1 is the 5′ nucleotide of the *mth* region as shown in Fig. 1A. The symbols > and < indicate the sense directions of the ORFs.
[c] Percent identity of the amino acid sequences of the homologous gene products from *M. thermoautotrophicum* strains ΔH and Winter.
[d] *orf1* and *orf4* are truncated in the analyzed sequences.
[e] The 5′ region of *orf3* encodes an amino acid sequence with similarity to the N-terminal region of a putative ABC transporter protein (see Table 3) but lacks an ATG, TTG, or GTG translation start codon as confirmed by sequencing of a DNA fragment that was PCR amplified directly from the *M. thermoautotrophicum* Winter genome. ND, not determined.
[f] Contains a frameshift at codon 156 leading to premature translation termination at codon 157. The percent identity of the N-terminal region of the *M. thermoautotrophicum* ΔH FLPC and the amino acid sequence encoded by the truncated *flpC* gene located 5′ to the frameshift in strain Winter is shown.

local variations over 300-bp stretches ranging from 68 to 93% identity and with short insertions and deletions (<100 bp) present in some intergenic regions. Eleven open reading frames (ORFs) that are initiated with an ATG, GTG, or TTG start codon and contain more than 100 codons were detected in the 11-kb region upstream of the *mvh* genes in *M. thermoautotrophicum* ΔH (Fig. 1A). All of these ORFs, except for *flpC* (see below), were also present and similarly organized in the Winter strain. Three additional ORFs at the 5′ end of the *M. thermoautotrophicum* Winter DNA that was cloned in λα32, which were not cloned from *M. thermoautotrophicum* ΔH in λH1, were sequenced (Fig. 1A). The locations and basic features of these ORFs and their predicted protein products are listed in Table 1, and the similarities of the products to previously identified protein sequences are described briefly in Table 2. The following ORFs warrant additional description.

The *flp* gene group. Located immediately upstream of the *mvh* operon are five closely linked ORFs, designated *flpECBDA*, that appear likely to form a single transcriptional unit (Fig. 1A). The *flpC* and *flpB* genes encode amino acid sequences that are 31% identical and 59% similar and 47% identical and 71% similar to the α and β subunits, respectively, of the molybdenum-containing formate dehydrogenase (FDH) from *Methanobacterium formicicum* and were therefore designated *flp* for FDH-like polypeptides. The FDH α subunit and other oxidoreductases that bind molybdenum, coordinated via a molybdopterin cofactor, have been reported to contain six conserved polypeptide domains (40), three of which are predicted to be present in FLPC (Fig. 2A). These include the N-terminally located domain I, in which three conserved cysteinyl residues are presumed either to be involved in molybdenum coordination or to form an iron-sulfur center (40).

FDHB, the β subunit of FDH, and the *flpB* gene product (FLPB) both have calculated molecular masses of ~44 kDa, and both polypeptides contain two CxxCxxCxxxCP motifs in their C-terminal regions that are likely to coordinate two [4Fe-4S] centers (Fig. 2C). FLPB and FDHB also have primary sequences in common with the β subunits of the F_{420}-reducing hydrogenases (FRHBs) from *M. thermoautotrophicum* ΔH (1) and *Methanococcus voltae* (10). Although these FRHB polypeptides lack the C-terminal ferredoxin-like motifs, an alignment with the FLPB and FDHB sequences revealed that all these sequences contain 27 conserved residues, including four cysteines and nine glycines (Fig. 2B). Since cofactor F_{420} is the electron acceptor for both FDH and FRH and both also bind flavin adenine dinucleotide, the regions conserved in FDHB, FRHB, and FLPB seem likely to be cofactor F_{420}- and/or flavin adenine dinucleotide-binding domains (1).

The fourth *flp* gene, *flpD*, encodes an amino acid sequence (FLPD) that is 65% identical and 84% similar to the sequence of MVHD, the *M. thermoautotrophicum* ΔH *mvhD* gene product, and 71% identical and 85% similar to VHCD, the product of the *vhcD* gene from *M. voltae* (Fig. 2C). The *mvhD* and *vhcD* genes are the promoter-proximal genes in operons that encode the subunits of methyl-viologen-reducing hydrogenases, but specific functions have not been assigned to MVHD and VHCD.

The *flpC* gene in the *M. thermoautotrophicum* Winter DNA cloned in λα32 contained a frameshift at codon position 156 that resulted in a stop codon at position 157. Sequence analysis of the corresponding DNA region that was PCR amplified directly from *M. thermoautotrophicum* Winter genomic DNA confirmed the presence of this frameshift mutation in the strain Winter genome. To examine the status of the *flpC* gene in a third *Methanobacterium* strain, the *flpC*-containing region was PCR amplified from genomic DNA of *M. thermoformicicum* Z-245, a close relative of *M. thermoautotrophicum* ΔH (20), and a 500-bp sequence from the area of interest was determined. The *flpC* gene from *M. thermoformicicum* Z-245 did not contain the frameshift, and its sequence was 97% identical to the sequence of the *flpC* gene from *M. thermoautotrophicum* ΔH. By contrast, the 280-bp intergenic sequences

TABLE 2. Similarities of the gene products encoded upstream from the *mvh* operon and in the *mtd* region in *M. thermoautotrophicum* strains ΔH and Winter to previously identified proteins

Gene[a]	Related protein(s) (accession number)	Identity (%)[b]
orf2	Red algae *Antithamnion* sp. chloroplast YCA3 protein encoded in the *atpA* 3′ region (Q02857)	27
	Mycobacterium leprae pps1 protein (U00013)	27
	M. leprae u1496a protein (U00013)	23
orf3	Diatom *Odontella sinensis* chloroplast *orf756* protein, part of ATPase operon and probable ABC family transport protein (Q00830)	38
	M. leprae ABC1 protein (U00013)	37
orf8	*M. leprae* biotin synthetase (U00010)	25
	E. coli biotin synthetase (P12996)	24
	Saccharomyces cerevisiae biotin synthetase (P32451)	23
mth	*M. thermoautotrophicum* Marburg H_2-forming methylene-H_4MPT dehydrogenase (MTH) (X59547)	96
	M. thermoautotrophicum Winter MTH (U19364)	97
	M. kandleri MTH (X60719)	59
flpC	*M. formicicum* FDH α subunit (J02581)	28
	A. eutrophus periplasmic nitrate reductase α subunit (X71385)	24
flpB	*M. formicicum* FDH β subunit (J02581)	49
	M. thermoautotrophicum ΔH F_{420}-reducing hydrogenase (FRH) β subunit (J02914)	30
	M. voltae FRH β subunit (S16724)	29
	M. voltae Se-cysteine-containing FRH β subunit (S24798)	27
flpD	*M. voltae* methyl viologen-reducing hydrogenase δ subunit (X61203)	71
	M. thermoautotrophicum ΔH methyl viologen-reducing hydrogenase δ subunit (J04540)	65
orf11	*E. coli cvaB* gene product probably involved in protein export (P22520)	27
mtd	*M. thermoautotrophicum* Marburg F_{420}-dependent methylene-H_4MPT dehydrogenase (L37108)	98
hisB	*Streptomyces coelicolor* imidazoleglycerol-phosphate dehydrogenase (P16247)	50
	Schizosaccharomyces pombe hisB gene product (U07831)	44
	Azospirillum brasilense hisB gene product (P18787)	43
fdxA	*Desulfovibrio desulfuricans* ferredoxin (P00211)	46
	Desulfovibrio vulgaris ferredoxin (P08813)	40
	Pelobacter carbinolicus putative ferredoxin (U01100)	39

[a] Gene designations as in Fig. 1.
[b] Based on optimum alignments of the encoded amino acid sequences.

located upstream of the *mth* genes in these two strains were only 91% identical, and they contained several nucleotide insertions or deletions.

***mth*.** The *mth* gene was located immediately upstream, but oriented in the opposite direction, from the *flp* genes in both *M. thermoautotrophicum* ΔH and Winter (Fig. 1A). Comparisons with the *mth* gene from *M. thermoautotrophicum* Marburg (37) revealed that these three *M. thermoautotrophicum* strains contain MTHs with amino acid sequences that are 94 to 97% identical and that are 57 to 59% identical to the sequence of the MTH from *Methanopyrus kandleri* (Table 2).

***orf8*.** The ORF (*orf8*) located immediately downstream from *mth* (Fig. 1A) encodes an amino acid sequence that is related to the sequences of biotin synthases, which catalyze the last step in the synthesis of the cofactor biotin. The *orf8* gene product (343 residues) and biotin synthases (~350 residues) are similar in size and, although their primary sequences are, at most, only ~25% identical (Table 2), they all have a central region of ~120 amino acids that contains 18 invariable residues (data not shown).

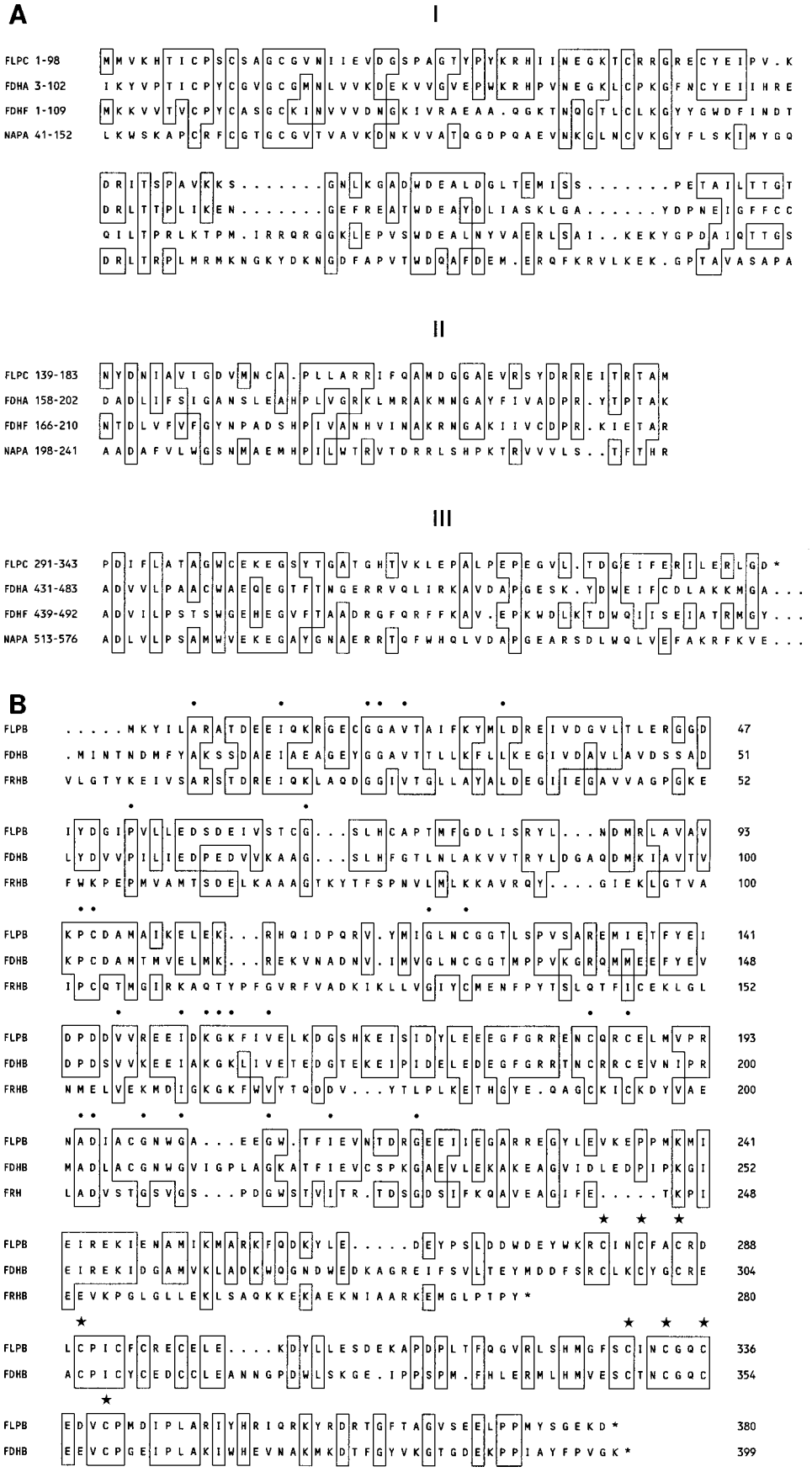
Cloning and analysis of the sequence of the *mtd* region from *M. thermoautotrophicum* ΔH. The *mtd* region from *M. thermoautotrophicum* ΔH was subcloned from two recombinant phages, designated λH13 and λH14, that were identified by hybridization with a ^{32}P-labeled *Hin*dIII-*Pst*I fragment (~800 bp) which contained part of the *mtd* gene from *M. thermoautotrophicum* Marburg (17). A 5,355-bp sequence that included the *mtd* gene, seven additional ORFs of >100 codons with ATG, GTG, or TTG initiation codons, and one shorter ORF, designated *fdxA*, that appears to encode a ferredoxin was obtained (Fig. 1B). The locations and basic features of these ORFs and their predicted gene products are listed in Table 3, and the similarities of the products to protein sequences in the databases are briefly described in Table 2.

The F_{420}-dependent methylene-H_4MPT dehydrogenase (MTD)-encoding *mtd* gene was the only methane gene determined to be present in this region of the *M. thermoautotrophicum* ΔH genome. It encodes an amino acid sequence that is 98% identical to that of the MTD in *M. thermoautotrophicum* Marburg (17). Immediately upstream of the *mtd* gene in these two *M. thermoautotrophicum* strains are ORFs, designated here *orf11* (Fig. 1B) and termed *orfX* for strain Marburg, (17), that encode polypeptides containing 142 amino acid residues with calculated molecular masses of ~15.5 kDa and 78% overall identity. These *orf11* and *orfX* gene products have low-level,

TABLE 3. Gene locations and features predicted for the gene products encoded in the *mtd* region of *M. thermoautotrophicum* ΔH

Gene	Size[a] of product	M_r of product (kDa)	pI of product	Start codon	Position[b]
orf9[c]	112	13.7	4.7		1>339
orf10	178	18.9	10.9	ATG	295<831
orf11	142	15.5	5.8	ATG	863>1291
mtd	276	29.6	4.4	ATG	1457>2287
orf12	123	14.6	5.1	ATG	2414<2785
orf13	128	14.4	10.2	ATG	2863<3249
hisB	194	21.1	5.6	ATG	3551<4135
fdxA	83	9.3	7.5	ATG	4092<4343
orf14	268	29.0	9.9	GTG	4431>5237

[a] Number of amino acid residues.
[b] Position 1 is the 5′ nucleotide of the *mtd* region shown in Fig. 1B. The symbols > and < indicate the sense directions of the ORFs.
[c] *orf9* is truncated in the analyzed sequence.


A
I
FLPC 1-98
FDHA 3-102
FDHF 1-109
NAPA 41-152
II
FLPC 139-183
FDHA 158-202
FDHF 166-210
NAPA 198-241
III
FLPC 291-343
FDHA 431-483
FDHF 439-492
NAPA 513-576
B
FLPB
FDHB
FRHB
FRH

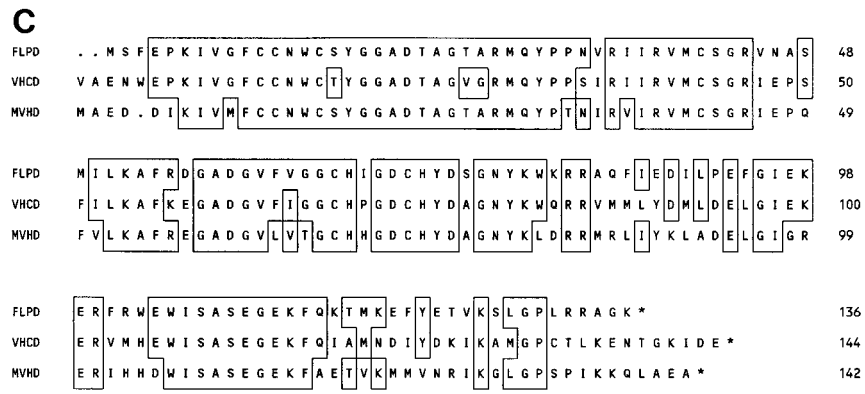


FIG. 2. Alignment of the amino acid sequences encoded by the *flpC*, *flpB*, and *flpD* genes from *M. thermoautotrophicum* ΔH with the sequences of previously identified proteins. Amino acid residues identical to those in the *flp* products are boxed. Numbers refer to amino acid residues. (A) Alignments of three amino acid regions of FLPC with regions conserved in molybdenum-pterin-binding proteins. FDHA, α subunit of FDH of *M. formicicum* (30); FDHF, FDH H of *E. coli* (43); NAPA, α subunit of nitrate reductase of *Alcaligenes eutrophus* H16 (31). (B) Alignment of FLPB with the β subunit of FDH (FDHB) from *M. formicicum* (30) and the β subunit of the F_{420}-reducing hydrogenase (FRHB) from *M. thermoautotrophicum* ΔH (1). The eight positions marked with asterisks are conserved cysteine residues that could participate in forming two [4Fe-4S] centers. The filled circles above the sequences indicate residues that are completely conserved when the β subunits of the two cofactor F_{420}-reducing hydrogenases from *M. voltae* are also added to the alignment (10) (data not shown). (C) Alignment of FLPD with VHCD from *M. voltae* (10) and MVHD from *M. thermoautotrophicum* ΔH (25).

but significant, similarity to the N-terminal regions of membrane proteins involved in signal sequence-independent secretion of proteins (8) (Table 2). Sequence alignment revealed that 23 residues were conserved in both of the *M. thermoautotrophicum* proteins and in the N-terminal regions of these membrane proteins (data not shown). The length determined for the *mtd* transcript indicates that *orf11* is not cotranscribed with the *mtd* gene (see Fig. 4). An ORF (*cobB*) localized ~500 bp further upstream from *orf11* appears to encode a cobyrinic acid *a*,*c*-diamide synthase (5), an enzyme involved in cobalamin biosynthesis (data not shown).

Located ~2 kb downstream, and in the opposite orientation from *mtd*, was a small ORF designated *fdxA*, which encodes 83 amino acid residues, including two CxxCxxCxxxCP motifs indicative of a ferredoxin (Fig. 1B). Otaka and Ooi (22) classified ferredoxins into five groups, and the *fdxA* gene product would, as expected, be placed in group 4, which contains the archaeal ferredoxins. The 3′ end of the *fdxA* gene overlaps by 34 bp the 5′ end of a gene, designated *hisB*, that is predicted to encode the histidine biosynthetic enzyme imidazoleglycerol-phosphate dehydrogenase (Fig. 1B; Table 2). Northern blot analyses of RNA preparations, isolated from fermentor-grown cells at different growth stages (see below), did not detect the presence of an *fdxA* transcript (data not shown).

Growth phase-dependent transcription of methane genes. Antisense oligonucleotides were synthesized and used to identify the *mth*, *mtd*, *mcr*, *mrt*, *frh*, and *mvh* transcripts on Northern blots of RNA preparations isolated from cells taken at different growth stages from an *M. thermoautotrophicum* ΔH culture (Fig. 3). The *mcrBDCGA*, *mrtBDGA*, *mvhDGAB*, and *frhADGB* genes are transcribed as single transcriptional units into polycistronic mRNAs (24), whereas the *mth* and *mtd* transcripts had lengths indicative of monocistronic transcripts. Pihl et al. (24) demonstrated that the *mrt* transcript was present only at early stages in the growth of an *M. thermoautotrophicum* ΔH culture and was then replaced, at later growth stages, by the *mcr* transcript. Essentially parallel transcription patterns were observed for the *mth* and *mtd* genes, respectively. Transcripts of the *mth* gene were present in the early RNA preparations that contained the *mrt* transcript, but as the cell density increased and the growth rate declined, these transcripts disappeared and were replaced by the *mtd* and *mcr* transcripts. In contrast, the *frh* and *mvh* transcripts were present in all the RNA preparations, although both transcripts were most abundant during the early growth stages and then decreased substantially at later growth stages.

Five of these six transcripts were most abundant at time point 3 (the *mcr* transcript was most abundant at time point 4) (Fig. 3), demonstrating that, although *mth*/*mrt* transcription was eventually completely replaced by *mtd*/*mcr* transcription, the switches from *mth* to *mtd* and from *mrt* to *mcr* transcription occurred gradually. All four promoters were active at time points 2 through 4, indicating that they either respond to different signals or respond differently to the same signal.

Sites of *mth*, *mtd*, and *frh* transcription initiation. The previously unidentified sites for *mth*, *mtd*, and *frhADGB* transcription initiation in *M. thermoautotrophicum* ΔH were mapped by primer extension reactions. Transcription of the *mth* and *mtd* genes was found to initiate 43 and 8 bp, respectively, upstream of their translation start codons, with the 5′ nucleotide of the *mtd* transcript coinciding with its putative ribosome binding site (Fig. 4A). Located 21 and 24 bp, respectively, upstream of the sites of *mth* and *mtd* transcription initiation are sequences that conform to the TATA box motif of an archaeal promoter (12). The 5′ terminus of the *frh* transcript was located 38 bp upstream of the *frhA* UUG translation start codon and 24 bp downstream from a TATA box motif (23).

Although the parallel patterns of *mth*/*mrt* and of *mtd*/*mcr* transcription suggest coordinated transcription initiation, there are no obviously conserved regulatory boxes located near the four transcription initiation sites (Fig. 4B). Similarly, comparisons of the intergenic regions upstream of the *mtd* and *frh* genes, which encode two F_{420}-dependent enzymes, and those upstream of the *mth* and *mvh* genes, which encode the corresponding two F_{420}-independent enzymes, did not reveal any clearly conserved boxes (Fig. 4B).

DISCUSSION

The physical map of the ~1.6-Mb genome of *M. thermoautotrophicum* Marburg, published in 1992 (33), indicated that most of the methane genes cloned at that time were located within an ~200-kb region. This raised the question of whether these genes might be very closely linked, in one contiguous

cluster, which seemed possible, since the *mrtBDGA* and *mvhDGAB* operons, and the *mcrBDCGA* and *mtrEDCBA* operons, were found to be directly adjacent on the *M. thermoautotrophicum* ΔH genome (24). With the results reported here, ~24 kb from the *mth* region have now been sequenced, and the *mth* gene and the putative methane genes *flpECBDA* have been added to the established *mvh* and *mrt* methane genes (Fig. 1A). However, this *mth-flp-mvh-mrt* cluster has been shown to be flanked by genes that do not encode enzymes directly involved in methanogenesis, and similarly, the *mtd* gene has also been shown to be isolated and not linked to other methane genes. Therefore, methane genes are not organized in just one methane gene region but are apparently more scattered in the genomes of *M. thermoautotrophicum* strains ΔH and Winter than earlier mapping results for *M. thermoautotrophicum* Marburg might have indicated.

On the basis of the similarity of the *flpC* and *flpB* gene products (FLPC and FLPB) to the α and β subunits of FDH, respectively (Fig. 2A and B), it appears likely that the *flp* genes encode a molybdenum- or tungsten-containing enzyme that probably uses F_{420} and/or flavin adenine dinucleotide as a cofactor in a redox reaction that involves a C_1 compound at the formyl oxidation level. The 78% identity of the *flpD* and *mvhD* gene products (FLPD and MVHD; Fig. 2C) and the observation that MVHD binds strongly to the *mvhB*-encoded polyferredoxin (23) suggest that FLPD might also interact with the polyferredoxin, perhaps as part of an electron transfer reaction involving the *flpECBA* gene products. Although the *flp* genes have the same size and organization in both *M. thermoautotrophicum* ΔH and Winter and are present in *M. thermoformicicum* Z-245, the *flpC* gene in the Winter strain contains a frameshift mutation. This mutated *flpC* gene could encode a functional but truncated polypeptide in *M. thermoautotrophicum* Winter; however, the existence of this mutation suggests rather that FLPC is not essential for the growth of the Winter strain, at least under laboratory conditions. Consistent with this, we have been unable to detect *flp* transcripts in RNA preparations from *M. thermoautotrophicum* ΔH cells grown either in the presence or in the absence of formate.

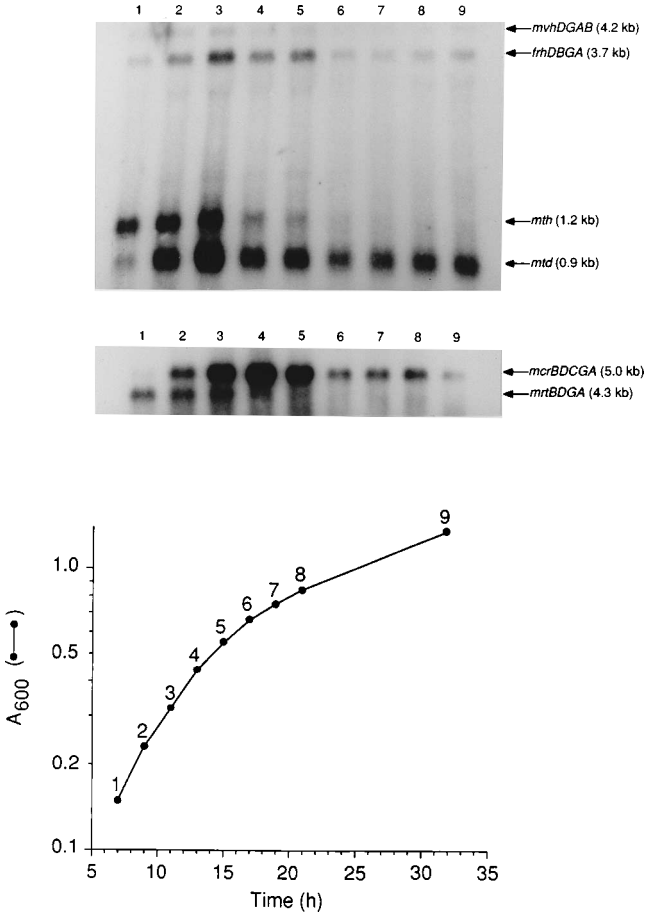


FIG. 3. Growth phase-dependent transcription of the *mvh*, *frh*, *mth*, *mtd*, *mcr*, and *mrt* genes in *M. thermoautotrophicum* ΔH cells. The growth of a culture was measured, as shown in the graph, by the increase of A_{600}, and at time points 1 through 9, cells were collected for RNA isolation. Aliquots (5 μg) of the RNA preparations isolated at each time point were used to obtain the Northern blots by hybridization with a mixture of ^{32}P-labeled oligonucleotides with sequences complementary to sequences in the *mvh*, *frh*, *mth*, and *mtd* transcripts (upper autoradiogram) or complementary to the *mcr* and *mrt* transcripts (lower autoradiogram) (24). The arrows identify the transcripts, and their approximate sizes are indicated.

The existence of the MRI and MRII isoenzymes and of the MTH and MTD pair of enzymes, which effectively catalyze the same reaction, suggests that the availability of these alternative enzymes must provide *M. thermoautotrophicum* strains with a selective advantage. Their growth phase-dependent synthesis indicates that this metabolic flexibility is used by the cells to respond to changes in their substrate supply, presumably to changes in the availability of hydrogen. The two (NiFe) hydrogenases, FRH and MVH, are present in *M. thermoautotrophicum* cells at all growth stages, but the third hydrogenase, MTH, is synthesized only when the H_2 supply is not limiting. Under these conditions, employing MTH instead of MTD should maximize the rate of H_2 uptake, and this must provide a growth advantage despite the fact that MTH has a twofold-lower V_{max} than MTD (34, 45). Reductant introduced into the cells by FRH, and possibly by MVH, is presumably available for general use, both in energy generation and anabolism, whereas MTH provides reductant for only a single step in methanogenesis. This activity of MTH should, however, reduce the methanogenesis demand for H_2F_{420} and therefore allow more of the H_2F_{420} generated by FRH to be used directly, or indirectly via reduced NAD derivatives (9), for biosynthetic reactions. MTH has a higher specific activity than FRH but a much lower affinity for H_2 (14, 35, 45), and therefore, FRH should continue to function at low external H_2 concentrations when MTH is no longer effective. Consistent with this, when the H_2 supply is limited, *M. thermoautotrophicum* cells replace MTH with MTD. This presumably increases the number of H_2F_{420}-consuming reactions, but it also ensures that all of the limited H_2 is made generally available and is not channeled into a single reaction by MTH.

M. thermoautotrophicum strains apparently use two different strategies to gain a growth advantage through metabolic flexibility. One has required the evolution of two unrelated enzymes, MTH and MTD, so that different reductants can be used for the same step in methanogenesis. The second strategy has taken advantage of gene duplication to acquire closely related isoenzymes, MRI and MRII, that catalyze identical reactions in methanogenesis but with different kinetic parameters. MRII has a higher V_{max} but lower substrate affinity than MRI (3), and consistent with this, MRII is predominant in cells when the H_2 supply is sufficient for rapid growth (4) and maximum rates of energy production are required. Under conditions of H_2 limitation, MRII is replaced by MRI, resulting in a reduced rate of methanogenesis. During the transition from a sufficient to insufficient H_2 supply, conditions under which MRII can synthesize CH_4 faster than H_2 can be recruited into the pathway must occur. One or more unloaded C_1 carriers

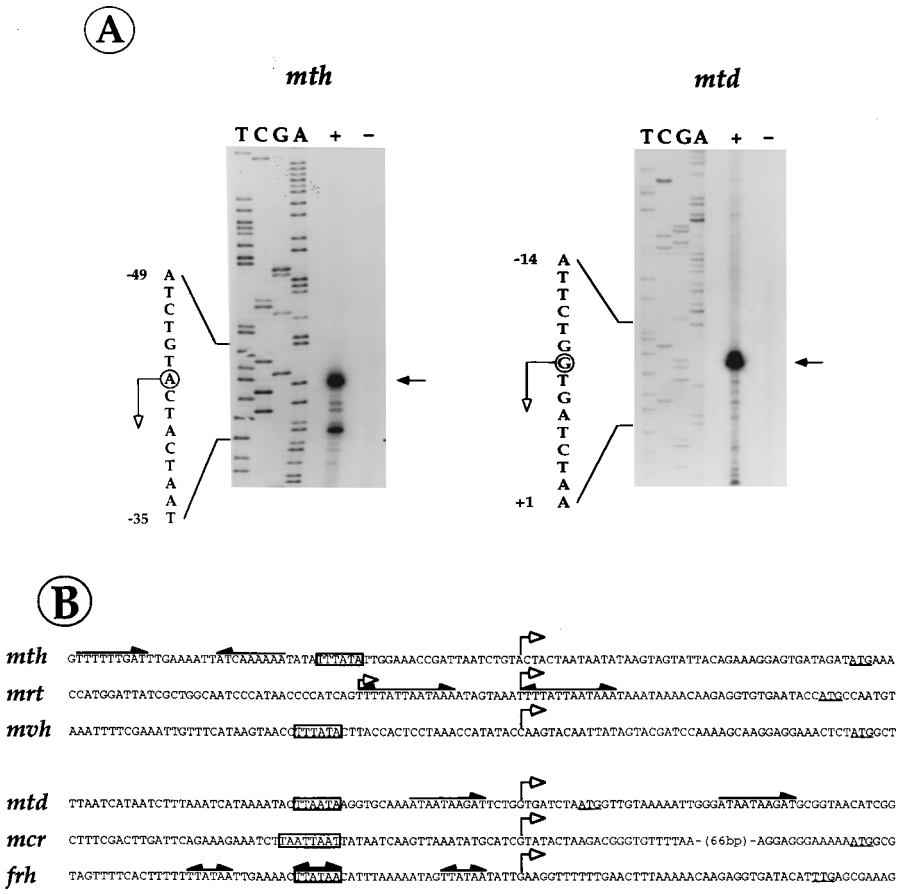


FIG. 4. Identification of the *mth* and *mtd* transcription initiation sites and an alignment of transcription initiation regions of methane genes in *M. thermoautotrophicum* ΔH. (A) The gels show the results of primer extension reactions, with (+) and without (−) reverse transcriptase, adjacent to DNA sequencing ladders obtained by using the same primer. The arrow on the right of each gel identifies the primer extension product that demonstrated transcription initiation at the nucleotide circled in the sequence shown to the left of that panel. (B) Sequences of the regions upstream of the methane genes aligned by their sites of transcription initiation, identified by the open arrowheads. Sites of translation initiation are underlined, and probable TATA box promoter elements (12) are boxed. Closed arrowheads indicate inverted and directly repeated sequences, and two-headed arrows identify the locations of repeated palindromic sequences.

(methanofuran, H_4MPT, and/or CoM) may then accumulate, and this could provide the intracellular signal needed to activate the switch from *mrt*/*mth* transcription to *mtd*/*mcr* transcription. Alternatively, the accumulation of an intracellular compound in a redox state, indicating a shortage of reductant, for example, nonreduced F_{420} or its adenylated derivative F_{390} (24, 36) or the degree of oxidation of a redox-sensitive protein such as the polyferredoxin, could signal this switch.

Transcription in *Archaea* conforms to the eukaryal paradigm (39). Transcription is activated by the interactions of specific transcription factors with a general transcription complex that binds to the TATA box promoter elements. The sequences surrounding these TATA boxes may not, therefore, contain readily identifiable and conserved regulatory boxes, as is often the case in the upstream regulatory regions in *Bacteria*. Comparison of the sequences upstream from the sites of *mth*, *mtd*, *mcr*, *mvh*, and *frh* transcription initiation has identified appropriately positioned TATA boxes (Fig. 4B) that are likely to bind the general transcription complex, but it has not identified any other conserved sequences. Transcription from the *mth* and *mrt* promoters, and from *mtd* and *mcr* promoters, may therefore be activated by transcription factors that do not, themselves, bind to specific DNA sequences but rather interact with an RNA polymerase-containing complex that is already bound to these TATA boxes. As in vitro transcription systems from methanogens have been established, it should now be possible to isolate these specific transcription factors and test this hypothesis.

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