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Cloning, Sequencing, and Growth Phase-Dependent Transcription of the Coenzyme F_{420}-Dependent N^5,N^{10}-Methylenetetrahydromethanopterin Reductase-Encoding Genes from *Methanobacterium thermoautotrophicum* ΔH and *Methanopyrus kandleri*

JÖRK NÖLLING,* TODD D. PIHL, AND JOHN N. REEVE
Department of Microbiology, The Ohio State University, Columbus, Ohio 43210

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The *mer* genes, which encode the coenzyme F_{420}-dependent N^5,N^{10}-methylenetetrahydromethanopterin reductases (CH_2=H_4MPT reductases), and their flanking regions have been cloned from *Methanobacterium thermoautotrophicum* ΔH and *Methanopyrus kandleri* and sequenced. The *mer* genes have DNA sequences that are 57% identical and encode polypeptides with amino acid sequences that are 57% identical and 71% similar, with calculated molecular masses of 33.6 and 37.5 kDa, respectively. In *M. thermoautotrophicum*, *mer* transcription has been shown to initiate 10 bp upstream from the ATG translation initiating codon and to generate a monocistronic transcript ~1 kb in length. This transcript was synthesized at all stages of *M. thermoautotrophicum* ΔH growth in batch cultures but was found to increase in abundance from the earliest stages of exponential growth, reaching a maximum level at the mid-exponential growth phase. For comparison, transcription of the *ftr* gene from *M. thermoautotrophicum* ΔH that encodes the formylmethanofuran:tetrahydromethanopterin formyltransferase (A. A. DiMarco, K. A. Sment, J. Konisky, and R. S. Wolfe, J. Biol. Chem. 265:472–476, 1990) was included in this study. The *ftr* transcript was found similarly to be monocistronic and to be ~1 kb in length, but, in contrast to the *mer* transcript, the *ftr* transcript was present at maximum levels at both the early and the mid-exponential growth stages.

The enzymes that catalyze the seven steps in the H_2-dependent reduction of CO_2 to CH_4 in the thermophilic archaeon *Methanobacterium thermoautotrophicum* have been isolated and characterized (see reference 38 for a review), and most of their encoding genes have been cloned and sequenced (6, 10, 13, 16, 24, 25, 27, 35, 40). These studies have revealed more complexity in the methanogenesis pathway in *M. thermoautotrophicum* than initially expected, since most of the reductive reactions in the ΔH and Marburg strains of this methanogen have been found to be catalyzed by isoenzymes or functionally equivalent enzymes. For example, the fourth reaction, the reduction of N^5,N^{10}-methenyltetrahydromethanopterin (methenyl-H_4MPT) to N^5,N^{10}-methylene-H_4MPT, is catalyzed by functionally equivalent H_2-dependent and cofactor F_{420}-dependent methylene-H_4MPT dehydrogenases, designated MTH and MTD, respectively (23, 40, 43, 44). Similarly, the final reaction that releases methane is catalyzed by two methylcoenzyme M reductase isoenzymes, designated MRI and MRII (2, 4, 18, 27, 29).

At the earliest growth stages of *M. thermoautotrophicum* batch cultures, MRII predominates but is replaced by MRI at later growth stages (2, 4, 29). This growth phase-dependent synthesis of the MR isoenzymes is regulated at the level of transcription initiation (27). At the earliest growth stages, transcription is almost exclusively from the *mrt* operon that encodes MRII and is then gradually replaced by transcription from the MRI-encoding *mcr* operon (27). A virtually identical pattern of growth phase-dependent transcription has also been established for the *mth* and *mtd* genes, which encode MTH and MTD, respectively (25). This growth phase-dependent transcription of methane genes apparently provides *M. thermoautotrophicum* with the flexibility to adjust advantageously to changes that must occur during growth in batch culture, such as changes in the availability of the substrates for methanogenesis, H_2 and CO_2 (33). At the earliest growth times, H_2 dissolution and diffusion rates are presumably adequate to accommodate the rate at which methane can be produced by the low density of cells present, but with increasing cell density, the capacity of the culture to synthesize methane may surpass the rate at which H_2 can be supplied (2–4, 33). This must limit growth and presumably activates the switches of transcription from *mth* to *mtd* and from *mrt* to *mcr* (25, 27), genes which control the synthesis of pairs of functionally equivalent enzymes that catalyze reductions in the methanogenesis pathway.

To extend this analysis, we have now cloned, sequenced, and investigated the transcription of the gene (*mer*) that encodes the F_{420}-dependent N^5,N^{10}-methylene tetrahydromethanopterin reductase (CH_2=H_4MPT reductase) from *M. thermoautotrophicum* ΔH (20, 36). This enzyme catalyzes the reduction at step 5 in the methanogenesis pathway by oxidizing reduced cofactor F_{420} ($H_2 \cdot F_{420}$) and therefore is analogous to the $H_2 \cdot F_{420}$-dependent MTD (23, 37), which catalyzes the reaction at the fourth step in methanogenesis. The step 4 reduction, however, is also catalyzed by the H_2-dependent enzyme MTH (40, 43, 44), and therefore this analogy predicts that there may also be an H_2-dependent enzyme that can catalyze the step 5 reduction. Although there is no direct biochemical evidence for an H_2-dependent, step 5-catalyzing enzyme (38), support for the existence of such an enzyme is provided by the analysis of the growth phase-dependent pattern of *mer* transcription described in this report. For comparison, the effects of growth phase on transcription of the *ftr*

* Corresponding author. Phone: (614) 292-6890. Fax: (614) 292-8120. Electronic mail address: jnolling@magnus.acs.ohio-state.edu.

gene, which encodes the nonreductive step 2-catalyzing enzyme formylmethanofuran:tetrahydromethanopterin formyltransferase (FTR) (6), have also been analyzed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. thermoautotrophicum* ΔH (DSM 1053) cultures were grown in a mineral salts medium in sealed and pressurized 20-ml bottles or in a 20-liter fermentor, supplied with CO_2 plus H_2 at a 1:4 input ratio, as previously described (27). *Escherichia coli* KW251 (Promega Corp., Madison, Wis.) and DH5α (9) were used to propagate λGEM11 (Promega Corp.)-based recombinant phages and pUC18-based recombinant plasmids (41), respectively. *E. coli* cultures were grown at 37°C in Luria-Bertani medium (30) that, when appropriate, was supplemented with 50 μg of ampicillin per ml or 5 μg of tetracycline per ml. *Methanopyrus kandleri* cells were generously provided as a frozen cell paste by K. O. Stetter (University of Regensburg, Regensburg, Germany).

Enzymes and chemicals. All enzymes used for nucleic acid manipulations were purchased from GIBCO/BRL (Gaithersburg, Md.) or from Boehringer Mannheim Biochemicals Co. (Indianapolis, Ind.). [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 Biochemicals, Inc. (Costa Mesa, Calif.) and from DuPont Co. (Hoffman Estates, Ill.). All other chemicals were purchased either from Sigma Chemical Co. (St. Louis, Mo.) or Jenneile Enterprises (Cincinnati, Ohio).

DNA manipulations and DNA sequencing. Standard DNA manipulations were performed as described by Sambrook et al. (30). Construction of the *M. thermoautotrophicum* ΔH genomic DNA library in λGEM11 has been described previously (25). The same protocol was used to construct an *M. kandleri* genomic DNA library in λGEM11 starting from a partial *Sau*3AI digest of *M. kandleri* genomic DNA. For subcloning of recombinant λ DNA, isolated as described by Meese et al. (22), into pUC18, both linearized plasmid vector DNA and λ DNA-derived restriction fragments were purified from agarose gels by using a GeneClean kit (Bio 101, La Jolla, Calif.). Double-stranded DNAs, purified as described by Yie et al. (42), were used as the templates in dideoxy chain termination DNA sequencing reactions (31) using the 7-deaza-GTP version of the Sequenase 2.0 kit (U.S. Biochemicals, Cleveland, Ohio). Some DNA sequences were determined directly from the recombinant phage DNAs by using the *fmol* DNA sequencing system (Promega Corp.). DNA sequences were analyzed by using the University of Wisconsin Genetics Computer Group package, version 7.0 (8), and the BLAST program (1).

RNA manipulations and Northern (RNA) blots. RNA preparations were isolated from *M. thermoautotrophicum* ΔH cells and used in Northern blot experiments to identify gene-specific transcripts and were used as templates in primer extension reactions to identify transcription initiation sites, following previously described protocols (25, 27). For stripping and reprobing of the Northern blots, the membrane (Biodyne Plus; Pall BioSupport Division, Port Washington, N.Y.) was incubated twice for 20 min in 0.1% sodium dodecyl sulfate at 95°C and then subjected to prehybridization for 15 min at 65°C, after which the new probe was added. The sequence of the oligonucleotide used as the hybridization probe to identify the *mer* transcript and as a primer to identify the site of *mer* transcription initiation is shown below (see Fig. 1). For the identification of the *ftr* transcript from *M. thermoautotrophicum* ΔH, an oligonucleotide with the sequence 5′-CAACGGAGACCACGACGATAAAACCG-3′, which is complementary to positions 94 through 119 of the *ftr* coding sequence (6), was used as the hybridization probe.

Nucleotide sequence accession numbers. The DNA sequences obtained for the *M. thermoautotrophicum* ΔH and *M. kandleri mer* genes and flanking regions have been assigned GenBank accession no. U31568 and U31567, respectively.

RESULTS

Cloning of the *mer* genes from *M. kandleri* and *M. thermoautotrophicum* ΔH. CH_2=H_4MPT reductases from *M. thermoautotrophicum* ΔH and Marburg (20, 36), *M. kandleri* (19), *Methanosarcina barkeri* (21), and the nonmethanogenic archaeon *Archaeoglobus fulgidus* (32) have been purified and characterized. All these enzymes are multimers of one polypeptide subunit which have apparent molecular masses ranging from 35 to 38 kDa and very similar N-terminal sequences (19, 32). Since the N-terminal sequence for the strain ΔH enzyme was not available, we initially screened the *M. thermoautotrophicum* ΔH phage library for clones carrying the *mer* gene by plaque hybridization, using a mixture of oligonucleotide probes with sequences derived from the N-terminal amino acid sequence of the CH_2=H_4MPT reductase from *M. thermoautotrophicum* Marburg (20). This approach was unsuccessful. We therefore cloned the *M. kandleri mer* gene to obtain a more reliable probe for cloning the corresponding *M. thermoautotrophicum* ΔH gene. As the *M. kandleri* genome is overall 60 mol% G+C, this results in a very strong preferential use of GC-rich codons (34, 44) which substantially reduces the redundancy that must be incorporated into oligonucleotide mixtures for use as hybridization probes. A mixture of oligonucleotides with the sequence 5′-GGCTTGTCGTCCGG(G/C)AG(G/C)AGCTC-3′, based on the N-terminal amino acid sequence of the *M. kandleri* CH_2=H_4MPT reductase (19), was therefore synthesized, ^{32}P end labeled, and used to screen the *M. kandleri* λGEM11 recombinant library for phages that contained the *mer* gene. DNA was isolated from a positive clone, designated λalv2, and Southern hybridization experiments demonstrated that the probe hybridized to an ~500-bp *Sst*I restriction fragment. Sequencing of this *Sst*I fragment, subcloned into pUC18, confirmed the presence of the 5′ terminus of the *mer* gene. This *Sst*I fragment was then used as the hybridization probe to subclone two adjacent *Sal*I-*Sma*I restriction fragments from the λalv2 DNA which, when sequenced, were found to contain most of the *mer* gene and ~700 bp of the upstream region. The remaining 3′ region of the *mer* gene and the immediately downstream region were then sequenced directly from the λalv2 DNA by using an oligonucleotide primer with a sequence derived from the already established *mer* sequence. The ~500-bp *Sst*I restriction fragment that contained part of the *M. kandleri mer* gene was also used as a probe to screen the *M. thermoautotrophicum* ΔH genomic library to identify clones that contained the *M. thermoautotrophicum* ΔH *mer* gene. Southern hybridizations to the DNA isolated from one such phage, designated λH12, demonstrated that the *mer* gene from *M. thermoautotrophicum* ΔH was located on overlapping *Sst*I-*Pst*I (~1.2-kb) and *Sst*I-*Xho*I (~1.8-kb) fragments. These fragments were therefore subcloned into pUC18, and the *mer* gene and immediately flanking regions were sequenced.

Comparison of the *mer* genes and gene products in *M. thermoautotrophicum* ΔH and *M. kandleri*. The 1,844-bp sequence determined from the *mer* region of the *M. thermoautotrophicum* ΔH genome is shown in Fig. 1. The sequence contains only one long open reading frame (ORF) which encodes 321 amino acid residues with an N-terminal sequence identical to that reported for the N-terminal sequence of the CH_2=H_4MPT reductase purified from *M. thermoautotrophicum* Marburg (20). The polypeptide encoded by this ORF has a calculated molecular mass of 33.6 kDa and a predicted pI of 4.4, values very close to the experimentally determined values of 35 kDa and pI 4.5 for the CH_2=H_4MPT reductase monomer purified from *M. thermoautotrophicum* Marburg (20). This ORF was therefore designated the *mer* gene.

The 1,819-bp region that was sequenced from the *M. kandleri* genome contained both the *M. kandleri mer* gene and 516 bp of a second, truncated ORF that was located on the opposite DNA strand and upstream from the *mer* gene. The amino acid sequence encoded by this second ORF has significant similarity to the amino acid sequence of IMP dehydrogenase from *Pyrococcus furiosus* (5). This ORF was not present at the corresponding location in the genomic DNA cloned from *M. thermoautotrophicum* ΔH. The polypeptide encoded by the *M. kandleri mer* gene contains 349 amino acid residues and has a calculated molecular mass of 37.5 kDa and a predicted pI of 4.4, values again very similar to the 38-kDa molecular mass and pI of 4.6 determined experimentally for the CH_2=H_4MPT reductase monomer purified from *M. kandleri* (19, 32). The N-terminal amino acid sequence encoded by the *M. kandleri mer* gene is identical to that determined directly from the purified *M. kandleri* enzyme (19), except that the

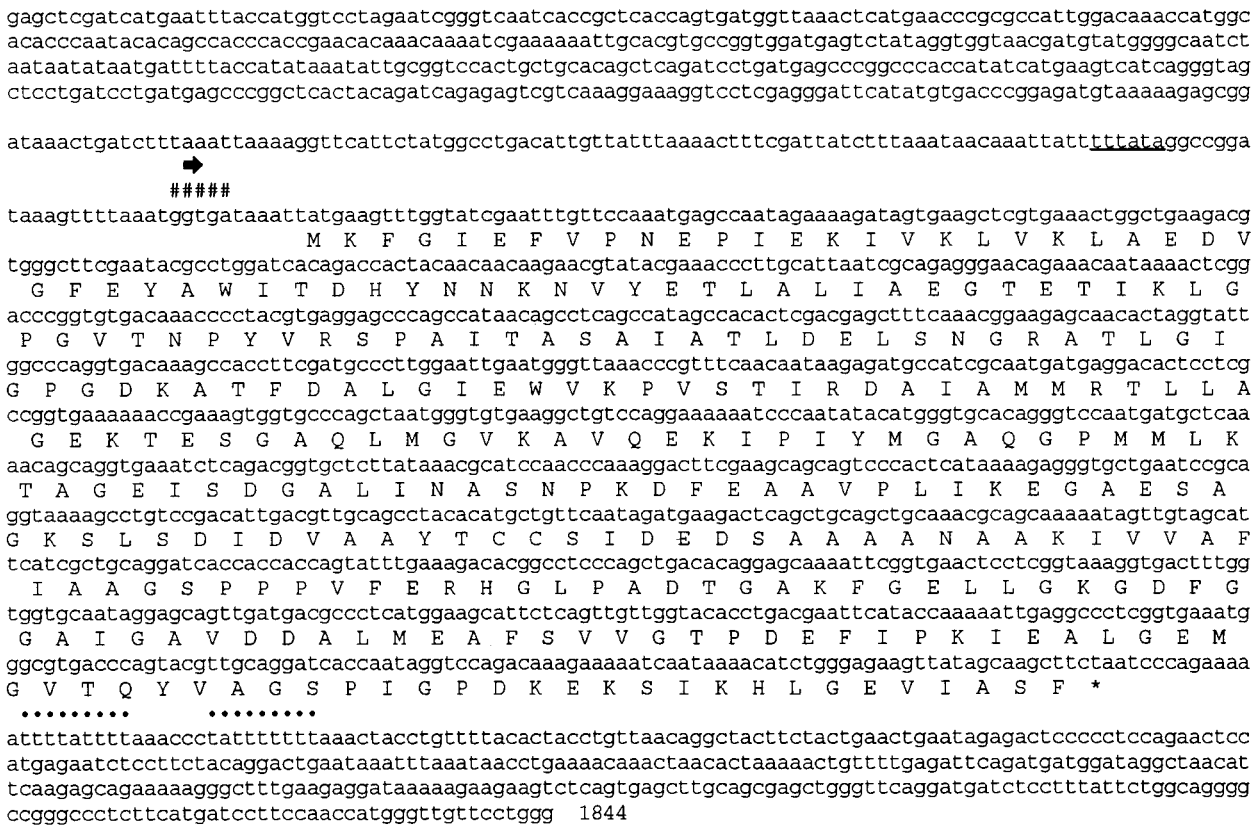

FIG. 1. Sequence of the *M. thermoautotrophicum* ΔH *mer* gene and the amino acid sequence predicted for the encoded CH_2=H_4MPT reductase. The site identified for *mer* transcription initiation (arrow) (see Fig. 3), a TATA-box sequence (11) that is located 21 bp upstream from this site (underlined), the sequence predicted to function as the ribosome binding site in the *mer* transcript (#####), and two oligo(dT) sequences that conform to sites of archaeal transcription termination (28) (dotted overline), located downstream from the *mer* gene, are indicated.

mature protein lacks an N-terminal methionyl residue. The CH_2=H_4MPT reductases from *M. thermoautotrophicum* ΔH and *M. kandleri* have amino acid sequences that are 57% identical and 72% similar (Fig. 2), although, on the basis of 16S rRNA analyses (17), these organisms are only very distantly related methanogens and there is no discernible conservation in the regions flanking the two *mer* genes. The central regions of the two CH_2=H_4MPT reductases are most dissimilar and include a block of 25 amino acid residues that are present in the *M. kandleri* enzyme but are not found in the *M. thermoautotrophicum* ΔH enzyme (Fig. 2). At least one insertion or deletion event therefore must have occurred since the divergence of the two *mer* genes, and the presence or absence of these additional codons should help trace the pathway of *mer* gene divergence in other methanogens.

Identification of the *mer* and *ftr* transcripts and location of the *mer* transcription initiation site in *M. thermoautotrophicum* ΔH. Oligonucleotides complementary to the 5′ ends of the *mer* and *ftr* transcripts from *M. thermoautotrophicum* ΔH were ^{32}P end labeled and used to generate Northern blots (see below). The *mer*-specific oligonucleotide was also used as the primer in primer extension reactions (Fig. 3). Transcription of the *mer* gene was found to initiate only 10 bp upstream from the ATG translation start codon and 21 bp downstream from a sequence, 5′-TTTATA-3′, that conforms to the consensus sequence for the TATA-box motif of archaeal promoters (Fig. 1 and 4) (11, 28). The 5′ nucleotide of the *mer* transcript surprisingly coincides with the second nucleotide in the sequence 5′-GGTGA-3′, which appears likely to be the *mer* ribosome binding site (Fig. 1 and 4). A similarly short 5′ leader sequence has also been documented for the *mtd* transcript from *M. thermoautotrophicum* ΔH (25) that encodes MTD, the enzyme that catalyzes step 4 in the methanogenesis pathway (Fig. 4). In addition, the intergenic regions located immediately upstream from the TATA-box motifs of the *mer* and *mtd* promoters show substantial sequence conservation (Fig. 4) consistent with coordinated transcription.

Northern blots demonstrated that the *mer* and *ftr* transcripts in *M. thermoautotrophicum* ΔH are both ~1 kb long (Fig. 5), consistent with monocistronic transcripts of the *mer* and *ftr* genes that are composed of 963 (Fig. 1) and 888 (6) bp, respectively. A TATA-box motif is located 38 bp upstream of the ATG translation initiation codon of the *ftr* gene (6), and *ftr* transcript initiated downstream from this promoter element would also have a very short 5′ leader sequence.

Transcription of the *mer* and *ftr* genes in cells at different growth stages in batch cultures. Northern blots of RNA preparations isolated at different growth stages from *M. thermoautotrophicum* ΔH cells growing in a 20-liter fermentor were hybridized to ^{32}P-labeled oligonucleotides synthesized with sequences complementary to regions within the transcripts of the *mer*, *ftr*, *mth*, and *mtd* genes. The *mer* and *ftr* transcripts were present at all growth stages, but the abundances of these transcripts differed considerably at the earliest phases of growth (Fig. 5). Whereas the *ftr* transcript was present at a constant and maximal level throughout the early exponential growth stage, the level of the *mer* transcript increased very substantially from the earliest time (time point 1) to a maximal level at

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M.t. ...MKFGIEFVPNEPIEKIVKLVKLAEDVGFEYAWITDHYNNKNVYETLA  47
M.k. MAEVSFGIELLPDDKPTKIAHLIKVAEDNGFEYAWICDHYNNYSYMGVLT  50

M.t. LIAEGTETIKLGPGVTNPYVRSPAITASAIATLDELSNGRATLGIGPGDK  97
M.k. LAAVITSKIKLGPGITNPYTRHPLITASNIATLDWISGGRAIIGMGPGDK 100

M.t. ATFDALGIEW..............VKPVSTIRDAIAMMRTLLAGEKTE..  131
M.k. ATFDKMGLPFPCKIPIWNPEAEDEVGPATAIREVKEVIYQYLEGGPVEYE 150

M.t. ......SGAQLMGVKAVQEKIPIYMGAQGPMMLKTAGEISDGALINASNP 175
M.k. GKYVKTGTADVKARSIQGSDIPFYMGAQGPIMLKTAGEIANGVLVNASNP 200

M.t. KDFEAAVPLIKEGAESAGKSLSDIDVAAYTCCSIDEDSAAAANAAKIVVA 225
M.k. KDFEVAVPKIEEGAKEAGRSLDEIDVAAYTCFSIDKDEDKAIEATKIVVA 250

M.t. FIAAGSPPPVFERHGLPADTGAKFGELLGKGDFGGAIGAVDDALMEAFSV 275
M.k. FIVMGSPDVVLERHGIDTEKAEQIAEAIGKGDFGTAIGLVDEDMIEAFSI 300

M.t. VGTPDEFIPKIEALGEMGVTQYVAGSPIGPDKEKSIKHLG.EVIASF     321
M.k. AGDPDTVVDKIEELLKAGVTQVVVGSPIGPDKEKAIELVGQEVLPHFKE  349
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FIG. 2. Alignment of the amino acid sequences of the *mer*-encoded CH_2=H_4MPT reductases in *M. thermoautotrophicum* ΔH (M. t.) and *M. kandleri* (M. k.) generated by the Genetics Computer Group program BESTFIT (8). Identical amino acids (lines), conservative changes (colons), and gaps introduced to maximize the homology (dots) are indicated. The numbers refer to the amino acid residues in each sequence. BLAST searches revealed that the N-terminal regions of these two enzymes have several residues (asterisks) that are conserved in the α-subunits of bacterial luciferases (12, 14, 15) and in the *lmbY* gene product of *Streptomyces lincolnensis* (26).

time point 3 (Fig. 5). This *mer* transcription pattern is essentially the same as that for *mtd* transcription in batch cultures (25), namely, a very low level of transcript at the earliest time point and then a substantial increase and maintenance at a relatively high level at later growth phases (Fig. 5).

DISCUSSION

The sequences of the methane genes encoding the enzymes that catalyze the second (*ftr*), fourth (*mth* and *mtd*), sixth (*mtrEDCBAFGH*), and seventh (*mcrBDCGA* and *mrtBDGA*) steps in the methanogenesis pathway from CO_2 plus H_2 in *M. thermoautotrophicum* have been reported previously (6, 10, 13, 16, 24, 25, 27, 35, 40), and growth phase-dependent transcription of the *mth*, *mtd*, *mtr*, *mcr*, and *mrt* genes in *M. thermoautotrophicum* ΔH has been documented (25, 27). We have now extended this work by adding the sequence of the *mer* gene, which encodes the step 5-catalyzing CH_2=H_4MPT reductase (20, 36), and by identifying and determining the relative abundances of the *mer* and *ftr* (step 2) transcripts at different growth stages in *M. thermoautotrophicum* ΔH batch cultures.

The *ftr* transcript was present at a relatively high and constant level in cells sampled from the earliest to the mid-exponential growth phases and was then maintained at a lower level at the later growth stages (Fig. 5). The abundance of the *ftr* transcript did not, therefore, alter substantially in response to the changes in growth rate which occur during the early to mid-exponential growth phases that presumably reflect changes in the supply of the reductant H_2 (2, 4, 33). This is consistent with the argument that the *ftr* gene product (FTR) does not directly

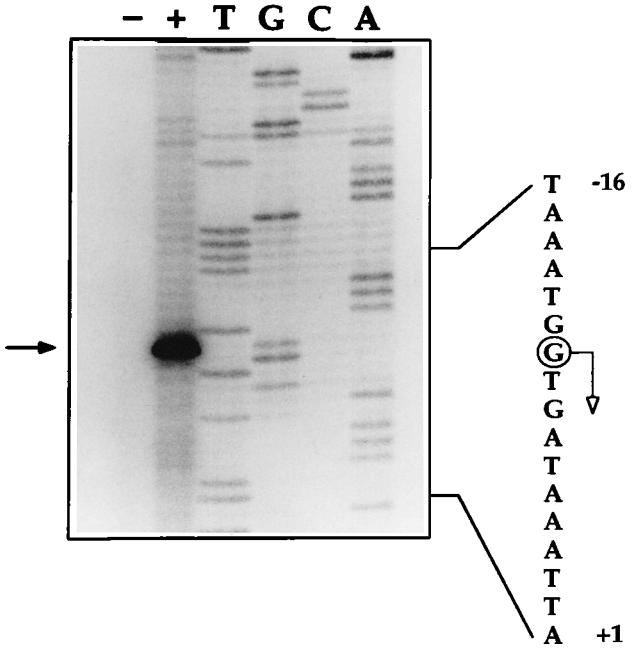

FIG. 3. Identification of the site of *mer* transcription initiation. The results of primer extension reactions with (+) and without (−) reverse transcriptase are shown adjacent to DNA sequencing ladders generated by using the same primer for DNA sequencing. The primer extension product that demonstrated that *mer* transcription is initiated at the circled base in the sequence shown to the right of the figure is indicated (arrow on the left). Nucleotide +1 is the A of the ATG translation-initiating codon (Fig. 1).

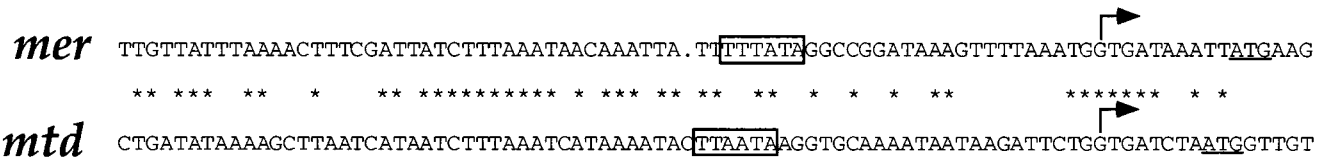

FIG. 4. Comparison of the sequences of the regions upstream from the *mer* and *mtd* genes (25) in *M. thermoautotrophicum* ΔH. The sites of transcription initiation (arrows), ATG translation-initiating codons (underlines), the presumed TATA boxes (boxes), and nucleotides common to the two sequences in the alignment shown (asterisks) are indicated.

catalyze a reduction or require a reductant for activity or stability (7), and therefore FTR synthesis need not be as sensitive to the availability of H_2. In contrast, the abundance of the *mer* transcript increased substantially in cells from the earliest to the mid-exponential growth stages, when the availability of H_2 is thought to become growth limiting (Fig. 5) (2–4, 33). The same transcription pattern has also been observed previously for the *mtd* gene and the *mcr* operon (25, 27) (Fig. 5), although the increases in the *mtd* and *mcr* transcripts, from the earliest to the later times, were larger than the increase observed in the *mer* transcript. We have argued previously that, as the *mth* and *mtd* gene products (MTH and MTD) catalyze the same reduction but oxidize different reductants (H_2 and $H_2 \cdot F_{420}$), they provide *M. thermoautotrophicum* cells with the flexibility to respond advantageously to variations in H_2 availability (25). MTH is synthesized and maximizes the use of H_2 for methanogenesis when excess H_2 is available, while MTD is synthesized and provides increased control over the apportionment of the more generally useful reductant $H_2 \cdot F_{420}$ when the availability of H_2 is limiting. The *mer* gene product also con-

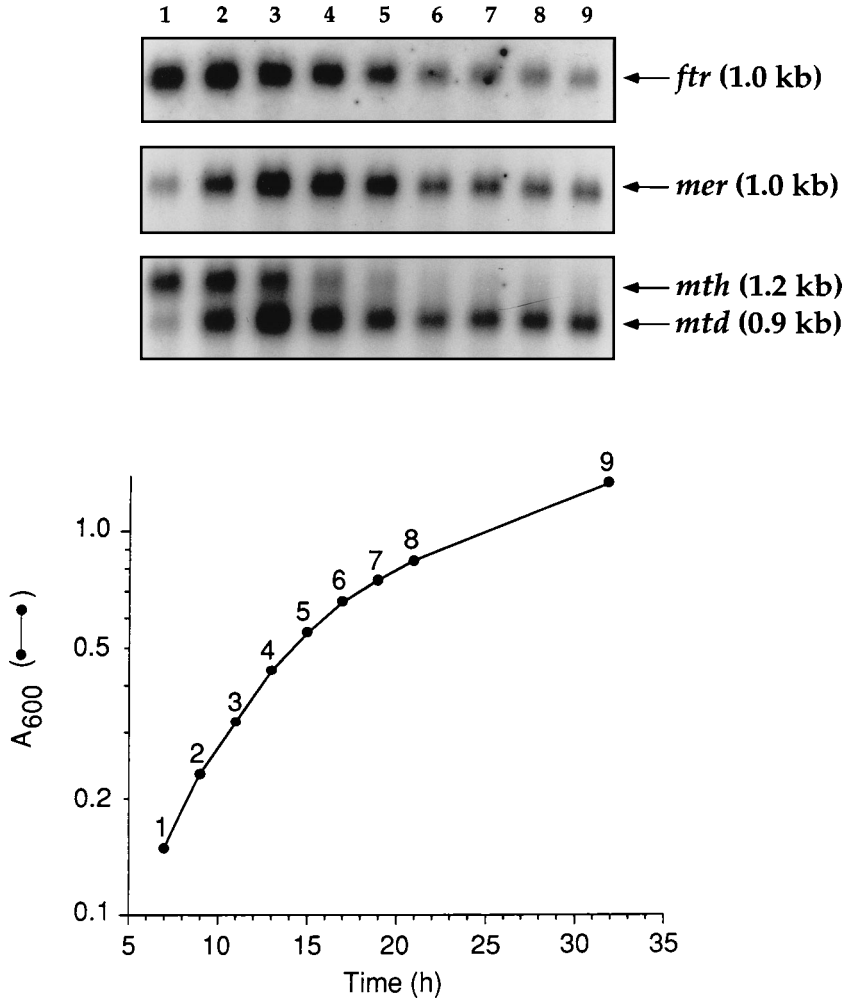

FIG. 5. Transcription of the *mer*, *ftr*, *mth*, and *mtd* genes in cells at different stages of growth in an *M. thermoautotrophicum* ΔH batch culture. The growth of the culture was measured, as shown in the graph, by the increase in A_{600}. At time points 1 through 9, cells were collected for RNA isolations. Aliquots (5 μg) of the RNA preparations isolated at each time point were used to generate the Northern blots shown by hybridization to ^{32}P-end-labeled oligonucleotides complementary to the *ftr*, *mer*, *mth*, and *mtd* transcripts. After hybridization to a mixture of the *mth* and *mtd* probes, the membrane was reused in separate hybridizations with the *mer* and *ftr* probes. The individual transcripts and their approximate sizes are indicated.

sumes $H_2 \cdot F_{420}$ at step 5 (20, 36), and therefore this enzyme would be expected to be synthesized in parallel with MTD if an H_2-consuming CH_2=H_4MPT reductase that can use H_2 directly in step 5 is also available in *M. thermoautotrophicum* cells under conditions of H_2 sufficiency. There is as yet no biochemical evidence for such an H_2-consuming step 5 enzyme, but the growth phase-dependent transcription pattern observed for the *mer* gene, paralleling that of the *mtd* gene (Fig. 5), does hint at its existence. A search for this activity in extracts of *M. thermoautotrophicum* ΔH cells harvested from batch cultures at the very earliest stages of growth is now needed. If such an H_2-consuming enzyme catalyzing the fifth reaction does exist, then all four reductions in the methanogenesis pathway from CO_2 would be catalyzed in *M. thermoautotrophicum* by pairs of functionally equivalent enzymes or by isoenzymes (38), providing strong support for the hypothesis that this methanogen has evolved alternative enzyme systems to respond competitively to variations in the supply of the reductant H_2.

ACKNOWLEDGMENTS

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We thank K. O. Stetter for the gift of *M. kandleri* cells.

ADDENDUM

During the review of this paper, the sequence of the *mer* gene from *M. thermoautotrophicum* Marburg was published (39). The deduced amino acid sequences of the strain ΔH and Marburg F_{420}-dependent CH_2=H_4MPT reductases are 98% identical.

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