JOURNAL OF BACTERIOLOGY, July 2002, p. 3476–3484 Vol. 184, No. 13
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.13.3476–3484.2002

Chloromethane-Induced Genes Define a Third C_1 Utilization Pathway in *Methylobacterium chloromethanicum* CM4

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Received 2 January 2002/Accepted 27 March 2002

Methylobacterium chloromethanicum **CM4 is an aerobic α-proteobacterium capable of growth with chloromethane as the sole carbon and energy source. Two proteins, CmuA and CmuB, were previously purified and shown to catalyze the dehalogenation of chloromethane and the vitamin B_{12}-mediated transfer of the methyl group of chloromethane to tetrahydrofolate. Three genes located near *cmuA* and *cmuB*, designated *metF*, *folD* and *purU* and encoding homologs of methylene tetrahydrofolate (methylene-H_4folate) reductase, methylene-H_4folate dehydrogenase–methenyl-H_4folate cyclohydrolase and formyl-H_4folate hydrolase, respectively, suggested the existence of a chloromethane-specific oxidation pathway from methyl-tetrahydrofolate to formate in strain CM4. Hybridization and PCR analysis indicated that these genes were absent in *Methylobacterium extorquens* AM1, which is unable to grow with chloromethane. Studies with transcriptional *xylE* fusions demonstrated the chloromethane-dependent expression of these genes. Transcriptional start sites were mapped by primer extension and allowed to define three transcriptional units, each likely comprising several genes, that were specifically expressed during growth of strain CM4 with chloromethane. The DNA sequences of the deduced promoters display a high degree of sequence conservation but differ from the *Methylobacterium* promoters described thus far. As shown previously for *purU*, inactivation of the *metF* gene resulted in a CM4 mutant unable to grow with chloromethane. Methylene-H_4folate reductase activity was detected in a cell extract of strain CM4 only in the presence of chloromethane but not in the *metF* mutant. Taken together, these data provide evidence that *M. chloromethanicum* CM4 requires a specific set of tetrahydrofolate-dependent enzymes for growth with chloromethane.**

Aerobic methylotrophic α-proteobacteria of the genus *Methylobacterium* can grow with single-carbon compounds such as methanol and methylamine as the carbon and energy source (15). After oxidation to formaldehyde, a central intermediate in methylotrophic metabolism (26), carbon is either assimilated via the serine cycle or completely oxidized to carbon dioxide (15). Historically, the oxidation of formaldehyde was thought to proceed via a linear pathway involving the sequential action of formaldehyde dehydrogenase and formate dehydrogenase. However, formaldehyde dehydrogenases described for *Methylobacterium* species are broad-range aldehyde dehydrogenases with often low specific activities with formaldehyde. The relevance of such a pathway for growth of *Methylobacterium* with C_1 compounds was therefore uncertain (3), and a pathway proceeding via pterin-dependent intermediates was suggested as an alternative (17). Indeed, two parallel pterin-dependent pathways were recently shown to be essential for growth with methanol in *Methylobacterium extorquens* AM1 (6) (Fig. 1). One of these pathways is tetrahydromethanopterin (H_4MPT)-dependent and appears to be present in most methylotrophic bacteria (40). The other is tetrahydrofolate (H_4folate) dependent and has so far been characterized in *M. extorquens* AM1 only. It is believed that the H_4MPT-dependent pathway operates predominantly in the oxidative direction but that the H_4folate-dependent pathway functions in either direction depending upon the cellular pools of C_1 intermediates available for biosynthesis and energy generation (6, 30, 39).

Methylobacterium chloromethanicum CM4 is distinct from other *Methylobacterium* species described thus far in its ability to grow with chloromethane as sole carbon and energy source (7). However, it is phylogenetically closely related (98% 16S ribosomal DNA sequence identity) to *M. extorquens* AM1 (23). Thus, central C_1 metabolism is expected to be similar in both strains. Physiological and genetic studies already demonstrated that the *cmuA* and *cmuB* genes of strain CM4 are essential for growth on chloromethane and that they encode the proteins responsible for the dehalogenation of this compound (37). The two corresponding proteins were recently purified and shown to catalyze the dehalogenation of chloromethane in vitro (34, 35). Dehalogenation occurs by methyl group transfer from chloromethane onto a vitamin B_{12} cofactor bound to the protein CmuA (34). The methyl group is then transferred from CmuA to H_4folate by CmuB (34). Importantly, the CmuA enzyme is unable to catalyze methyl transfer to H_4MPT and is therefore specific for H_4folate (35).

It appeared possible that methyl-H_4folate generated from chloromethane by the conjugated action of CmuA and CmuB in strain CM4 is oxidized to formate via H_4folate-linked intermediates and not oxidized via formaldehyde as other C_1 growth substrates of *Methylobacterium* (6, 26, 42). In support of this idea, genes designated *metF*, *folD*, and *purU*, which encode homologs of methylene-H_4folate reductase, methylene-H_4folate dehydrogenase–methenyl-H_4folate cyclohydrolase and formyl-H_4folate hydrolase, respectively, were found near *cmuA* and *cmuB* in the CM4 genome (36). This suggested that the H_4folate-dependent chloromethane utilization pathway in

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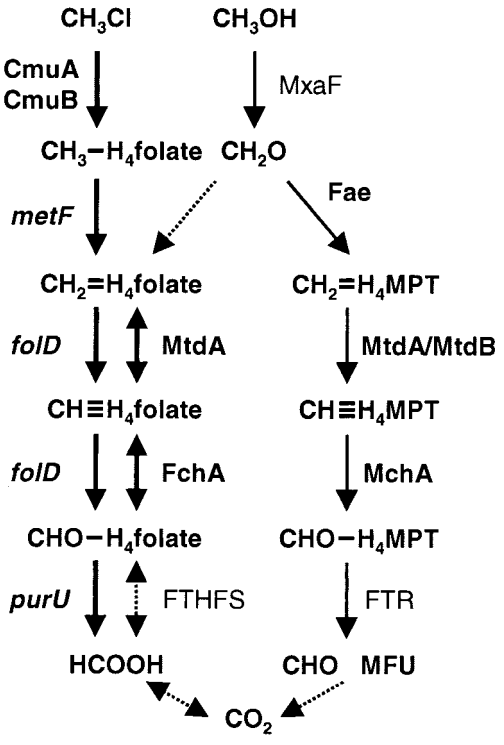

FIG. 1. Proposed model of pterin-dependent C_1 metabolism in *M. chloromethanicum* CM4. Transformations shown by light arrows indicate reactions involved in growth of *M. extorquens* AM1 with methanol (6). Transformations shown by heavy arrows are proposed to be specific to the chloromethane-degrading strain *M. chloromethanicum* CM4. CmuA, chloromethane-corrinoid methyltransferase (34); CmuB, methylcobalamin-H_4folate methyltransferase (35); *metF*, methylene-H_4folate reductase; *folD*, bifunctional methylene-H_4folate dehydrogenase–methenyl-H_4folate cyclohydrolase; *purU*, 10-formyl-H_4folate hydrolase; MtdA, NADP-dependent methylene-H_4folate–H_4MPT dehydrogenase (39); FchA, methenyl-H_4folate cyclohydrolase; (30); FTHFS, formyl-H_4folate synthetase; Fae, formaldehyde-activating enzyme (41); MtdB, NAD(P)-dependent methylene-H_4MPT dehydrogenase (10); Mch, methenyl-H_4MPT cyclohydrolase; (30) FTR, formylmethanofuran-H_4MPT formyltransferase (29). Enzymes indicated in boldface were detected in strain CM4 at the DNA level (reference 13 and PCR data not shown).

strain CM4 may be distinct from the corresponding methanol utilization pathway recently defined in *M. extorquens* AM1 (6), since the latter involves two proteins, methylene-H_4folate dehydrogenase MtdA (39) and methenyl-H_4folate cyclohydrolase FchA (30), which are unrelated to the bifunctional methylene-H_4folate dehydrogenase–cyclohydrolase homolog encoded by *folD* found near *cmuA* in strain CM4.

In the present study, we report experimental evidence for the existence of a C_1 oxidation pathway specific for chloromethane in *M. chloromethanicum* CM4, obtained by transcriptional analysis, gene inactivation studies, and enzyme measurements.

MATERIALS AND METHODS

Materials. Restriction endonucleases, T4 ligase and T4 polymerase were obtained from MBI Fermentas, and *Pfu* DNA polymerase was from Stratagene. All chemicals were from Fluka, Sigma, or Merck except where noted.

Bacterial strains and plasmids. *M. chloromethanicum* CM4 wild-type (7, 23) and the *cmuA*, *cmuB*, and *purU* mutants (37) were described previously. *Escherichia coli* strains DH5α (4) and CC118(λpir) (11), pBluescript II KS(+) vector (Stratagene), the broad-host plasmid pCM62 (18), the *xylE* promoter probe vector pCM130 (18), and the gene inactivation plasmid pKNOCK-Km (1) were used for cloning. Conjugation of plasmids into *M. chloromethanicum* CM4 was performed with *E. coli* S17-1 (33) or S17-1(λpir) (25) and selection of transconjugants was performed as described previously (12).

Media and growth conditions. Luria-Bertani medium (24) was used for growth of *E. coli*. *M. chloromethanicum* was grown in 500-ml rubber-stoppered serum bottles filled with 100 ml of phosphate mineral medium (37). Chloromethane gas was added with a syringe through the rubber stopper to a final concentration of 4.5 mM (2% [vol/vol]), corresponding to an initial concentration of 1.4 mM in the liquid phase, assuming a Henry constant of 0.43 (9). Methanol (sterile filtered) was added to a concentration of 20 to 40 mM. Ampicillin (100 μg/ml, final concentration) and kanamycin and tetracycline (25 μg/ml) were used where required.

DNA manipulations. Preparation of total and plasmid DNA, recombinant DNA work, and Southern analysis were performed according to standard protocols (4). All cloning junctions and cloned PCR products were checked by sequencing by using Dye-Terminator chemistry on an ABI 310 Genetic Analyzer (Perkin-Elmer). Transcriptional *xylE* fusions of the *folD* and *purU* genes were obtained by the insertion of cloned DNA from strain CM4 (36) into the *xylE* reporter plasmid pCM130 (Fig. 2) (18). A 1.4-kb *Eco*RI fragment containing *purU* and part of the *folD* gene (Fig. 2A) was cloned into pBluescript (plasmid pME1798) and subcloned as a *Bam*HI-*Hin*dIII fragment into pCM130 digested with the same restriction enzymes, yielding plasmid pME8251. A 0.7-kb *Hin*dIII/*Xho*I fragment of pME1798 with the *Xho*I site filled in was cloned into pCM130 sequentially digested with *Pst*I, blunted, and digested with *Hin*dIII to yield plasmid pME8252. Plasmid pME8253 was constructed by digestion of plasmid pME8251 with *Hin*dIII and *Xho*I, filling in, and religation.

The *purU-orf414* intergenic region was cloned into promoter probe vector pCM130 (18) as a PCR fragment generated with primers (5′ to 3′) TTCCGCC A**TCTAGA**GATTCC (nucleotides [nt] 4841 to 4860 in database entry AJ011316, *Xba*I site [in boldface] introduced by two nucleotide sequence changes [underlined]) and GGCGA**CA**TATGACGGCACC (nt 5635 to 5617 in AJ011316, introducing an *Nde*I site). The resulting 779-bp PCR fragment was digested with *Xba*I and *Nde*I, its ends were filled in, and it was ligated with *Hin*dIII digested and blunted pCM130, yielding plasmids pME1790 (*purU′-′xylE* fusion) and pME1791 (*orf414′-′xylE* fusion).

The *cmuB′-′xylE* fusions in plasmids pME1799 and pME8250 (Fig. 2B) were constructed by cloning a 1.7-kb *Hin*dIII/*Xho*I genomic fragment and a 0.65-kb *Cla*I/*Xho*I genomic fragment as blunt-ended fragments into CM130 as described above. The *orf219-metF* intergenic region was PCR-amplified with primers GCT TTCGGATCCATCAGACG, (nt 3332 to 3351 in AJ011317, with two nucleotide changes introducing a *Bam*HI site) and CCACATATGC**GGATCC**GTC, (nt 3512 to 3494 in AJ011317). The obtained 159-bp PCR product was cloned as a *Bam*HI fragment into pCM130 digested with the same enzyme, resulting in plasmid pME1796 (*orf219′-′xylE* fusion) and pME1797 (*metF′-′xylE* fusion).

The *folD* gene of strain CM4 mutant was mutagenized by single-crossover recombination of plasmid pME1774, a derivative of pKNOCK-Km containing the 0.5-kb *Eco*RI-*Bam*HI internal fragment of the *folD* gene. The *metF* gene of strain CM4 was mutagenized in the same way with pME1781 containing the 597-bp *Sma*I/ClaI internal fragment of *metF* in pKNOCK-Km (see Fig. 5). Derivatives of plasmid pCM62 (18) constructed for complementation studies contained the genes of interest under P_{lac} control and the following genes (see Fig. 2B): *purU* (2.8-kb *Bam*HI-*Sac*II fragment, plasmid pME1776), *metF* (1.2-kb *Kpn*I-*Fsp*I fragment, pME1789), or *metF-cmuB-cmuC* and the 5′ ends of *orf219* and *orf361* (pME1793, 4.5-kb *Hin*dIII-*Sma*I fragment).

PCR detection of C_1 utilization genes. PCR was performed according to standard protocols with addition of dimethyl sulfoxide to a final concentration of 4% (vol/vol), in a volume of 50 μl with 10 ng of total DNA and 30 pmol of each primer, by using 30 cycles of 1 min in each step of denaturation (94°C), annealing (54°C), and elongation (72°C). The primer sequences used corresponded to known genes of *M. extorquens* AM1, i.e., TGTGGCGCTCGTCGTCAAGG and GAGCTTCAAGCCGCCGATGC for *mtdA* (product: 454 bp) and TGGTCTC GATGGTGTGTAACC and CGAGATGACGTTGAGATTGC (326 bp) for *fchA*, and to known genes of *M. chloromethanicum* CM4, i.e., CCCTGTGAAT GCGGGTTTGC and ACCTCCGGGGCACGTATTGC for *folD* (504 bp), GCT CGAGCAACTTGTCCTC and CAATATCGTCGAAGCGAAGC for *purU* (386 bp), and CAAGAAGCTGTCTCCTACGC and GCTCGCGAAACTATA TCTGC for *metF* (910 bp), respectively.

XylE activity measurements. *M. chloromethanicum* CM4 cultures containing derivatives of *xylE* fusion reporter plasmid pCM130 (18) were grown at 30°C and harvested in late exponential phase (A_{600} of 0.6 to 0.7). Samples (1.5 ml) were centrifuged and resuspended in 0.2 ml of cold catechol-2,3-dioxygenase (XylE) assay buffer (50 mM potassium phosphate [pH 7.5], 10% acetone). A 50-μl aliquot was added to 1 ml of assay buffer, the reaction was initiated by addition

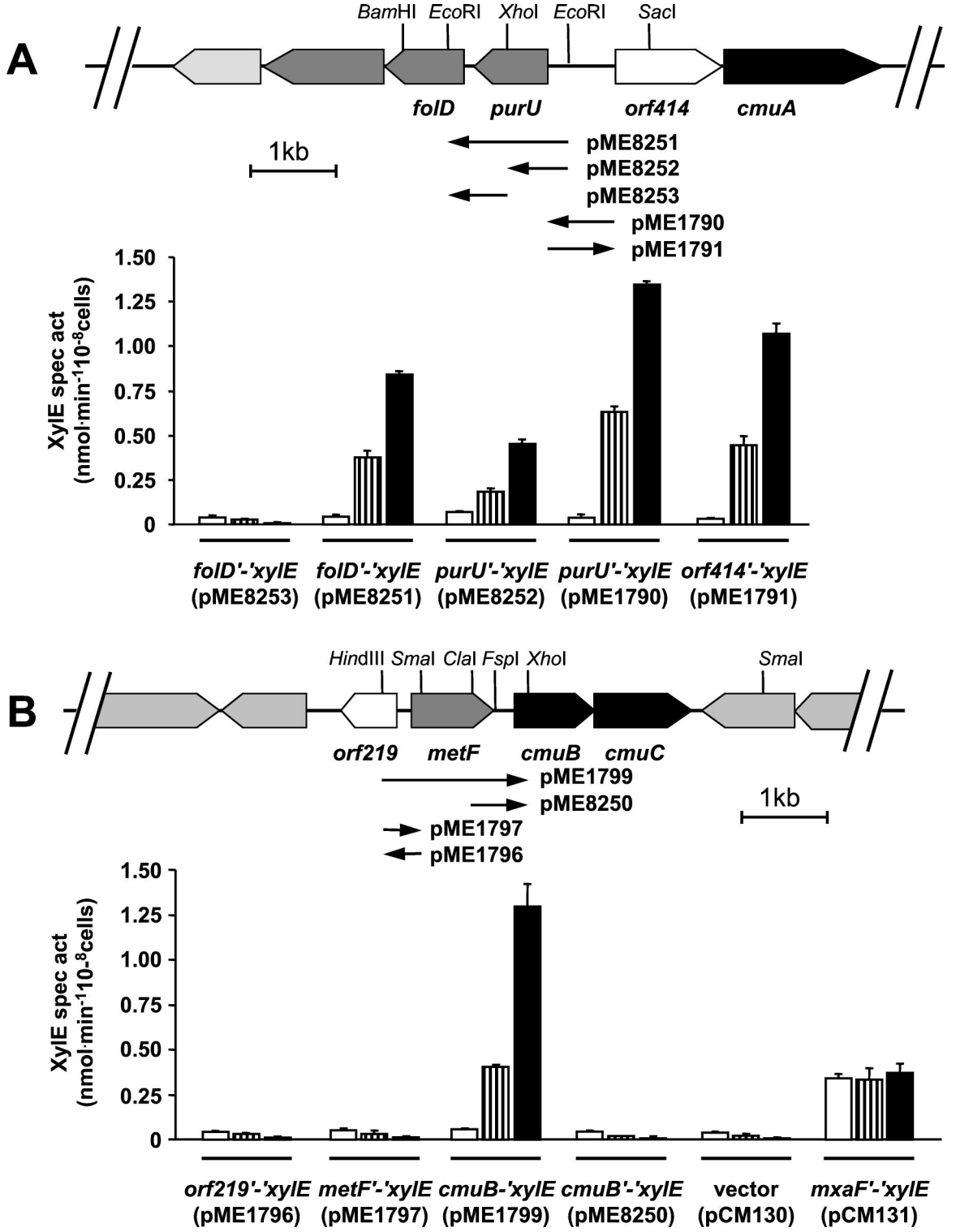

FIG. 2. Expression of plasmid-borne *xylE* fusions in *M. chloromethanicum* CM4. Genetic organization of the gene clusters I (A) and II (B) involved in chloromethane utilization in *M. chloromethanicum* CM4 (36). Genes encoding methyltransferases are shown in black, genes encoding putative H_4folate-dependent enzymes in C_1 metabolism are shown in dark gray, vitamin B_{12} biosynthesis genes are shown in light gray, and genes of unknown function are shown in white. Bar diagrams show catechol dioxygenase activity in transconjugants of wild-type *M. chloromethanicum* CM4 with different intergenic DNA sequences fused to the promoterless *xylE* gene of vector pCM130 (18) grown on methanol (□), a mixture of methanol and chloromethane (hashed bars), or chloromethane (■). Plasmid constructs (see Materials and Methods) are schematically indicated below the sequence, with the orientation of the *xylE* gene indicated by a black arrowhead.

of 10 μl of 0.1 M catechol-1,2(dihydroxybenzene), and the change in absorbance at 375 nm was recorded at 25°C. A molar extinction coefficient (ε_{375}) of 4.4×10^4 M^{-1} cm^{-1} was used for calculating the specific activity of XylE (31), which was expressed as nmol product liberated per min per 10^8 cells assayed. An A_{600} of 1 was shown to correspond 4×10^8 cells per ml (unpublished observations).

RNA isolation. Total RNA was isolated from *M. chloromethanicum* CM4 cells grown to an A_{600} of 0.6 to 0.7 as described previously (38). A culture aliquot (25 ml) was added to 20 ml of frozen and crushed 20 mM Tris chloride buffer [pH 7.5] containing 5 mM $MgCl_2$ and 20 mM sodium azide. Cells were harvested by centrifugation (20,000 × *g*, 15 min) and resuspended in 2.5 ml of ice-cold 1 mM EDTA in 20 mM sodium acetate buffer (pH 5.5), and the aqueous phase was extracted with 2.5 ml of prewarmed (65°C) acidic phenol (pH 5.5) containing 0.5% (wt/vol) sodium dodecyl sulfate. The aqueous phase was further extracted with 2.5 ml of phenol-chloroform-isoamyl alcohol (49.5:49.5:1) and with 2.5 ml of dichloromethane. The RNA was precipitated with 2.5 volumes of ethanol and dissolved in water which had been treated with 0.1% diethyl pyrocarbonate.

Mapping of transcriptional start sites. *M. chloromethanicum* CM4 was grown with either 40 mM methanol or 5% chloromethane, and RNA isolated as described above. Approximately 20 μg of RNA and 2×10^5 to 3×10^5 cpm of radiolabeled primer was used for primer extension experiments, which were performed as previously described (5). Extension products were purified by phenol extraction, followed by ethanol precipitation before separation on 6% denaturing polyacrylamide gels. The primers used were 5′-CGCACCTGAAACGGCAGC GACGATGC-3′ (nt 4800 to 4775 in AJ011316) for *purU*, 5′-CGACAGACCC GAACCTCGCCATTGG-3′ (nt 5509 to 5533 in AJ011316) for *orf414*, and 5′-GGGAGACCTCCAATGACAGATCGCG-3′ (nt 3627 to 3603 in AJ011317) for *metF*. Sequencing reactions were carried out with the same primers and a suitable plasmid as the template by using the fmol Cycle Sequencing kit (Promega).

Methylene-H_4folate reductase assay. Bacteria were grown to late exponential phase (A_{600} of 0.6 to 0.8) with 40 mM methanol, 5% chloromethane, or a mixture of both carbon sources or were grown with methanol and induced with 2% chloromethane for 8 h. Enzyme measurements were performed in cell extracts prepared as described previously (36). Labeled 5-[^{14}C]-methyl-H_4folate (barium salt [Amersham Biosciences], 57 mCi/mmol) was added to unlabeled (6*R*,*S*)-methyl-H_4folate (Sigma) in 8 mM ascorbate buffer to a final stock concentration of 5 mM methyl-H_4folate (925 dpm of ^{14}C label/nmol) as described previously (32). Methylene-H_4folate reductase activity was measured at 37°C according to previously reported methods (20, 32) with ca. 60 μg of total cell extract protein in 0.75 ml of 100 mM potassium phosphate, 0.6 mM EDTA, 0.67% bovine serum albumin buffer (pH 6.7), 0.15 ml of saturated menadione solution in 20% aqueous methanol, 0.36 ml of labeled methyl-H_4folate stock solution (final concentration, 1.2 mM), and water to a total volume of 1.5 ml. Aliquots (0.25 ml) were taken at time points up to 5 min, quenched by the addition of 75 μl of 1 M sodium acetate acid containing 3 mg of dimedone/ml, and incubated at 100°C for 2 min. The formaldehyde released from methylene-H_4folate and derivatized with dimedone was extracted into toluene (1 ml) by vortexing for exactly 2 min. After centrifugation (Jouan A14 centrifuge) at maximal speed (17,500 × *g*) for 1 min, an aliquot of the toluene phase (0.25 ml) was added to 1 ml of scintillation fluid (Microscint 40; Packard) and counted (Beckman LS1801). The reported data represent the average of three independent determinations.

RESULTS

Identification of genes encoding pterin-dependent C_1 interconverting enzymes in *M. chloromethanicum* CM4. Genomic DNA from *M. extorquens* AM1, which is unable to grow with chloromethane, was hybridized with probes for the *purU*, *folD*, and *metF* genes of *M. chloromethanicum* CM4. These genes encode homologs of methylene-H_4folate reductase, methylene-H_4folate dehydrogenase–methenyl-H_4folate cyclohydrolase, and formyl-H_4folate hydrolase, respectively, and were previously proposed (36) to define a specific oxidation pathway for chloromethane in strain CM4 (Fig. 1). No specifically hybridizing DNA fragments were detected (data not shown). The absence of these genes was further suggested by PCR analysis with primer pairs specific to the sequences of these genes from strain CM4 (see Materials and Methods; data not shown). That *M. extorquens* AM1 lacked homologs of *metF*, *folD*, and *purU* was also indicated by analysis of the already available sequences from the *M. extorquens* AM1 genome sequencing project (L. Chistoserdova and M. E. Lidstrom, unpublished data; also see http://pedant.gsf.de/cgi-bin/wwwfly.pl?Set=Mextorquens&Page=index). Conversely, however, hybridization analysis with probes corresponding to the *mtdA*, *fae*, and *mchA* genes of *M. extorquens* AM1 representing both branches of C_1 utilization described in *M. extorquens* AM1 (6, 39, 41) (Fig. 1) showed that these genes were also present in strain CM4 and in the dichloromethane-degrading strain *Methylobacterium dichloromethanicum* DM4 (13). In the latter strain, sequence analysis revealed very strong sequence conservation of these genes (>96% sequence identity at the DNA level) with their homologs of strain AM1 (13). This analysis was confirmed and extended by obtaining the expected PCR products for *mtdA* (454 bp; see Materials and Methods) and *fchA* (326 bp), which define the H_4folate-dependent branch of C_1 characterized in *M. extorquens* AM1 (Fig. 1), by using total DNA from strain CM4 as a template and primers derived from the sequences of these genes that were reported for strain AM1 (data not shown). Taken together, therefore, our data indicate that strain CM4 not only possesses both the H_4folate- and H_4MPT-dependent pathways of C_1 metabolism present in *M. extorquens* AM1 but also an additional set of genes encoding a third H_4folate-dependent pathway for chloromethane degradation.

Analysis of transcriptional *xylE* fusions. In order to gain insight into chloromethane-dependent gene regulation in *M. chloromethanicum* CM4, plasmids harboring transcriptional *xylE* fusions of the *metF*, *folD*, *purU*, *cmuB*, and *orf414* genes (Fig. 2) were constructed and conjugated into wild-type strain CM4. Transconjugant strains were grown with methanol, with chloromethane, or with a mixture of both these carbon sources. Catechol dioxygenase activity was measured in exponentially growing bacteria. The controls used in these experiments were *M. chloromethanicum* CM4 harboring the low-background *xylE* promoter-probe vector pCM130 (18) or plasmid pCM131, in which the *xylE* gene is constitutively expressed under the control of the promoter of the large subunit methanol dehydrogenase gene (*mxaF*) from strain AM1 (18). The previously noted (18) high expression of the latter reporter fusion during growth on methanol was verified (Fig. 2B). Expression of XylE from both *purU′-′xylE* and *folD′-′xylE* fusions (plasmids pME8251 and pME8252) was induced in the presence of chloromethane (Fig. 2A). In contrast, the reporter *folD′-′xylE* construct pME8253 lacking the DNA sequence upstream of *purU* showed no chloromethane-induced catechol dioxygenase activity. This suggested that expression of *purU* and *folD* was induced by chloromethane from a common promoter located upstream of the *purU* gene (Fig. 2A). Further, a DNA fragment including the entire intergenic region beween *purU* and *orf414* also led to high chloromethane-induced XylE activity for the *purU′-′xylE* fusion construct (plasmid pME1790, Fig. 2A). Construct pME1791, which contains the same insert as pME1790 but in the reverse orientation, was used as a promoter probe vector for monitoring expression of *orf414* and *cmuA* genes as a *orf414′-′xylE* fusion (Fig. 2A). The catechol dioxygenase activity measured with these two last constructs were in the same range as for the other *purU′-′xylE* and *folD′-′xylE* reporter fusions.

Regarding the genes found in cluster II, the data obtained with the two transcriptional fusions *metFcmuB′-′xylE* and *cmuB′-′xylE* (plasmids pME1799 and pME8250, Fig. 2B) indi-

cated that expression of *cmuB* is controlled by a promoter located upstream of the *metF* gene rather than in the noncoding region between *metF* and *cmuB*. Interestingly, however, the short intergenic sequence between the start codons of the divergent *orf219* and *metF* genes was by itself insufficient to induce XylE expression in either direction (Fig. 2B, plasmids pME1796 and pME1797).

Determination of chloromethane induced transcription initiation sites in *M. chloromethanicum* CM4. Expression studies with transcriptional fusions suggested the presence of at least three chloromethane-inducible promoters located upstream of *purU*, *metF*, and *orf414*, respectively (Fig. 2). Transcriptional start sites were mapped by primer extension with RNA isolated from wild-type *M. chloromethanicum* CM4 grown with either chloromethane or methanol. Specific elongation products were obtained with RNA from chloromethane-grown cells that were absent in RNA from cultures grown with methanol, and their 3′ ends mapped upstream of genes *purU*, *metF*, and *orf414* (Fig. 3). In contrast, no extension products were obtained with primers designed to detect transcriptional start sites upstream of *cmuA*, *folD*, and *orf219* (data not shown). The mapping of a transcriptional start site 340 nt upstream of the translational start of *orf414* revealed a previously overlooked short open reading frame of 165 nt, *orf55*, which could be transcriptionally coupled with *orf414* since the stop codon of *orf55* overlaps with the predicted start codon of *orf414* (Fig. 3). The putative promoter sequences upstream of the chloromethane-induced transcripts exhibited striking sequence conservation (Fig. 4). Their −35 region was identical to the minus −35 region of the dichloromethane dehalogenase promoter of *M. dichloromethanicum* DM4 (14) and that of the *E. coli* consensus promoter. Conservation was less extensive in the −10 region, and the deduced consensus had no similarity to the −10 promoter regions previously identified for *Methylobacterium* genes (Fig. 4).

Analysis of gene disruptions in *purU*, *folD*, and *metF*. The previously obtained minitransposon *purU* mutant (37), although unable to grow with chloromethane, exhibited wild-type chloromethane dehalogenase activity (36). In comparison, *cmuA* mutants (36) were unable to dehalogenate chloromethane but showed the same growth phenotype as the *purU* mutant. The growth yield of a *cmuA* mutant with a mixture of methanol and chloromethane was only two-thirds that of the wild-type CM4 (Table 1), since the *cmuA* mutant metabolized the methanol but not the chloromethane present in the medium (Table 1). In contrast, the growth yield of the *purU* mutant was in the same range as that of the wild type during growth on a mixture of chloromethane and methanol (Table 1). This indicated that the *purU* mutant was able to assimilate carbon derived from the dehalogenation of chloromethane in the presence of methanol even if it did not grow with chloromethane as the sole carbon source. Providing the *purU* gene in *trans* by ways of plasmid pME1776 was sufficient to restore growth of the *purU* mutant with chloromethane. Southern blot analysis confirmed that plasmid pME1776 was stably maintained in strain CM4 and did not recombine into the chromosome (data not shown).

A mutant in *metF*, encoding a methylene-H_4folate reductase homolog suspected to represent the next enzyme of the chloromethane oxidation pathway after dehalogenation of chloromethane to methyltetrahydrofolate (34, 36), was also constructed by insertional mutagenesis with a derivative of the suicide broad-host-range vector pKNOCK-Km (1) containing an internal fragment of the *metF* gene. Southern blot analysis demonstrated that the gene knockout plasmid had inserted into the CM4 genome as expected, causing a disruption of *metF* (Fig. 5 and data not shown). The growth rate of the *metF* mutant on methanol was in the same range as that of the wild type, whereas no growth was observed on chloromethane or, interestingly, on a mixture of chloromethane and methanol (data not shown). This suggested a toxic effect of chloromethane dehalogenation in this mutant. Growth on chloromethane was restored by mating the *metF* mutant with derivatives of plasmid pCM62 (18) containing the *metF* gene. Plasmid pME1793 containing *metF*, together with the downstream *cmuB* and *cmuC* genes, was stably maintained in the *metF* mutant growing with chloromethane. However, plasmid pME1789 containing only the *metF* gene recombined into the CM4 genome upon cultivation of the transconjugant on chloromethane (data not shown). Taken together, these observations confirmed not only that the observed growth phenotype of the *metF* mutant was caused by disruption of the *metF* gene but also that the genes *cmuB* and *cmuC* essential for growth of strain CM4 with chloromethane (36) were cotranscribed with *metF*.

The *folD* gene, encoding a homolog of the bifunctional enzyme methylene-H_4folate dehydrogenase–methenyl-H_4folate cyclohydrolase and suggested from experiments with reporter gene fusions to be cotranscribed with *purU*, was disrupted by the same strategy as for the *metF* mutant. The resulting *folD* mutant strain was shown by hybridization analysis to lack an intact *folD* gene as a consequence of the recombination of the plasmid into the chromosome (data not shown). This mutant, however, grew with chloromethane as well as the wild type (Table 1).

Measurements of methylene-H_4folate reductase activity. Cell extracts of wild-type CM4, its *metF* mutant, and the *metF* mutant complemented by expression of *metF*, *cmuB*, and *cmuC* genes from plasmid pME1793 were assayed for methylene-H_4folate reductase activity by measuring the conversion of [^{14}C]methyl-H_4folate to methylene-H_4folate by a previously described method (19, 32). This provided unequivocal evidence for chloromethane-induced methylene-H_4folate reductase activity of MetF (Table 2). Cell extracts of wild-type CM4 grown with chloromethane showed significant methylene-H_4folate reductase activity, unlike those of the strain grown with methanol. Strain CM4 grown on a mixture of both growth substrates showed an intermediate level of activity. Importantly, low but significant activity was also detected in an extract of strain CM4 grown with methanol and then induced with chloromethane for 8 h. In contrast, the corresponding extract of the *metF* mutant showed no detectable activity under the same conditions (Table 2). Finally, the very high and specific induction of enzyme activity in the complemented mutant grown in the presence of chloromethane (Table 2) confirmed that the *metF* gene from strain CM4 encodes a methylene tetrahydrofolate reductase whose expression is specifically regulated by chloromethane.

DISCUSSION

The data obtained with *xylE* reporter fusions of *metF*, *purU*, and *folD* (Fig. 2) and the chloromethane-regulated transcriptional units that were identified (Fig. 3 and Fig. 4) provide

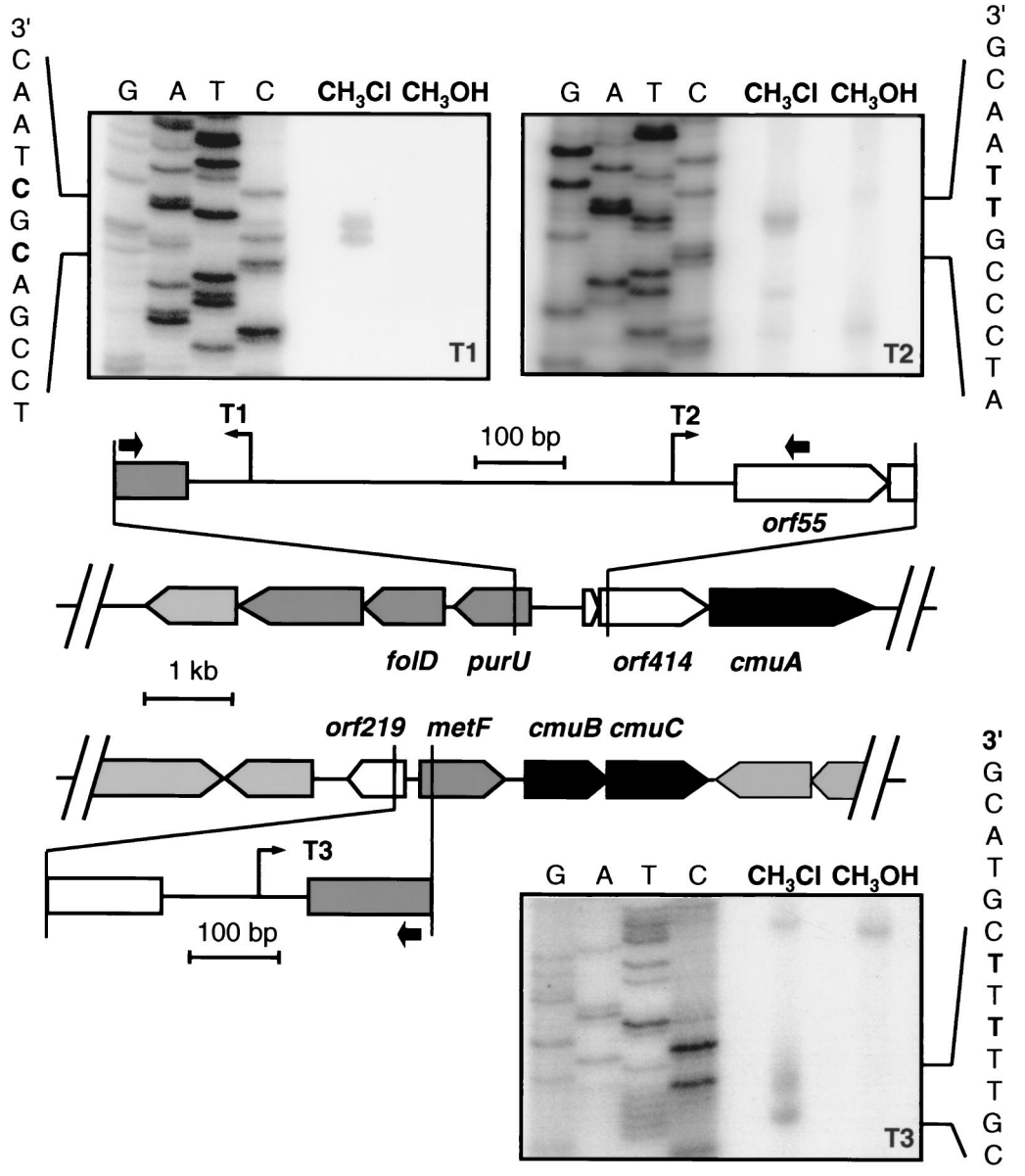

FIG. 3. Transcriptional start sites of chloromethane induced genes in *M. chloromethanicum* CM4. RNA was isolated from *M. chloromethanicum* CM4 grown with chloromethane or methanol and reverse transcribed with the primers indicated by black arrows on the schematic view of the two gene clusters (see Fig. 2). Lanes A, T, C, and G show the sequencing ladders obtained with the same primers. The identified transcriptional start sites are marked in boldface (hooked arrows; T1, AJ011316, nt 4918 and 4920; T2, AJ011316, nt 5383 and 5384; and T3, AJ011317, nt 3483 and 3485).

evidence for the existence of a chloromethane-induced H_4folate-dependent pathway for the oxidation of chloromethane that involves *metF*, *folD*, and *purU* (Fig. 1). This pathway runs alongside to that defined by methylene-H_4folate dehydrogenase MtdA (39) and methenyl-H_4folate cyclohydrolase FchA (30), which are both essential for growth of strain AM1 with methanol and, at the genetic level at least, are also present in strain CM4.

A database search using the promoter consensus proposed in Fig. 4 as a pattern failed to detect any other such sequence motifs in available sequences from *M. chloromethanicum* CM4, in the gene upstream sequences available for the chloromethane degraders *Aminobacter* sp. strain IMB-1 (*metF*, *cmuB*, and *cmuA* homologs [44]) and *Hyphomicrobium chloromethanicum* CM2 (*folD* homolog [22]) or indeed in any other *Methylobacterium* sequences deposited in databases. Thus, the identified promoter consensus is probably neither utilized for the regulation of chloromethane genes in methylotrophic bacteria nor for driving gene expression in *Methylobacterium* species in general. However, the existence of a consensus promoter sequence for chloromethane-regulated genes in strain CM4 suggests that the expression of these genes could be driven by a common regulator. Experiments aiming at the identification of such a regulator protein are in progress.

Evidence for the specific involvement of *metF* and *purU* in a chloromethane oxidation pathway to formate in strain CM4 stems from the phenotypic properties of the corresponding

```
                    -35                          -10
P orf414            GATTGACAGCCTTACCCATAAATGGAATCGTACGTTAA..
P purU              GCTTGACAAAGCGAGCGG-CACATTAATCATACGTTAGCG
P metF              CATTGACATTCAAAAATCACACCTTAA-CGTACGAAAAA.
Consensus             TTGACA                  AA C TACG

P dcmA(DM4)         GCTTGACAGAGATGCATAGCCTTGTATAGAACTAGCCC

P mxaF(AM1)         TAAAGACATCGCGTCCAATCAAAGCCTAGAAAATATAGG
P mxaF(XX)          TAAAGACATCTCCTTCAATCAACGCCTAGAAACGATA

σ70(E.coli)       ..TTGACA.....N(16-18).....TATAAT....↓..
```

FIG. 4. Alignment of chloromethane specific promoter regions in *M. chloromethanicum* CM4. The putative −35 and −10 regions are indicated in boldface, and experimentally determined transcription initiation sites are underlined. A likely consensus promoter is shown. Promoter regions of previously identified *Methylobacterium* genes involved in one-carbon metabolism are also shown for comparison. P_{dcmA}, dichloromethane dehalogenase promoter of *M. dichloromethanicum* (14); $P_{mxaF(AM1)}$, methanol dehydrogenase promoter of *M. extorquens* AM1 (2); $P_{mxaF(XX)}$, methanol dehydrogenase promoter of *Methylobacterium organophilum* XX (16). The *E. coli* σ70 consensus promoter sequence (43) is also given for comparison.

mutants and the absence of these genes in strain AM1. The observation that the *metF* mutant of strain CM4 was not only unable to grow with chloromethane but was sensitive to its presence during growth with methanol is worth emphasizing. We speculate that in the *metF* mutant, folate accumulates as methyl-H_4folate upon dehalogenation of chloromethane and that this is detrimental for the function of biosynthetic pathways that rely on C_1 moieties provided by H_4folate. Such a "methyl trap" model was already proposed for the clinical picture observed in human patients with pernicious anemia, in which the presence of a dysfunctional methionine synthase leads to the accumulation of methyl-H_4folate (19) and to poor availability of the H_4folate cofactor for other important physiological processes. Strikingly, MetF of strain CM4 displays only low sequence similarity to the majority of known methylene-H_4folate reductases (see Fig. 6). The MetF homolog of the halomethane-utilizer *Aminobacter* sp. strain IMB-1 (44) is more closely related to the CM4 MetF rather than that encoded in the *M. extorquens* AM1 genome sequence (26% sequence identity [Chistoserdova and Lidstrom, unpublished, and Fig. 6]). This might reflect the fact that in chloromethane degradation, the role of MetF is to enable the efficient oxidation of methyl-H_4folate to methylene-H_4folate (Fig. 1, Table 2). The main function of MetF is usually in the opposite direction, affording one-carbon precursors for the synthesis of methionine from homocysteine, as in *E. coli*, for example (21).

Like the *metF* mutant, the *purU* mutant is unable to grow with chloromethane alone. However, its growth yield on a mixture of chloromethane and methanol is undistinguishable from that of the wild-type (Table 1), indicating that the tetrahydrofolate-bound carbon derived from chloromethane can be metabolized in a productive fashion despite the lack of this gene. No *purU* gene could be detected in strain AM1 (our DNA hybridization and PCR data; Chistoserdova and Lidstrom, unpublished), and it appears that the functionally homologous enzyme involved in the interconversion of formyl-H_4folate and formate in strain AM1 is not related to PurU (J. Vorholt, unpublished data). We therefore assume at present that in CM4 *purU* encodes a specific chloromethane-induced formyl-H_4folate hydrolase and that in the absence of *purU* formyl-H_4folate can be transformed to formate by another enzyme common to strain AM1 and CM4 which is expressed

TABLE 1. Growth yields of *M. chloromethanicum* CM4 wild-type and mutants[a]

CM4 strain	Growth yield (g [dry weight] mol of C^{-1})		
	MeOH	MeOH-CH_3Cl	CH_3Cl
Wild type	10.0 ± 0.2	9.5 ± 0.2	10.6 ± 0.7
cmuA	9.7 ± 0.3	6.2 ± 0.2	NG
purU	9.9 ± 0.3	9.6 ± 0.2	NG
folD	8.9 ± 0.6	9.1 ± 0.2	11.3 ± 1.1

[a] The average of three independent cultures grown with either 20 mM methanol (MeOH), 20 mM methanol and 10 mM chloromethane (MeOH-CH_3Cl), or 10 mM chloromethane (CH_3Cl) is given. NG, no growth.

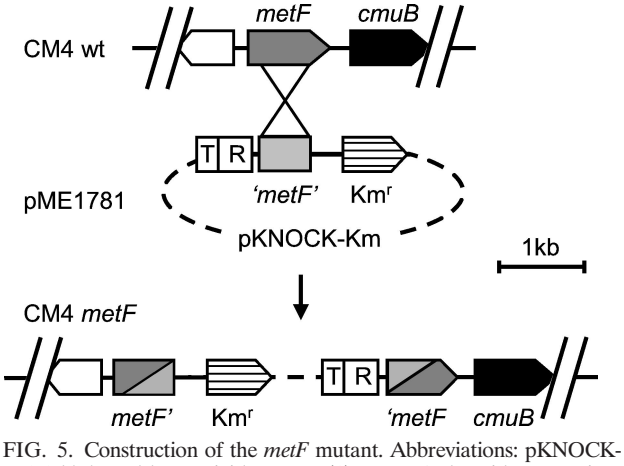

FIG. 5. Construction of the *metF* mutant. Abbreviations: pKNOCK-Km, 2.1-kb broad-host suicide vector (1); T, RP4 plasmid *oriT* region; R, R6K γ-origin of replication; Km^r, kanamycin resistance gene.

TABLE 2. Methylene-H_4folate reductase activity in cell extracts of bacteria grown with different C_1 sources

CM4 strain	Sp act (nmol min^{-1} mg^{-1})		
	MeOH	MeOH-CH_3Cl	CH_3Cl
Wild type	<0.7	158 ± 20	245 ± 44
		6.5 ± 0.6[a]	<0.5[b]
metF	<0.4	<0.5[a]	NG[c]
metF(pME1793)	8.5 ± 0.9	931 ± 52	1,378 ± 77

[a] Grown with 20 mM methanol and induced with 2% CH_3Cl for 8 h.
[b] Extract was boiled for 5 min before measurement.
[c] NG, no growth.

during growth in the presence of methanol but not during growth with chloromethane alone.

The evidence for a role of *folD* in the chloromethane-degrading pathway of *folD*, suggested by its sequence to be a methylene-H_4folate dehydrogenase–methenyl-H_4folate cyclohydrolase, is more indirect. Indeed, the retained ability of the *folD* mutant to grow with chloromethane, as well as the complementation of the growth phenotype of the *purU* mutant by an intact copy of *purU* in *trans* without the need for the downstream *folD* gene, actually shows that the *folD* gene is dispensable for chloromethane metabolism. However, its coinduction with *purU* in strain CM4 (Fig. 2) and its location near the chloromethane dehalogenase gene *cmuA* in *H. chloromethanicum* CM2 (22) support the idea that *folD* is involved in C_1 oxidation during chloromethane utilization. Moreover, no *folD* homolog was yet detected in strain AM1 (unpublished data and Chistoserdova and Lidstrom, unpublished). At present, therefore, we assume that in the CM4 *folD* mutant growing with chloromethane, the two reactions from methylene-H_4folate dehydrogenase to formyl-H_4folate are performed by MtdA (39) and FchA (30) since the corresponding genes are also present in strain CM4 and that this explains the lack of

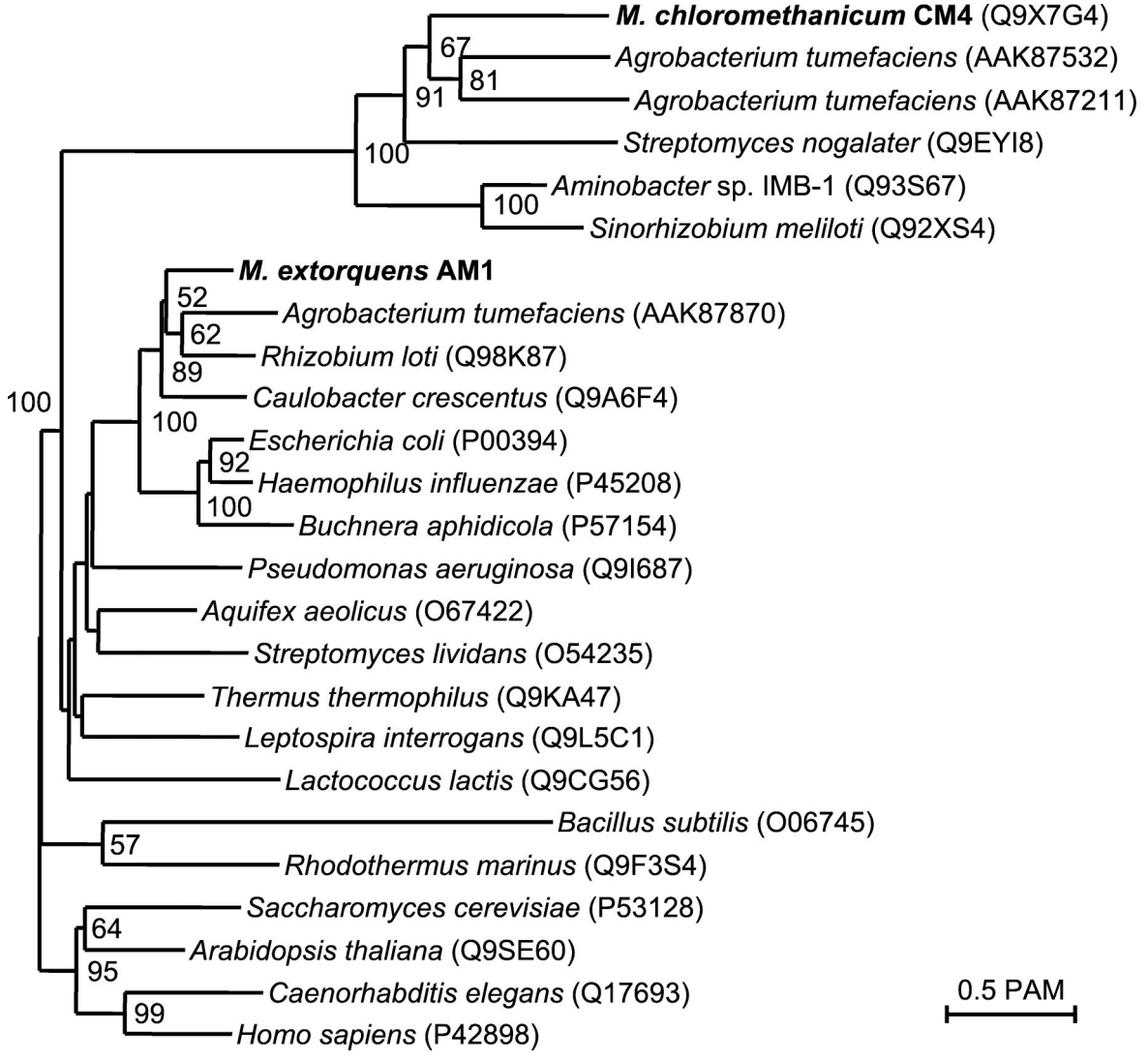

FIG. 6. Phylogenetic analysis of MetF proteins from bacteria. A multiple alignment of representative MetF sequences produced with T-Coffee (27) was used to generate a phylogenetic tree (325 aligned positions) with FITCH from PHYLIP (8). The tree was drawn with NJPlot (28). Nodes confirmed by bootstrapping analysis are indicated by the percent conservation, and the accession numbers of protein sequences are given in parentheses.

phenotype of the *folD* mutant. The construction of *mtdA* and *fchA* mutants in both the background of strain CM4 and that of its *folD* mutant should help to shed light on this question.

In conclusion, the evidence presented here for an additional C_1 utilization pathway specific for chloromethane in strain CM4 adds to the emerging complexity of C_1 metabolism in *Methylobacterium* species. On the other hand, the existence of such an additional pathway in strain CM4 may allow us to obtain and investigate, in the background of strain CM4, mutants in C_1 utilization genes previously found to be essential in *M. extorquens* AM1 if these can be complemented by homologous genes of the chloromethane-specific pathway.

ACKNOWLEDGMENTS

A.S. and C.M. contributed equally to this work.

We thank Ludmila Chistoserdova and Mary Lidstrom for access to unpublished sequence information from the *M. extorquens* AM1 genome, Julia Vorholt for discussions, and Chris Marx and Mikhail Alexeyev for generous gifts of plasmids.

This work was supported by ETH grant 0-20436-97 to T.L.

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