Distribution of Tetrahydromethanopterin-Dependent Enzymes in Methylotrophic Bacteria and Phylogeny of Methenyl Tetrahydromethanopterin Cyclohydrolases

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The methylotrophic proteobacterium *Methylobacterium extorquens* AM1 possesses tetrahydromethanopterin (H_4MPT)-dependent enzymes, which are otherwise specific to methanogenic and sulfate-reducing archaea and which have been suggested to be involved in formaldehyde oxidation to CO_2 in *M. extorquens* AM1. The distribution of H_4MPT-dependent enzyme activities in cell extracts of methylotrophic bacteria from 13 different genera are reported. H_4MPT-dependent activities were detected in all of the methylotrophic and methanotrophic proteobacteria tested that assimilate formaldehyde by the serine or ribulose monophosphate pathway. H_4MPT-dependent activities were also found in autotrophic *Xanthobacter* strains. However, no H_4MPT-dependent enzyme activities could be detected in other autotrophic α-proteobacteria or in gram-positive methylotrophic bacteria. Genes encoding methenyl H_4MPT cyclohydrolase (*mch* genes) were cloned and sequenced from several proteobacteria. Bacterial and archaeal Mch sequences have roughly 35% amino acid identity and form distinct groups in phylogenetic analysis.

Aerobic methylotrophs are gram-negative or gram-positive bacteria capable of growth on reduced C_1 compounds such as methanol or methane as their carbon and energy source (24). Formaldehyde is formed as a central intermediate in methylotrophic metabolism. At least four different enzyme systems have been proposed as C_1 pathways for the oxidation of formaldehyde to CO_2 in methylotrophic bacteria (see also 13). The first of these is a linear pathway involving thiol-linked formaldehyde dehydrogenase, a hydrolase and formate dehydrogenase. This pathway is found in the autotrophic α-proteobacterium *Paracoccus denitrificans* and the nonsulfur purple bacteria *Rhodopseudomonas acidophila* and *Rhodobacter sphaeroides*, which possess a glutathione-dependent formaldehyde dehydrogenase (2, 3, 20, 32, 36). Similarly, the gram-positive methylotroph *Amycolatopsis methanolica* that contains the ribulose monophosphate cycle was shown to contain a mycothiol-dependent formaldehyde dehydrogenase (27, 28), which was previously called NAD factor-dependent formaldehyde dehydrogenase (15, 30). As a second route, tetrahydrofolate (H_4F)-dependent enzymes have been discussed as being involved in formaldehyde oxidation in serine cycle methylotrophs (10, 26). A third proposed route for formaldehyde oxidation entails the cyclic ribulose monophosphate pathway involving 6-phosphogluconate dehydrogenase (1, 4, 18). This pathway has been proposed for β-proteobacterial methylotrophs that contain the ribulose monophosphate cycle. However, only low activities of this pathway could be detected in the other group containing the ribulose monophosphate cycle, the obligate methanotrophic bacteria that belong to the γ-proteobacteria class (48). Therefore, the formaldehyde oxidation pathway in these methanotrophs has been uncertain.

Recently, we described a fourth pathway for formaldehyde oxidation, involving tetrahydromethanopterin (H_4MPT)-dependent enzymes in the α-proteobacterium *Methylobacterium extorquens* AM1, a serine cycle methylotroph (11, 31, 47). These enzymes were previously thought to be restricted to methanogenic and sulfate-reducing archaea (for a review, see reference 43). Three H_4MPT-dependent and H_4F-dependent enzymes from *M. extorquens* AM1 have been purified and characterized (31, 47): a methenyl H_4MPT cyclohydrolase, a methenyl H_4F cyclohydrolase, and an NADP-dependent methylene H_4MPT dehydrogenase, which also catalyzes the dehydrogenation of methylene H_4F (47). Both pterin cofactors have been shown to be present in similar amounts (47). It therefore appears that in *Methylobacterium extorquens* AM1, two independent C_1 transfer pathways are simultaneously operative, one involving H_4MPT (in a dephospho form) and one involving H_4F (11, 47). On the basis of mutant and enzyme studies, the H_4MPT-dependent pathway was proposed to be the main pathway for oxidation of formaldehyde to CO_2 (11, 31, 47).

In this study, we investigated the distribution of H_4MPT-dependent enzyme activities in methylotrophic representatives from different phylogenetic and physiological groups. For those bacteria that showed H_4MPT-dependent enzyme activities, we cloned one of the encoding genes to obtain sequence data and gain insights into the origin and phylogeny of H_4MPT-dependent enzymes. We concentrated on methenyl H_4MPT cyclohydrolase, which is also present in methanogenic archaea, rather than NAD- and NADP-dependent methylene H_4MPT dehydrogenases, which are novel enzymes not found in archaea (47). The gene encoding methenyl H_4MPT cyclohydrolase (*mch*) from *M. extorquens* AM1 has been identified by N-terminal sequencing of the purified protein and shown to consist of only one type of subunit of molecular mass 33 kDa (31). Mch from *M. extorquens* AM1 possesses about 36% amino acid sequence identity to homologues from methanogenic archaea and *Archaeoglobus fulgidus* (11).

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MATERIALS AND METHODS

Organisms. The following are laboratory strains (University of Washington, Seattle) that were used in this study: *Methylobacterium extorquens* AM1 (29), *Methylosinus trichosporium* OB3b (5), *Xanthobacter flavus* H4-14 (33), *Methylococcus capsulatus* Bath (5), *Methylococcus thermophilus* IIIp (25), *Methylomicrobium album* BG8 (5), *Methylomonas rubra* 15sh (35), and *Shewanella putrefaciens* MR-1 (34). *Methylobacterium organophilum* XX (DSM 760), *Hyphomicrobium methylovorum* GM2 (DSM 5458), *Xanthobacter autotrophicus* (DSM 432), *Paracoccus denitrificans* (DSM 413), *Rhodopseudomonas acidophila* (DSM 145), *Methylobacillus flagellatum* KT (DSM 6875), *Methylophilus methylotrophus* AS1 (DSM 46235), *Amycolatopsis methanolica* (DSM 44096), and *Methanobacterium thermoautotrophicum* Marburg (DSM 2133) were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *Rhodobacter sphaeroides* 2.4.1 (44) was obtained from S. Kaplan (University of Texas). Cell mass of *Bacillus methanolicus* MGA3 (38) was kindly provided by S. Pluschkell, University of Minnesota.

Growth of bacteria. *Methylobacterium extorquens* AM1, *Methylobacterium organophilum* XX, *H. methylovorum* GM2, *Methylobacillus flagellatum* KT, and *Methylophilus methylotrophus* AS1 were grown on methanol on minimal medium as described previously or on succinate (0.5%) on the same medium (17). The methanotrophic strains *Methylosinus trichosporium* OB3b, *Methylococcus capsulatus* Bath, *Methylococcus thermophilus* IIIp, *Methylomicrobium album* BG8, and *Methylomonas rubra* 15sh were grown on methane as described previously (19). The following bacteria were grown on the media described by the DSMZ (12a). *X. autotrophicus* and *X. flavus* H4-14 were grown on nutrient broth medium plus methanol (100 mM) or on methanol (100 mM) in minimal medium 81 supplemented with 0.01% yeast extract, *P. denitrificans* was grown on methanol (100 mM) in minimal medium 81 supplemented with 0.01% yeast extract, and *Rhodopseudomonas acidophila* was grown on medium 26 plus methanol (100 mM) under anoxic conditions in the light. *Amycolatopsis methanolica* was also grown on methanol (100 mM) or glucose (0.4%) in the minimal medium described previously (21), and *R. sphaeroides* 2.4.1 was grown on Luria-Bertani medium (37) containing 5 g (instead of 10 g) NaCl per liter.

Rhodopseudomonas acidophila was grown at room temperature, *M. thermophilus* IIIp was grown at 42°C, and all other bacteria were grown at 30°C.

Coenzymes. Tetrahydromethanopterin (H$_4$MPT) and methenyl H$_4$MPT were purified from *Methanobacterium thermoautotrophicum* Marburg (7). H$_4$F was purchased from Sigma.

Preparation of cell extracts. Frozen cells (0.5 g) were resuspended in 1 ml of 50 mM morpholinepropanesulfonic acid (MOPS)-KOH (pH 7.0) at 4°C and passed two times through a French pressure cell at 1.2×10^8 Pa. Cell debris and unbroken cells were removed by centrifugation in an Eppendorf microcentrifuge. The resulting supernatant is referred to as cell extract.

Protein concentration was determined by the Bradford assay (6) by using the Bio-Rad reagent with bovine serum albumin as the standard.

Determination of enzyme activities. The assays were routinely performed at room temperature in 1-ml cuvettes (diameter, 1 cm) in a total volume of 0.7 ml.

Methenyl H$_4$MPT cyclohydrolase activity was determined photometrically at 335 nm as described before (31). NADP- and NAD-dependent methylene H$_4$MPT dehydrogenase and NADP-dependent methylene H$_4$F dehydrogenase activities were monitored at 340 nm (47). Glutathione-linked formaldehyde dehydrogenase was measured at 340 nm as described before (32).

Standard molecular biology techniques. All standard procedures were performed as described by Sambrook et al. (37). Enzymes were used in accordance with recommendations of the manufacturers (Boehringer Mannheim and New England Biolabs).

PCR amplification. The heterologous primers *mch-1* (5′-TTCTTCGCCMTG GGKTCKGGKCC), *mch-2a* (5′-TGCCTCGGCTCKCAATATGCYGGBTG G), *mch-2b* (5′-TGCCTCGGCAGCCAGWAYGCSGGCTGG), *mch-3* (5′-GC GTCGTTKGTKCKBCCCAT), and *mch-4* (5′-AACAGCGCTCCGTCKATYT TGTARAARTC) were derived from four sequence parts of Mch conserved in the sequences from *Methylobacterium extorquens* AM1, methanogenic archaea, and *Archaeoglobus fulgidus*. The primer combination *mch-2a*–*mch-3* was used to amplify a PCR product of approximately 420 nucleotides with chromosomal DNA from *Methylococcus capsulatus* Bath, *Methylomonas rubra* 15sh, *Methylococcus thermophilus* IIIp, and *Methylobacillus flagellatum* KT. From the newly obtained Mch sequences, additional heterologous primers were designed, namely, *mch-5* (5′-GAGGAGCTSTACAAGGAGCTSGGBTA) and *mch-6* (5′-TGSGCCTTGTGYAGYGCBACYTC). PCR products were obtained for *Methylophilus methylotrophus* AS1 (primer combination *mch-4*–*mch-5*, 489-nucleotide product) and *H. methylovorum* GM2 (primer combination *mch-1*–*mch-6*, 264-nucleotide product). Homologous primers were derived from part of *mch* from both organisms: *m1* (5′-GGCCTTGAACACTTCAGC) and *m2* (5′-CAA AGACAGTGCTGATGC), *h1* (5′-GGCGATCTGCACGGTGCC) and *h2* (5′-GTGGAAGACCTCTTCAAGG). With template DNA from *Methylophilus methylotrophus* AS1, *m1* and *mch-2a* were used to amplify an additional PCR fragment. With template DNA from *H. methylovorum* GM2, two additional PCR fragments were cloned, generated with primers *h1* and *mch-4* as well as *h2* and *mch-2b*.

PCR amplifications were carried out at annealing temperatures of 35, 38, and 42°C by using *Taq* polymerase (Boehringer Mannheim). PCR products were

cloned into the pCR2.1 vector with the Invitrogen TA cloning kit and identified by sequencing.

Cloning of *mch* genes. Southern blots indicated the presence of the *mch* gene in chromosomal digests of *Methylobacterium organophilum* XX DNA by using *mch-2* as a heterologous oligonucleotide probe, the presence of the *mch* gene in chromosomal digests of *X. autotrophicus* DNA by using *mch-6* as a heterologous oligonucleotide probe, and the presence of the *mch* gene in chromosomal digests of *Methylosinus trichosporium* OB3b by using *mch-3* as a heterologous oligonucleotide probe. Hybridizations were carried out at 55°C, and washing steps were performed at 55°C and with 5× SSC (0.75 M NaCl plus 0.075 M sodium citrate). For cloning of the *mch* gene from *Methylobacterium organophilum* XX, chromosomal DNA was digested by *Sal*I, eluted from the gel at approximately 2.1 kb (Qiagen gel extraction kit), and ligated to pAYC63 (9) predigested with *Sal*I. For cloning of the *mch* gene from *X. autotrophicus*, an *Sph*I digest was used and DNA at approximately 4 kb was ligated to pAYC63 (9) predigested with the same enzyme. For cloning of the *mch* gene from *Methylosinus trichosporium* OB3b, a *Sal*I digest was used and DNA at approximately 0.9 kb was ligated to pAYC63 (9) predigested with the same enzyme. Positive clones were obtained from the partial clone banks, which were identified by colony hybridizations (37) by using the probes mentioned and by sequencing. The positive clone obtained for *Methylosinus trichosporium* OB3b contained only the 3′ region of the *mch* gene. To obtain the sequence further upstream, the oligonucleotides *ob3b-6* (5′-GGAAT AGATGATCGCGTC) and *ob3b-7* (5′-CAGGCGAGACGTTCCGTGC) were used for inverted PCR. Chromosomal DNA of *Methylosinus trichosporium* OB3b was digested with *Bgl*II and self-ligated. This DNA was used as a template for amplification employing the oligonucleotides *ob3b-6* and *ob3b-7*. The band of interest (approximately 1.8 kb) was identified by Southern hybridization using the oligonucleotide *ob3b-4* (5′-GTCGACGGCATCGCCACG) as a probe and cloned into pCR2.1 (Invitrogen) and sequenced. The insert was shown to contain 449 nucleotides of new sequence.

The complete *mch* gene from *Methylococcus capsulatus* Bath was cloned by using an oligonucleotide probe (*MC-1* 5′-CCACCAAGATCAAGGACGGCAA CG) derived from the sequence of the PCR product obtained as described above. Chromosomal DNA was digested by *Eco*RI, eluted at approximately 1.6 kb, and ligated to pAYC63 (9) predigested with *Eco*RI. The blots were washed at 50°C and with 2× SSC. Positive clones were obtained from the partial *Eco*RI clone bank and identified by colony hybridizations and sequencing. The complete *mch* gene from *Methylobacillus flagellatum* KT was cloned in a similar fashion, from a partial chromosomal library in pAYC63 (9) containing *Sac*I inserts of approximately 2.7 kb. The PCR product described above was used as a probe.

Oligonucleotide labeling, hybridizations, and detections were done with the digoxigenin oligonucleotide tailing kit and by following the protocol provided by Boehringer Mannheim.

DNA sequencing and analysis. DNA sequencing was performed at the sequencing facility of the University of Washington. Nucleotides and inferred peptide sequences were aligned by the Clustal method (DNAStar package) and manually. Dendrograms were constructed by using PUZZLE version 4.0 (42) and the programs from the PHYLIP version 3.5c package (16).

Nucleotide sequence accession numbers. The gene sequences have been deposited in the GenBank database under accession no. AF139592, AF139593, AF142649 to AF142655, and AF162786.

RESULTS

Occurrence of H$_4$MPT-dependent enzyme activities in cell extracts of methylotrophic bacteria. Cell extracts from different methylotrophic bacteria were tested for the presence of methenyl H$_4$MPT cyclohydrolase, NADP-dependent methylene H$_4$MPT dehydrogenase, and NAD-dependent methylene H$_4$MPT dehydrogenase activities, which were shown to involve three different enzymes in *Methylobacterium extorquens* AM1 (31, 47). The results are shown in Table 1. Cells were grown on the C$_1$ substrates indicated. Additionally, most facultative methylotrophic bacteria were grown under a nonmethylotrophic growth condition for induction studies. All of the methanotrophic bacteria tested were found to exhibit H$_4$MPT-dependent enzyme activities in the range of 0.1 to 1.7 U/mg (Table 1). The methanotrophs are either α-proteobacteria containing the serine pathway for assimilation (*Methylosinus trichosporium* OB3b) or γ-proteobacteria containing the ribulose monophosphate pathway (*Methylococcus capsulatus* Bath, *Methylococcus thermophilus* IIIp, *Methylomicrobium album* BG8, and *Methylomonas rubra* 15sh). The methanotrophic bacteria are all obligate methylotrophic bacteria growing only on methane or, in some cases, also on methanol. *Methylomicrobium album* BG8 also exhibited a comparable activity of

TABLE 1. Activities of methenyl H_4MPT cyclohydrolase, NAD-dependent methylene H_4MPT dehydrogenase, and NADP-dependent methylene H_4MPT dehydrogenase in cell extracts of different methylotrophic bacteria

Organism	Major assimilation pathway[a]	Grown on:	Activity (U/mg)[b]		
			Methenyl H_4MPT CH	Methylene H_4MPT DH (NAD)	Methylene H_4MPT DH (NADP)
α-Proteobacteria:					
Methylobacterium extorquens AM1	Serine	Methanol	0.7	0.6	5.1
		Succinate	0.3	0.5	1.0
Methylobacterium organophilum XX	Serine	Methanol	0.3	0.1	1.3
		Succinate	0.1	0.1	0.2
Hyphomicrobium methylovorum GM2	Serine	Methanol	0.4	0.1	1.3
Methylosinus trichosporium OB3b	Serine	Methane	0.7	0.7	1.0
Xanthobacter autotrophicus	CBB	Methanol	0.6	0.1	0.2
		N^h + Methanol	0.3	0.03	0.2
Xanthobacter flavus H4-14	CBB	N + Methanol	0.2	0.2	0.4
Paracoccus denitrificans[c]	CBB	Methanol	<0.01	<0.01	<0.01
Rhodopseudomonas acidophila[d,e]	CBB	Succinate + Methanol	<0.01	<0.01	<0.01
Rhodobacter sphaeroides 2.4.1[f]	CBB	N	<0.01	<0.01	<0.01
β-Proteobacteria:					
Methylobacillus flagellatum KT	RuMP	Methanol	1.0	1.1	1.8
Methylophilus methylotrophus AS1	RuMP	Methanol	0.6	0.2	0.4
γ-Proteobacteria:					
Methylococcus capsulatus Bath	RuMP	Methane	0.7	0.5	1.7
Methylococcus thermophilus IIIp	RuMP	Methane	0.2	0.1	0.3
Methylomicrobium album BG8	RuMP	Methane	0.4	0.3	0.7
Methylomonas rubra 15sh	RuMP	Methane	0.6	0.4	0.6
Gram-positive bacteria:					
Bacillus methanolicus MGA3	RuMP	Methanol	<0.01	<0.01	<0.01
Amycolatopsis methanolica[g]	RuMP	Methanol	<0.01	<0.01	<0.01
		Glucose	<0.01	<0.01	<0.01

[a] CBB, Calvin-Benson-Bassham; RuMP, ribulose monophosphate.
[b] Enzyme activities were measured under standard assay conditions at room temperature. CH, cyclohydrolase; DH, dehydrogenase.
[c] Contains a glutathione-linked formaldehyde dehydrogenase (32).
[d] Contains a glutathione-linked formaldehyde dehydrogenase (36).
[e] Cells were grown anaerobically in the light.
[f] Contains a glutathione-linked formaldehyde dehydrogenase (2, 3).
[g] Contains a mycothiol-linked formaldehyde dehydrogenase (27, 28).
[h] N, nutrient agar.

methenyl H_4MPT cyclohydrolase when growing in the presence of methanol (data not shown). No significant activities (<0.01 U/mg) of a pyridine-dependent methylene H_4F dehydrogenase could be detected in methanotrophic bacteria.

The ratio of NADP- to NAD-dependent H_4MPT dehydrogenase activity in methanotrophic bacteria appears to be more balanced than that in other methylotrophic bacteria. This might reflect the higher demand for NADH for methane monooxygenase, which uses NADH rather than NADPH (12).

Hyphomicrobium methylovorum GM2 and the facultative methylotroph *Methylobacterium organophilum* XX contain the serine pathway for assimilation. Cell extracts of both α-proteobacteria were found to exhibit H_4MPT-dependent enzyme activities. Like *Methylobacterium extorquens* AM1, *Methylobacterium organophilum* XX showed C_1-inducible activities of methenyl H_4MPT cyclohydrolase and NADP-dependent methylene H_4MPT dehydrogenase and no distinct induction of NAD-dependent methylene H_4MPT dehydrogenase (Table 1). The NADP-dependent methylene H_4F dehydrogenase activity in cell extracts of *H. methylovorum* GM2 (0.13 U/mg) and *Methylobacterium organophilum* XX (0.03 U/mg) was found to be very low (see also reference 26).

The β-proteobacteria *Methylobacillus flagellatum* KT and *Methylophilus methylotrophus* AS1 contain the ribulose mono-

phosphate pathway for formaldehyde assimilation and were found to exhibit rather high activities of H_4MPT-dependent enzymes of 0.2 to 1.8 U/mg.

In contrast to these proteobacteria, no H_4MPT-dependent enzyme activities could be detected in the gram-positive methylotrophic bacteria *Amycolatopsis methanolica* and *Bacillus methanolicus* MGA3.

Five autotrophic bacteria were tested for H_4MPT-dependent cyclohydrolase and dehydrogenase activities, *Xanthobacter autotrophicus* and *Xanthobacter flavus* H4-14, *Paracoccus denitrificans*, *Rhodobacter sphaeroides* 2.4.1, and *Rhodopseudomonas acidophila*. The latter organism was grown on methanol anaerobically in the light, while *R. sphaeroides* was grown aerobically on rich medium. Interestingly, it was found that cell extracts of the *Xanthobacter* strains contain H_4MPT-dependent enzyme activities. However, in cell extracts of the other autotrophic bacterial strains tested, these enzyme activities could not be detected. *P. denitrificans*, *Rhodopseudomonas acidophila*, and *Rhodobacter sphaeroides* were previously shown to contain glutathione-dependent formaldehyde dehydrogenase (2, 3, 32, 36), which was measured as a control enzyme (data not shown).

Besides the organisms shown in Table 1, cell extracts of *Shewanella putrefaciens* MR1 were assayed for H_4MPT-depen-

dent enzyme activities. *S. putrefaciens* is a γ-proteobacterium that can grow anaerobically on formate as the sole carbon source and has been shown to contain hydroxypyruvate reductase activity, the key enzyme of the serine pathway (39). The bacterium was not grown on C_1 substrates but on minimal medium supplemented with glucose or in rich media. In cell extracts of *S. putrefaciens*, no H₄MPT-dependent enzyme activities could be detected under these growth conditions and it was not investigated further.

Cloning of methenyl H₄MPT cyclohydrolase genes (*mch*). The known sequences of methenyl H₄MPT cyclohydrolases (Mch) from *Methylobacterium extorquens* AM1 (11), methanogenic archaea (8, 41, 45, 46), and *Archaeoglobus fulgidus* (23) were used to deduce heterologous primers for PCR (for an alignment, see reference 11). PCR products of approximately 430 nucleotides (corresponding to bp 279 to 706 of the *mch* gene from *Methylobacterium extorquens* AM1) were obtained by using template DNA from *Methylococcus capsulatus* Bath, *Methylomonas rubra* 15sh, *Methylococcus thermophilus* IIIp, and *Methylobacillus flagellatum* KT. The PCR products were cloned and sequenced. They showed sequence identities to other Mch proteins, with highest identity to Mch from *Methylobacterium extorquens* AM1. From the newly obtained bacterial Mch sequences, additional heterologous primers were designed and used for PCR. Using these new primers, positive PCR products were obtained for *Methylophilus methylotrophus* AS1 and *H. methylovorum* GM2 (see Materials and Methods).

The *mch* genes of *Methylobacterium organophilum* XX and *X. autotrophicus* were cloned directly from the chromosome as complete genes by using heterologous oligonucleotide probes for Southern hybridization and constructing partial clone banks. The *mch* gene of *Methylosinus trichosporium* OB3b was cloned in part directly from the chromosome by using a heterologous oligonucleotide probe and in part by inverted PCR. For *Methylococcus capsulatus* Bath and *Methylobacillus flagellatum* KT, the complete *mch* genes were cloned and sequenced after the partial sequence was obtained by PCR (see Materials and Methods).

Phylogenetic analysis of Mch sequences. In Fig. 1, an alignment of partial Mch fragments from bacteria and archaea is shown. Approximately 15% of the amino acids are conserved in the region studied for all bacterial and archaeal fragments, while approximately 30% of the amino acids are conserved in all bacterial fragments.

In Fig. 2, the result of a phylogenetic analysis by PUZZLE (42) of Mch fragments, based on the alignment shown in Fig. 1, is given. Phylip (16) was also used for analysis, and it resulted in a similar phylogenetic tree (data not shown). The exact positions of *H. methylovorum* GM2 and *X. autotrophicus* are uncertain according to the programs used. An alignment of the known complete Mch sequences including the five new bacterial sequences reported here is shown in the inset to Fig. 1 and was based on an alignment by the Clustal method (DNAStar). The alignment of Mch sequences as well as the phylogenetic tree shows that all the sequences are related to one another, with a strict separation between the bacterial and archaeal sequences.

The highest degree of divergence within the groups of bacteria analyzed is found among the α-proteobacteria. Although the fragments from the two *Methylobacterium* species show 84% identity, the other pairwise comparisons in this group show only 48 to 63% identity. In contrast, the identities between the fragments for the two β-proteobacteria are 97% and those for the four γ-proteobacteria are 71 to 86%. The lowest sequence identity between different proteobacteria was found to be 48% between an α-proteobacterium (*Methylosinus tricho-*

sporium OB3b) and a γ-proteobacterium (*Methylococcus thermophilus* IIIP) as well as two α-proteobacteria (*Methylosinus trichosporium* OB3b and *H. methylovorum* GM2). Positioning of some α-proteobacterial sequences turned out to be uncertain, consistent with the greater divergence of sequence identities in this group. However, the sequences of the β- and γ-proteobacteria form distinct clusters within the bacterial sequences. Within the group of archaea, sequence identities from 52 to 73% between different species were found. The identity between the bacterial and the archaeal Mch sequences is in the 26 to 42% range.

For those *mch* genes which were cloned from the chromosome, the genomic arrangement was determined and found to be conserved with respect to *Methylobacterium extorquens* AM1 for *M. organophilum* XX and the γ-proteobacterium *Methylococcus capsulatus* Bath. Upstream of *mch*, an as-yet-unidentified but conserved open reading frame designated *orfY* (11) was found with identities only to open reading frames from methanogenic archaea. Downstream of *mch*, an open reading frame (*orf5*) with similarity to that coding for the ribosomal S6 modification protein was found. The gene arrangement is different in *X. autotrophicus* in that *orf5* overlaps *mch* by 64 nucleotides, while upstream of *mch* a gene similar to *mtdA* (10, 47) was found. In *M. extorquens* AM1, *mtdA* was shown to encode the NADP-dependent methylene H₄MPT dehydrogenase and is located approximately 35 kb upstream of *mch*. These results show that the genomic arrangement of genes for H₄MPT-dependent enzymes is not strictly conserved among all methylotrophic proteobacteria. In contrast to the clustered occurrence of genes that are proposed to be involved in a H₄MPT-dependent formaldehyde oxidation pathway in bacteria, in methanogenic archaea and the sulfate-reducing archaeon *Archaeoglobus fulgidus*, those genes are widely scattered over the chromosome.

DISCUSSION

In this report, we demonstrate that the H₄MPT-dependent enzymes involved in formaldehyde oxidation are not restricted to serine cycle methylotrophs like *Methylobacterium* species. These enzymes were also found in the methylotrophic proteobacteria assimilating formaldehyde by the ribulose monophosphate cycle. Even autotrophic *Xanthobacter* strains were found to contain H₄MPT-dependent enzyme activities and the respective genes.

The methylotrophs tested in this study that are known to contain thiol-dependent formaldehyde oxidation systems did not contain detectable levels of H₄MPT-dependent enzyme activities. These include *Rhodopseudomonas acidophila*, *Rhodobacter sphaeroides*, and *Paracoccus denitrificans*, all α-proteobacterial methylotrophs that grow autotrophically on methanol via the Calvin-Benson-Bassham cycle and that contain a glutathione-dependent formaldehyde dehydrogenase (3, 32, 36). In the latter two cases, this enzyme has been shown to be required for methylotrophic growth (3, 32). In addition, the gram-positive methylotroph *Amycolatopsis methanolica* contains a mycothiol-dependent formaldehyde dehydrogenase (27, 28). The other methylotrophs in which H₄MPT-dependent enzyme activities could not be detected have not yet been tested for thiol-dependent formaldehyde oxidation systems, and so the extent of this correlation is not yet known.

Genes similar to the H₄MPT-dependent enzymes were not present in other nonarchaeal microbial genome sequences that are currently available, which include a yeast genome and representatives from 10 of the major branches of the bacteria. Therefore, a H₄MPT-dependent C_1 pathway does not appear
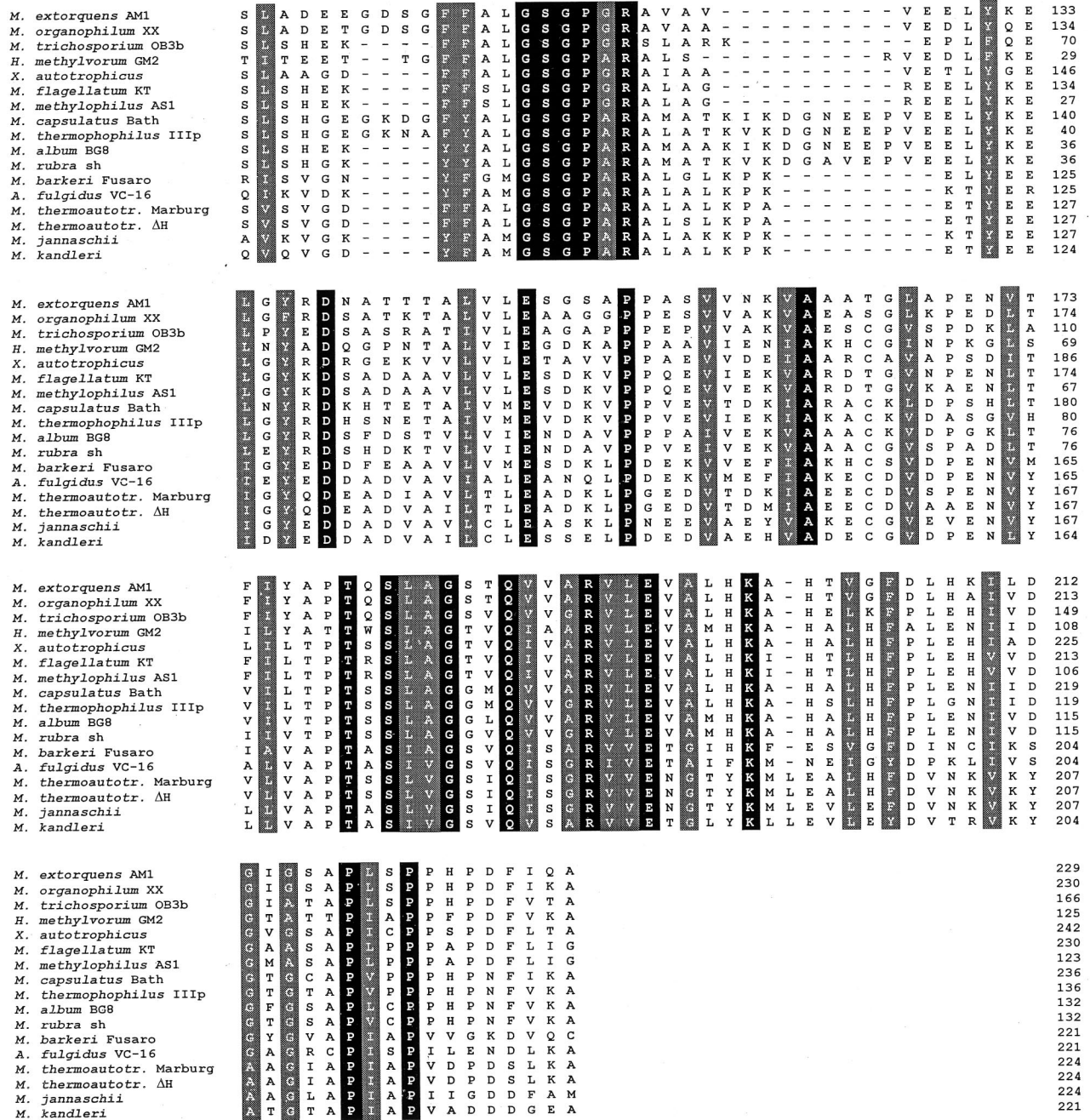
FIG. 1. Alignment of predicted amino acid sequences corresponding to partial *mch* genes from bacteria and archaea by the Clustal method (DNASTAR). Sequences were obtained by cloning the gene directly from the chromosome from *Methylobacterium organophilum* XX, *Xanthobacter autotrophicus*, *Methylobacillus flagellatum* KT, and *Methylococcus capsulatus* Bath. Sequences were retrieved by PCR from *Hyphomicrobium methylovorum* GM2, *Methylophilus methylotrophus* AS1, *Methylococcus thermophilus* IIIp, *Methylomicrobium album* BG8, and *Methylomonas rubra* 15sh. Sequences from *Methylobacterium exotorquens* AM1 (11), *Methanosarcina barkeri* Fusaro (Vaupel and Thauer, EMBL accession no. Y08843), *Archaeoglobus fulgidus* VC-16 (23), *Methanobacterium thermoautotrophicum* Marburg (45), *Methanobacterium thermoautotrophicum* ΔH (41), *Methanococcus jannaschii* (8), and *Methanopyrus kandleri* (46) were known before. Amino acid positions conserved in all bacterial and archaeal sequences are shown in black boxes; positions where chemically similar amino acids were found are shown in grey boxes.

to be widespread in bacteria as a whole but is present in most of the proteobacterial methylotrophs tested. The broad distribution of H_4MPT-dependent enzymes in methylotrophic proteobacteria leads to speculation on the potential function of this pathway. In *Methylobacterium extorquens* AM1, the H_4MPT-dependent formaldehyde oxidation system appears to be the main dissimilatory pathway during growth on C_1 sub-

strates (11, 47). This pathway is indispensable for methylotrophic growth, and the enzymes are present at high activities in methylotrophically grown cells as compared to cells grown on succinate (11). *Methylobacterium extorquens* AM1 also contains a functionally analogous H_4F-linked pathway, albeit with much lower enzyme activities (11). Mutant evidence suggests that in *Methylobacterium extorquens* AM1, both pathways are involved
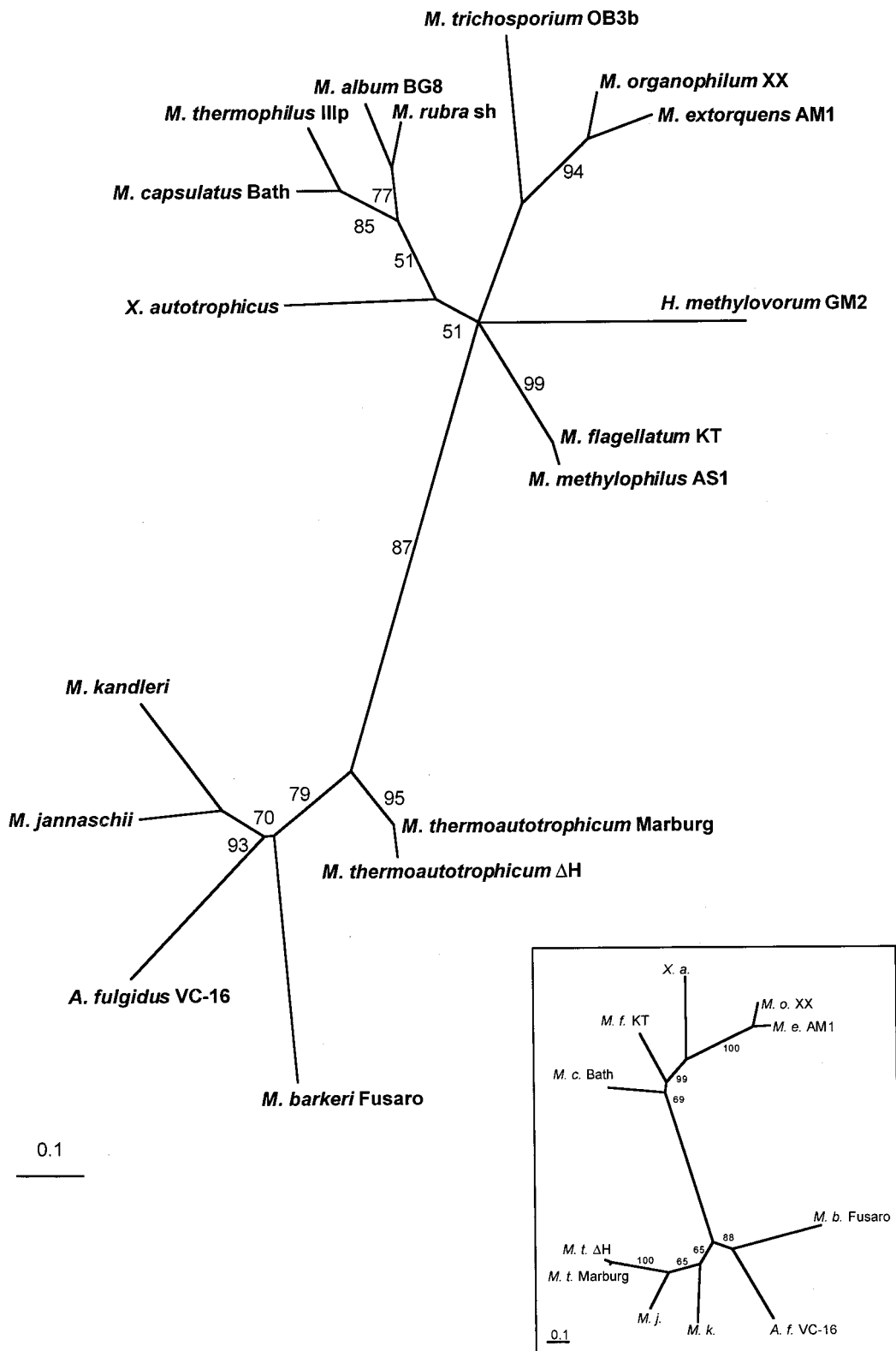
FIG. 2. Phylogenetic analysis of the partial Mch amino acid sequences from bacteria and archaea based on the alignment shown in Fig. 2. The tree shows the result of an analysis with PUZZLE (42); the BOOTSTRAP values above 50% from 1,000 replicates are shown. The bar scale stands for numbers of substitutions per site. The inset shows a phylogenetic analysis from Mch sequences which are known completely.

in methylotrophy (unpublished data), and so the H_4F-linked pathway may also contribute to formaldehyde dissimilation or detoxification. It seems likely that the H_4MPT-dependent formaldehyde oxidation system plays a similar role in other serine cycle methylotrophs and in the *Xanthobacter* strains, since they do not contain sufficiently high activities of enzymes from other potential formaldehyde oxidation pathways to account for their growth rate on C_1 substrates. A similar argument follows for the methanotrophic bacteria containing the ribulose monophosphate cycle, since they also lack sufficient enzyme activities of alternative oxidative pathways (48). However, the presence of the H_4MPT-dependent formaldehyde oxidation system in the β-proteobacteria is surprising, since these bacteria have been proposed to employ a cyclic route of formaldehyde oxidation (1, 4). The results reported here show that like the serine cycle methylotrophs, these ribulose monophosphate cycle methylotrophs contain two possible formaldehyde oxidation pathways. Mutagenesis studies will be necessary to address the role of these two pathways in the β-proteobacterial methylotrophs. The potential role of the H_4MPT-dependent oxidation pathway in formaldehyde dissimilation in methylotrophs is consistent with the apparent absence of this pathway in methylotrophs that contain a thiol-linked formaldehyde oxidation system. These results suggest that methylotrophic bacteria have either one or the other of these formaldehyde oxidation pathways but may also have a second pathway as in the β-proteobacterial methylotrophs (cyclic oxidation pathway) and the serine cycle nonmethanotrophs (H_4F-linked pathway). However, we cannot rule out completely the possibility that the absence of H_4MPT-dependent enzyme activities might be due to the presence of a modified pterin in these methylotrophic bacteria that is not functional with the H_4MPT that was used in this study, which was isolated from the methanogenic archaeon *Methanobacterium thermoautotrophicum*. *Methylobacterium extorquens* AM1 was shown to contain a modified H_4MPT that lacks the α-hydroxyglutaryl phosphate unit, although enzyme activity was detected with both pterins (11, 47).

The data presented here show the presence of functional H_4MPT-dependent enzymes in three branches of the proteobacteria. Phylogenetic analysis revealed that the bacterial and archaeal branches in the tree of *mch* genes are clearly separated but that bacterial and archaeal *mch* genes are clearly related. H_4MPT and H_4MPT-dependent enzymes were previously thought to be a specific attribute of archaea. Their sparse occurrence among the bacteria in general and the proteobacteria in particular raises the question of the evolutionary process underlying this phylogenetic distribution. In principle, it is possible that the common ancestor of archaea and bacteria possessed H_4MPT, Mch, and other H_4MPT-dependent enzymes and that countless parallel losses have occurred in independent lineages. This would mean that these enzymes and their corresponding cofactors were retained throughout the trunk of bacterial and archaeal evolution but have only been retained in selected lineages that use these pathways for C_1 metabolism today.

Alternatively, it is possible that the genes for these enzymes and cofactors have been transferred between bacteria and archaea by horizontal gene transfer (14, 22). The information available at this time is not sufficient to distinguish between these possibilities. However, it is noteworthy that Mch and H_4MPT are ubiquitous among methanogenic archaea, where they are integral to the ecologically specialized energy metabolism of this group, while in contrast, results to date suggest that only some proteobacteria possess this gene and the cofactor. This finding led to the suggestion that proteobacteria may

have acquired Mch and functionally related genes of typically methanogenic C_1 metabolism through lateral transfer from archaeal donors (11). If so, the small sample of sequences studied here would be more supportive of a single rather than multiple transfer events, because the bacterial and archaeal genes are distinct rather than interleaved in the tree. It was previously noted that bacterial Mch is activated 20-fold by 1.2 M potassium phosphate (31). This is an unusual property for α-proteobacterial enzymes but is a property of some enzymes from methanogenic archaea, which require such salt concentrations for activity (40). Activation of Mch by 1.2 M potassium phosphate would be consistent with the hypothesis of lateral transfer from methanogens to proteobacteria. However, at this time we can state with confidence only that the two major lineages of these genes, bacterial and archaeal, show separate evolution.

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