MtdC, a Novel Class of Methylene Tetrahydromethanopterin Dehydrogenases

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Novel methylene tetrahydromethanopterin (H₄MPT) dehydrogenase enzymes, named MtdC, were purified after expressing in *Escherichia coli* genes from, respectively, *Gemmata* sp. strain Wa1-1 and environmental DNA originating from unidentified microbial species. The MtdC enzymes were shown to possess high affinities for methylene-H₄MPT and NADP but low affinities for methylene tetrahydrofolate or NAD. The substrate range and the kinetic properties revealed by MtdC enzymes distinguish them from the previously characterized bacterial methylene-H₄MPT dehydrogenases, MtdA and MtdB. While revealing higher sequence similarity to MtdA enzymes, MtdC enzymes appear to fulfill a function homologous to the function of MtdB, as part of the H₄MPT-linked pathway for formaldehyde oxidation/detoxification.

Formaldehyde detoxification is a metabolic function essential to all of life, due to the extreme toxicity of formaldehyde (4, 12). However, in methylotrophic bacteria formaldehyde oxidation is a part of their central metabolism (1). One of the most widespread modes of formaldehyde oxidation in methylotrophs is the pathway that involves tetrahydromethanopterin (H₄MPT) as a cofactor (28, 30). Most of the enzymes involved in this pathway are homologous to the enzymes involved in methanogenesis by the *Archaea* (6, 9). However, one enzyme in the pathway, an NAD(P)-linked methylene-H₄MPT dehydrogenase (MtdB), is unique to *Bacteria* (14, 15). MtdB has evolved independently of the archaeal functional counterparts that are linked to H₂ or cofactor F₄₂₀ (26), based on the lack of sequence similarity (9, 15). Enzyme properties and mutant analyses have demonstrated that MtdB fulfills a dual physiological role in methylotrophic metabolism, in energy generation (in the form of NADH) and in formaldehyde detoxification (9, 14, 15, 21, 28). In some methylotrophs, so far only in methylotrophs employing the serine cycle for formaldehyde assimilation, a paralog of MtdB is present, an NADP-linked methylene-H₄MPT/methylene-tetrahydrofolate (H₄F) dehydrogenase (MtdA) (7, 10, 28, 29). Experiments with *Methylobacterium extorquens* AM1, including protein purification and analysis, mutant analysis, and flux analysis, all suggested that in vivo, the main function of MtdA is in reducing methenyl-H₄F to methylene-H₄F, which feeds into the serine cycle (7, 23, 25, 28, 29). MtdA also has a secondary function, in general metabolism (purine biosynthesis, etc.) in the serine cycle methylotrophs that do not possess F₄₂₀ (7, 23), the enzyme fulfilling this function in most bacteria and eukaryotes (20). Heterologously expressed *folD* was shown to complement the function of *mtdA* in general metabolism, but not in methylotrophy (23).

The origin and the evolutionary history of MtdA and MtdB remain poorly understood. While MtdA reveals low levels of sequence similarity to F₄₂₀ enzymes (15% identity at the amino acid level [7, 29]), MtdB shares no similarity with F₄₂₀ (15). However, the two paralogs reveal a significant level of similarity to each other (about 30% at the amino acid level), pointing to their common origin (15). In this work we identify and characterize a third class of bacterial methylene H₄MPT dehydrogenases, MtdC, that possess higher sequence similarity toward MtdA but appear to be functional homologs of MtdB.

MATERIALS AND METHODS

Identification of *mtdA*-like genes, sequencing, and sequence analysis. The sequences translated from the *mtdA* and *mtdB* genes of *Methylobacterium extorquens* AM1 (7, 15) were used as queries in BLAST analyses to identify *mtdA*/*mtdB* gene orthologs in the genomes of *Gemmata* sp. strain Wa1-1 (6) and *Methylobium petroleophilum* (http://genome.jgi-psf.org/finished_microbes/metpe/metpe.home.html). A single ortholog was previously identified in the genomes of *Rhodopirellula baltica* and *Gemmata obscuriglobus* (2, 13). A divergent ortholog of *mtdA* (*env97*) was identified in our previous study (16). To obtain the complete sequence of this gene and the surrounding DNA region, we analyzed a metagenomic library of Lake Washington (unpublished results) by PCR with primers specific for *env97* and identified a positive clone containing *env97* (LWBAC10-10). The region surrounding *env97* was sequenced from primers originating in *env97*. Two other clones in the metagenomic library, named LWBAC-L1N9 and LWBAC10-4, were identified by hybridization with, respectively, divergent *fae* and divergent *fhcD* genes identified in our previous studies (16, 17). Two additional divergent *mtdA*-like genes employed in this study were identified via sequencing of the regions surrounding, respectively, the divergent *fae* gene in clone LWBAC-L1N9 and the divergent *fhcD* gene in clone LWBAC10-4.

Mutant complementation. *mtdA*-like genes were PCR amplified from the *Gemmata* sp. strain Wa1-1 chromosome and from the DNA of the fosmid clone LWBAC10-10 and cloned into the *Methylobacterium* expression vector pCM80, as previously described (22). A *fae* gene was amplified from clone LWBAC-L1N9 and cloned into pCM80 in a similar fashion. The *Escherichia coli* JM109 (Invitrogen) strains harboring the plasmids expressing *mtdA*-like genes were used as donors in matings with both *mtdA* and *mtdB* mutants of *M. extorquens* (15, 23), as previously described (22). The plasmid expressing *fae* from LWBAC-L1N9 was mated into the *fae* mutant of *M. extorquens* (31) in a similar fashion. Progenies were selected on succinate medium supplemented with 1 mM methanol or on succinate medium with the precaution of avoiding methanol vapors, respectively, as described before (8, 18, 21). The resulting transconjugants were tested

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for growth on succinate, methanol, or succinate in the presence of methanol vapors, as described before, to test for complementation of the specific phenotypes of the *mtdA* and *mtdB* mutants (8, 18, 21).

Expression and purification of recombinant MtdA-like enzymes. *mtdA*-like genes were PCR amplified from chromosomal DNA of *Gemmata* sp. strain Wa1-1 and from the DNA of clones LWBAC-L1N9 and LWBAC10-10. The following primers were employed: Gem-NdeF (5'-GGAATTCATATGTCCG AAAAACCACGATCC-3') and Gem-XhoR (5'-CCGCTCGAGCGGGAGC TGCAGCGCAGACTGTAC-3') to amplify the *mtdA* ortholog from *Gemmata* sp. strain Wa1-1; L1N9-NdeF (5'-GGAATTCATATGCGACCGCTTCTCCT GCAG-3') and L1N9-NotR (5'-ATAAGAATGCGGCCGCCACCATCCCC GCGGATATC-3') to amplify the *mtdA* ortholog from clone LWBAC-L1N9; and LWBAC10-NdeF (5'-GGAATTCATATGCGAACACTTCTTCTCC-3') and LWBAC10-NotR (5'-ATAAGAATGCGGCCGCCCTCGGGGCGGAG ACAGCGTG-3') to amplify the *mtdA* ortholog from clone LWBAC10-10. The PCR product amplified from *Gemmata* sp. strain Wa1-1 was digested with NdeI and XhoI and ligated into the pET15b expression vector (Novagen). The PCR products amplified from clones LWBAC-L1N9 and LWBAC10-10 were digested with NdeI and NotI and ligated into pET24a (Novagen). The resulting constructs were transformed into *E. coli* JM109. Sequencing of the cloned inserts revealed that no sequence errors were generated during PCR, and these constructs were used to transform *E. coli* BL21(DE3) (Novagen). The construct containing the *mtdA* ortholog amplified from clone LWBAC-L1N9 resulted in insoluble expressed protein (data not shown). Thus, this insert was cloned into the pET15b vector using NdeI/XhoI restriction sites, and the resulting construct was transformed into *E. coli*. Cells were grown to an optical density at 600 nm of approximately 0.6 and then induced by 1 mM isopropyl- β -thiogalactoside, followed by additional 3-hour incubation at 24°C (optical density at 600 nm of 1.0 to 1.2). MtdA-like proteins were purified via three chromatographic steps as follows. Frozen cells were resuspended in 50 mM sodium phosphate (pH 8.0) buffer containing 0.5 M NaCl, 10 mM imidazole, and 10 mM β -mercaptoethanol and passed two times through a French pressure cell at 1.2×10^8 Pa. Centrifugation was performed at $15,000 \times g$ for 30 min at 4°C to remove cell debris. The supernatants (25 ml) were mixed with 1 ml Ni-nitrilotriacetic acid-agarose, incubated for 1 h on ice, and purified using a QIAexpress type IV kit as described by the manufacturer (QIAGEN). Proteins were eluted with 5 ml of 250 mM imidazole, 0.5 M NaCl, 1 mM dithiothreitol, and dialyzed against 50 mM HEPES buffer, pH 8.0 (LWBAC-L1N9 and LWBAC10-10) or pH 7.5 (*Gemmata* sp. strain Wa1-1). The dialyzed extracts were applied onto 1-ml HiTrap DEAE-FF columns (Amersham Biosciences). LWBAC-L1N9 and LWBAC10-10 proteins were eluted with 1 M NaCl in HEPES (pH 8.0). The *Gemmata* sp. strain Wa1-1 protein was collected in the flowthrough fractions, which were pooled, dialyzed against 50 mM bis-Tris pH 5.0 buffer, and subjected to cation-exchange chromatography on a 1-ml HiTrap SP FF column (Amersham Biosciences) equilibrated with the same buffer. The protein was eluted with 1 M NaCl. The fractions containing MtdA-like proteins were pooled, desalted, and concentrated using Amicon Ultra-4 centrifugal filter devices (Millipore), replacing the respective buffers with 25 mM HEPES (pH 7.5), 0.5 M NaCl, 2 mM dithiothreitol, and 5% glycerol. Preparations were frozen in liquid nitrogen and stored at -80°C. Preparations used for determination of kinetic parameters of the enzymes contained at least 95% pure MtdA-like enzymes (Fig. 1). In addition, enzyme activities were tested in pure protein preparations obtained after an additional purification step, size-exclusion chromatography using HiLoad 26/60 Superdex 75 (Amersham Biosciences), to ensure that the H₄F-linked activity was not a result of the presence of contaminating FoLD (data not shown).

Coenzymes, enzyme assays, and kinetic analysis. H₄MPT was purified from *Methanobacterium thermoautotrophicum* Marburg (3) and was a gift of R. K. Thauer. H₄F was purchased from Sigma. The anoxic 120 mM potassium phosphate buffer (pH 6.0) was prepared as follows. Buffer was boiled, and dithiothreitol was added (final concentration, 2 mM). The gas phase over the buffer was then changed to nitrogen. The anoxic buffer was used to make stock solutions of H₄MPT and H₄F to prevent the preparations from oxygen damage. Assays were performed routinely at room temperature in 1-ml cuvettes (depth, 1 cm) in a total volume of 0.7 ml (this volume was used due to limited amounts of H₄MPT available). The reactions were monitored spectrophotometrically by measuring the increase in absorbance at 340 nm. For the calculations, ϵ_{340} values of $27 \text{ mM}^{-1} \text{ cm}^{-1}$ for methylene H₄MPT dehydrogenation with NAD(P) and $27.9 \text{ mM}^{-1} \text{ cm}^{-1}$ for methylene H₄F dehydrogenation with NAD(P) were used (29). Units of enzyme activities are defined as 1 $\mu\text{mol/min}$ per mg of protein at room temperature. Methylene H₄MPT dehydrogenation with NAD(P) was measured in 120 mM potassium phosphate, pH 6.0, at a methylene H₄MPT concentration of 40 μM and an NAD(P) concentration of 0.3 mM. Methylene H₄F was measured in the same way, whereby the concentration of methylene H₄F was 70

FIG. 1. Electrophoresis of purified MtdC enzymes expressed from *Gemmata* sp. strain Wa1-1 (lane 1), clone LWBAC-L1N9 (lane 2), and clone LWBAC10-10 (lane 3) in a gradient (4 to 20%) sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad). Proteins were stained with Coomassie brilliant blue R250 (Amersham Biosciences). Bands of high molecular weights are minor contaminants. Bands below the MtdC bands are products of degradation. Lane MW, Bio-Rad kaleidoscope prestained standards.

μM . Methylene H₄F and methylene H₄MPT were generated by spontaneous reaction of formaldehyde (3 mM) with H₄F and H₄MPT, respectively. K_m and V_{max} values were deduced from reciprocal plots of the initial rates versus the concentration of one substrate at different fixed concentrations of the second substrate.

Protein alignment and phylogenetic analysis. Translated amino acid sequences were aligned using the ClustalW program (27). For phylogenetic analyses, the Phylip package (11) was used. Maximum likelihood, distance, and parsimony methods were employed, and 1,000 bootstrap analyses were performed.

Nucleotide sequence accession numbers. The sequences of the fosmid inserts used in this study have been deposited with GenBank under accession numbers DQ084247, DQ084250 and DQ084248.

RESULTS

Identification of novel *mtdA* gene orthologs. Planctomycetes have recently emerged as the third major microbial group to possess the genes encoding reactions of the H₄MPT-linked C₁ transfer pathway (2, 6, 13). Analysis of 16 genes conserved between *Bacteria* and *Archaea* has revealed the deeply branching nature of the planctomycete sequences, pointing toward the antiquity of this group and to the long evolutionary history of the C₁ reactions linked to H₄MPT (2, 6). In this work we analyzed the *mtdA* gene orthologs in the three available planctomycete genomes. A single ortholog was identified in each genome via BLASTP analysis using either MtdB or MtdA sequences as queries. When compared to the data in the non-redundant sequence database (NCBI), translated orthologs from planctomycetes revealed significant divergence from both MtdA and MtdB polypeptides but possessed higher similarity to the former (43 to 53% and 28 to 32% at the amino acid level, respectively). This finding was surprising, as in methylotrophic *Proteobacteria* MtdB has been shown to serve as the main enzyme in H₄MPT-linked C₁ transfer (9, 14, 15, 21, 28), while the major function of MtdA seems to be in providing methylene-H₄F to the serine cycle (24), in addition to its function in general metabolism (7, 23). The presence of a single

FIG. 2. Phylogenetic analysis of MtdA, MtdB, and MtdC polypeptides. The Phylip package (11) was used for this analysis. The bootstrap values (percentages) are shown for maximum likelihood/distance/parsimony analyses. One thousand bootstrap analyses were run for each method.

ortholog in planctomycete genomes and the presence of traditional *fold* orthologs in these genomes (reference 13 and unpublished data) suggested that the function of the MtdA protein orthologs in planctomycetes could be more similar to the function of MtdB than to the function of MtdA. Gene orthologs significantly diverging from both *mtdB* and *mtdA* were also detected in environmental samples from Lake Washington via direct PCR amplification (16) or via metagenome analysis, as described in Materials and Methods. Phylogenetically, *mtdA* orthologs from planctomycetes and *mtdA* orthologs from uncharacterized microbes fell into two distinct groups that were clearly separated from both MtdA and MtdB enzymes (Fig. 2).

Mutant complementation tests. Mutants of *M. extorquens* that contain lesions in, respectively, *mtdB* and *mtdA* possess characteristic phenotypes: an *mtdB* mutant is negative for growth on methanol and is highly sensitive to methanol vapors (15, 21), while an *mtdA* mutant is methanol negative and requires an added C_1 substrate (methanol or formate) for growth on succinate but is not sensitive to methanol vapors (7, 23). To test for the potential of the proteins encoded by the *mtdA* orthologs to fulfill either MtdB or MtdA function, genes from *Gemmata* sp. strain Wa1-1 and from one environmental clone (LWBAC10-10) were overexpressed in these mutants, and the transconjugants were checked for phenotypic complementation. Neither of the novel genes could complement either *mtdA* or *mtdB* mutants. For comparison, expression of other genes of *Gemmata* sp. strain Wa1-1 (6) or the divergent *fae* from clone LWBAC-L1N9 (this work) using the same expression system resulted in complementation of the respective mutants of *M. extorquens*. We tested whether the failure in complementation was caused by the lack of expression in the system used or by

different substrate specificities of the enzymes encoded by the novel genes. Activities were measured in cell extracts of *E. coli* and in *M. extorquens* MtdA and MtdB mutant backgrounds, using different combinations of cofactors. Considerable activity of methylene- H_4 MPT dehydrogenase with NADP as a cofactor was measured in *E. coli* (0.6 U/mg for the LWBAC10-10 gene and 0.9 U/mg for the *Gemmata* sp. strain Wa1-1 gene), while none was measured with NAD as a cofactor. The LWBAC10-10 gene was responsible for an increase over the background in the methylene- H_4 F dehydrogenase (NADP) activity (0.01 U/mg), but no activity over the background was detected with the *Gemmata* sp. strain Wa1-1 gene construct. Similarly, an increase in the NADP-linked methylene- H_4 MPT dehydrogenase activity was observed in cell extracts of *M. extorquens* background (up to 0.1/mg U for the LWBAC10-10 gene and up to 5.2 U/mg for the *Gemmata* sp. strain WA-1-1 gene). None to a very low activity increase was observed with NAD as a cofactor (<0.001/mg and 0.003 U/mg, respectively), and only a small increase of NADP-dependent methylene- H_4 F dehydrogenase activity of 0.002 U/mg was observed upon complementation with the *Gemmata* sp. strain WA-1-1 gene, while no activity was detected with the LWBAC10-10 gene. These data point toward the novel enzymes having substrate specificities differing from those of MtdA or MtdB. Likely, these differences and not the lack of expression were responsible for negative complementation results.

Substrate specificity and kinetic parameters of the novel enzymes. We expressed in *E. coli* three novel MtdA orthologs, one from *Gemmata* sp. strain Wa1-1 and two from the yet-unidentified species present in Lake Washington. Each was purified and tested for the substrate specificity and kinetic

TABLE 1. K_m and V_{max} values and V_{max}/K_m ratios for MtdA, MtdB, and MtdC enzymes

Enzyme	Source	Substrate	K_m (mM)	Cofactor	K_m (mM)	V_{max} (U mg ⁻¹)	V_{max}/K_m
MtdA ^a	<i>M. extorquens</i>	Methylene-H ₄ MPT	0.02	NADP ⁺	0.03	600	20,000
		Methylene-H ₄ MPT		NAD ⁺			
		Methylene-H ₄ F	0.03	NADP ⁺	0.01	30	3,000
MtdB ^b	<i>M. extorquens</i>	Methylene-H ₄ F		NAD ⁺			
		Methylene-H ₄ MPT	0.10	NADP ⁺	0.02	950	47,500
		Methylene-H ₄ MPT	0.05	NAD ⁺	0.20	1100	5,500
		Methylene-H ₄ F		NADP ⁺			
		Methylene-H ₄ F		NAD ⁺			
MtdC	<i>Gemmata</i> sp. strain Wal-1	Methylene-H ₄ MPT	0.02	NADP ⁺	0.08	130	1,650
		Methylene-H ₄ MPT	0.07	NAD ⁺	2.2	0.3	0.1
		Methylene-H ₄ F	1.1	NADP ⁺	0.30	2	6.7
		Methylene-H ₄ F		NAD ⁺			
MtdC	LWBAC10-10	Methylene-H ₄ MPT	0.02	NADP ⁺	0.05	200	4,000
		Methylene-H ₄ MPT	0.04	NAD ⁺	5.0	1	0.2
		Methylene-H ₄ F	0.39	NADP ⁺	0.06	2	33
		Methylene-H ₄ F		NAD ⁺			
MtdC	LWBAC-LIN9	Methylene-H ₄ MPT	0.02	NADP ⁺	0.005	130	26,000
		Methylene-H ₄ MPT	0.11	NAD ⁺	1.7	180	106
		Methylene-H ₄ F	0.71	NADP ⁺	0.05	1	20
		Methylene-H ₄ F		NAD ⁺			

^a Values are from reference 29.^b Values are from reference 15. Activity measurements were performed at 30°C for MtdA and MtdB and at 20°C for MtdC enzymes.

properties. The purified enzymes were highly active in catalyzing the methylene-H₄MPT dehydrogenase reaction using NADP as a cofactor but were much less efficient in catalyzing methylene-H₄MPT dehydrogenation with NAD (Table 1). They also revealed low efficiency in catalyzing the reaction of dehydrogenation of methylene-H₄F with NADP as a cofactor (Table 1). The kinetic parameters (K_m and V_{max}) were determined for the new enzymes for different combinations of cofactors, and these, along with the V_{max}/K_m ratios (the indicator of enzyme efficiency), are shown in Table 1 in comparison to the values previously reported for MtdA and MtdB enzymes from *M. extorquens*. Based on these values, the catalytic properties of the novel enzymes are different from the properties of the previously characterized MtdA and MtdB enzymes. While the K_m and V_{max} values varied significantly between the three new enzymes, all three possessed much lower efficiencies in catalyzing NAD-linked dehydrogenation of methylene-H₄MPT (52- to 55,000-fold lower than for MtdB), and they showed very low efficiencies in catalyzing NADP-linked dehydrogenation of methylene-H₄F (91- to 448-fold lower than for MtdA). The novel enzymes thus differed from MtdA and MtdB enzymes by their broader substrate specificities. While MtdA revealed no detectable affinity for NAD and MtdB revealed no detectable affinity for H₄F, the novel enzymes revealed affinities for NAD, NADP, H₄F, and H₄MPT, with the highest affinity for the H₄MPT/NADP couple. It seems likely that the latter represents the physiologically relevant combination of cofactors for these enzymes, in agreement with the failure to complement either *mtdA* or *mtdB* mutants (see above). Based on the properties distinguishing them from both MtdA and MtdB, we named the new enzymes MtdC. As is the case with MtdA and MtdB, MtdC enzymes did not catalyze detectable dehydrogenation of methylene-H₄F with NAD.

Active site conservation. The sequences of the newly characterized MtdC enzymes, as well as representative sequences of MtdB enzymes from proteobacteria, were aligned with the

sequence of MtdA from *M. extorquens*, whose crystal structure has been determined with and without bound NADP (10). In general, the MtdA active site residues showed more conservation with MtdC than with MtdB. For example, arginine 152 of MtdA, which is involved in binding of the 2' phosphate of NADP, is conserved in MtdC but not in MtdB (data not shown). This may contribute to the strong preference of MtdC enzymes for NADP as opposed to NAD. In MtdB enzymes, the arginine is replaced by a histidine, which likely enables interaction with both NADP and NAD. Since no crystal structure of MtdA has been determined with H₄MPT bound, the amino acid residues involved in H₄F versus H₄MPT specificity remain unknown.

DISCUSSION

Here we describe a novel class of bacterial methylene-H₄MPT dehydrogenases, named MtdC, possessing high specificity toward methylene-H₄MPT and NADP. This specificity distinguishes MtdCs from the previously characterized bacterial methylene-H₄MPT dehydrogenases, MtdB and MtdA, with MtdB being specific to methylene-H₄MPT in combination with either NAD or NADP and MtdA being specific to NADP in combination with either H₄MPT or H₄F. So far, genes encoding MtdA, whose main function in *M. extorquens* is in supplying methylene-H₄F into the serine cycle (25), have only been found in bacterial genomes encoding the serine cycle enzymes (*Methylobacterium* species *M. capsulatus* and *M. petroleophilum*) (7, 19, 32; this study). Moreover, in those genomes, *mtdA* genes are physically linked to *fch* genes encoding methenyl-H₄F cyclohydrolase and, in *Methylobacterium* species and *M. petroleophilum*, these are parts of larger serine cycle gene clusters (5, 19, 32; this study). On the contrary, genes encoding MtdB, whose function in *M. extorquens* is in the oxidation of methylene-H₄MPT with energy generation and also in formaldehyde detoxification (9, 15, 21, 28), are widely

FIG. 3. Chromosomal location of *mtdC* genes. *fae*, *fhcD*, *orf9*, and *orfY* are all involved in the H₄MPT-linked C₁ transfer pathway (5, 8). Open reading frames with no designation indicate hypothetical genes.

distributed within the *Proteobacteria* possessing the H₄MPT-linked formaldehyde oxidation pathway (16, 18), and in many cases they are physically linked to other genes specifically involved in the pathway (5, 18, 19, 24).

MtdC genes so far have been found in planctomycetes (2, 6, 13; this study) and in yet-unidentified species residing in Lake Washington. Sequencing of fosmid inserts in clones LWBAC-L1N9, LWBAC10-4, and LWBAC10-10 (25, 40, and 34 kbp, respectively) revealed a low degree of similarity between the genes on the fosmids and the sequences of known microbes (data not shown), suggesting that these species may represent deeply branching phyla as well. In all known cases, *mtdC* genes are linked on the chromosomes to other genes involved in the H₄MPT-linked formaldehyde oxidation pathway and most consistently to *fae*, the gene encoding a reaction preceding the methylene-H₄MPT dehydrogenase reaction (31) (Fig. 3). On the other hand, no homologs for the serine cycle genes were found in the planctomycete genomes. These data point toward MtdC being a part of the H₄MPT-linked formaldehyde oxidation/detoxification pathway, i.e., fulfilling a function homologous to the function of MtdB, with which it shares lower sequence similarity, as opposed to the function of MtdA, with which it shares higher similarity (Fig. 2). Confirmation of this hypothesis will require mutant analysis, which is not feasible at this time either with planctomycetes or of course with the uncultured environmental species.

Compared to MtdA and MtdB, MtdC enzymes reveal broader substrate specificity. This substrate "promiscuity" is consistent with an ancestral role of MtdC with respect to both MtdA and MtdB. Testing this hypothesis will require identification of more divergent *mtdA/B* sequences and possibly identification of species carrying *mtdC* in combination with *mtdA* or *mtdB*.

In conclusion, we here describe a fifth class of microbial methylene-H₄MPT dehydrogenases, of which two are found in the *Archaea* (Mtd and Hmd) and three are found in the *Bacteria* (MtdA, MtdB, and MtdC). While archaeal and bacterial analogs are not evolutionarily related, the three bacterial enzymes must originate from a common ancestor.

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