Characterization of Two Methanopterin Biosynthesis Mutants of *Methylobacterium extorquens* AM1 by Use of a Tetrahydromethanopterin Bioassay†

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An enzymatic assay was developed to measure tetrahydromethanopterin (H₄MPT) levels in wild-type and mutant cells of *Methylobacterium extorquens* AM1. H₄MPT was detectable in wild-type cells but not in strains with a mutation of either the *orf4* or the *dmrA* gene, suggesting a role for these two genes in H₄MPT biosynthesis. The protein encoded by *orf4* catalyzed the reaction of ribofuranosylaminobenzene 5′-phosphate synthase, the first committed step of H₄MPT biosynthesis. These results provide the first biochemical evidence for H₄MPT biosynthesis genes in bacteria.

*Methylobacterium extorquens* AM1 is a facultative methylotrophic bacterium capable of growth on succinate and one-carbon (C₁) compounds. Growth on C₁ compounds requires several clusters of genes found on the chromosomal DNA (5, 6), and a number of these genes code for enzymes which have archaeal homologs that depend on tetrahydromethanopterin (H₄MPT) or structurally related coenzymes (6, 7, 24, 25). Previously, these coenzymes had been found only in methanogenic or hyperthermophilic sulfur-dependent archaea (9, 19, 22, 29, 32).

*M. extorquens* cells contain a form of H₄MPT called dephospho-H₄MPT (7). Although it has been assumed that this bacterium produces dephospho-H₄MPT biosynthetic enzymes, these proteins have not yet been identified, and their evolutionary relationship to archaeal enzymes is unknown. In archaea, the genes encoding only 4 of the 18 putative H₄MPT biosynthesis enzymes have been identified (14, 15, 28, 33, 34). One of these enzymes, ribofuranosylaminobenzene 5′-phosphate (RFAP) synthase, catalyzes the first committed step of H₄MPT biosynthesis (26, 28). In *M. extorquens*, a gene encoding an RFAP synthase homolog (*orf4*, also called *mpG*) has been found clustered among several genes encoding H₄MPT-dependent enzymes (6, 7). The *orf4* gene product is 29% identical to RFAP synthase from *Archaeoglobus fulgidus* (28). The protein encoded by a second putative H₄MPT biosynthesis gene (*dmrA*) shows homology to bacterial dihydrofolate reductase and has been proposed by Marx et al. (21) to encode dihydrofolate reductase, which would catalyze the first committed step of H₄MPT biosynthesis. The *dmrA* mutant cannot grow on C₁ compounds and exhibits a methanol- and formaldehyde-sensitive phenotype characteristic of mutants deficient in H₄MPT-dependent metabolism.

To test the hypotheses that *orf4* and *dmrA* encode H₄MPT biosynthesis enzymes, we have developed an enzymatic assay to measure H₄MPT levels in *M. extorquens* mutants. The assay is based on the NAD⁺-reducing activity of methylene-H₄MPT dehydrogenase B (MtdB) (16) (Fig. 1). Here, we provide the initial biochemical evidence for two H₄MPT biosynthetic genes in *M. extorquens* and demonstrate that the protein encoded by *orf4* has RFAP synthase activity.

**Methods.** *Methanosarcina thermophila* cells were grown anaerobically on acetate as previously described (28). *M. extorquens* AM1 wild-type and mutant strains were generously provided by the laboratory of Mary Lidstrom. It has previously been shown that the *orf4*, *dmrA*, and *fae* mutants are unable to grow on methanol and that complementation of each mutant with the corresponding plasmid-borne gene restores the wild-type phenotype, indicating that the mutant phenotype is not due to a polar effect (7, 21, 31). Wild-type *M. extorquens* cells were grown at 30°C on modified minimal medium at pH 7.0 with 20 mM succinate or 0.5% (vol/vol) methanol as previously described (1) except that the concentration of CaCl₂·2H₂O was 2.5 mg per liter. *M. extorquens* AM1 is naturally resistant to rifamycin, which was routinely added to wild-type and mutant cultures at 50 μg per ml to prevent contamination by other microorganisms. Cultures of the *orf4*, *dmrA*, and *fae* mutants were grown on succinate, rifamycin, and kanamycin (50 μg per ml). When the cultures reached an optical density at 600 nm (OD₆₀₀) of 0.6, either 10 ml of 1 M succinate (pH 7.0) or 5 ml of 100% methanol was added. At an OD₆₀₀ between 0.8 and 1.0, the cells were harvested by centrifugation and washed with 50 mM TES [tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (pH 7.0; Fisher Scientific, Suwanee, Ga.), 10 mM...
FIG. 1. Reaction of MtdB. The R group represents the side chain of H₄MPT, which consists of ribitol, ribofuranosyl phosphate, and hydroxylglutaryl groups. H₄SPT from *Methanosarcina thermophila* contains an additional glutamate residue, while dephospho-H₄MPT from *M. extorquens* lacks the phosphate and hydroxylglutaryl groups of H₄MPT. Formaldehyde addition can occur nonenzymatically; however, in cells of *M. extorquens*, the reaction is catalyzed enzymatically by the formaldehyde-activating enzyme (31).

MgCl₂, and either 10 mM succinate (for cells grown on succinate) or 1% methanol. Cells were stored in liquid N₂.

For high-level expression of MtdB, the *mtdB* gene (16) was amplified by the PCR (27) for cloning into the *NdeI* and *BamHI* sites of pET28b (Novagen, Inc., Madison, Wis.). This vector introduces an N-terminal six-histidine (His₆) tag. The template was plasmid pALS8 (7), and the primers were 5'-G GACGTCCATATGGCCCGCTCGATCTGCACA and 5'-GAAGGATCCCTATCCGGCAGTCTCGAC. After amplification with *Pfu* polymerase (Stratagene, La Jolla, Calif.), the PCR product was purified with a PCR purification kit (QIAGEN, Valencia, Calif.), cut with *NdeI* and *BamHI* (New England Biolabs, Beverly, Mass.), and ligated (T4 DNA ligase; New England Biolabs) into pET28b cut with the same enzymes. The DNA was used to transform electrocompetent *Escherichia coli* DH1. The sequence of the insert was verified by dyeoxy sequencing (27), and the plasmid was transformed into *E. coli* BL21(DE3):RIL cells (Stratagene). The expression cell line was called SW11.

For overproduction of *His₆*-MtdB, SW11 cells were grown in Luria–Bertani medium with kanamycin (50 μg per ml) at 37°C. When cells reached an OD₆₀₀ of 0.8, expression was induced with isopropylthiogalactoside (IPTG; Inalco Pharmaceuticals, San Luis Obispo, Calif.) at 1 mM. Cells were harvested after 40 min. The supernatant (cell extract) was stored in liquid N₂.

For determination of H₄MPT concentrations, M. *extorquens* cell extracts by using ion-exchange and hydrophobic-interaction chromatography in an anaerobic chamber (10). To filtered cell extract (12 to 16 ml), an equal volume of buffer A (50 mM MOPS [pH 6.8], 1 % [vol/vol] 2-mercaptoethanol) was added. The mixture was loaded onto a 1-ml column of DEAE-Sephadex A25-125 (Sigma). Although H₄MPT did not bind to the column, some contaminants bound to the column and were removed. H₄MPT was concentrated on a 0.5-ml Serdolit Pad I column (Serva, Heidelberg, Germany) equilibrated with buffer B (1.4% [vol/vol] formic acid [pH 3], 10 mM 2-mercaptoethanol). The column was washed with 2 ml of buffer B, followed by a methanol gradient of 1 ml each of 15, 25, and 50% (vol/vol) in buffer B. The pH of each fraction was adjusted to 7. Formaldehyde (2 μl of a 37% [vol/vol] solution) was added to 800 μl of the fractions, and the mixtures were incubated at room temperature for 10 min. After the solutions were transferred to a 3-ml glass cuvette, 1.1 ml of assay buffer (120 mM KH₂PO₄ [pH 6.8], 3 mM formaldehyde) and 20 μl of Ni-NTA-purified *His₆*-MtdB were added. The absorbance at 340 nm (λ₅₄₀) was monitored for 25 s, and the reaction was initiated with 100 μl of 2 mM NAD⁺. The amount of NADH produced was estimated by using an extinction coefficient of 340 nm of 6.22 per μM NADH per cm (8).

To prepare samples containing tetrahydrosarcinapterin (H₄SPT) from *Methanosarcina thermophila* TM1, cells (5 g) were sealed in a stoppered serum vial and purged with H₂ gas for 5 min. H₂ treatment was required for the enzymatic reduction of the oxidized forms of sarcinapterin to H₄SPT. Anaerobic acetate buffer (10 ml of 30 mM sodium acetate [pH 4.0], 200 mM 2-mercaptoethanol) was added, and the cells were autoclaved for 15 min. The autoclaved cell extract was centrifuged anaerobically at 13,000 × g for 20 min to remove precipitated proteins. The supernatant containing H₄SPT was stored in anaerobic vials at −80°C. For the measurement of H₄SPT, the assay mixture contained 1.8 ml of assay buffer (120 mM KH₂PO₄ [pH 6.8], 3 mM formaldehyde), 20 μl of Ni-NTA-purified His₆-MtdB, and 100 μl of heat-treated cell extract. The reaction was initiated with 100 μl of 2 mM NAD⁺.

PCR was used to amplify the *orf4* gene from plasmid pALS8 (7). The primers (5'-GATCCATATGGACCCGGCCGAGGTCCCG and 5'-CATGGGATCCCTAAACTTCCGCAA CCGAG; Genosys) introduced a 5' *NdeI* site and a 3' *BamHI* site for cloning into pET15b (Novagen), which provides an anaerobically in a glass vial covered with foil to protect H₄MPT from light inactivation.
H4MPT relative to those of methanogens (7, 13), the assay between H4MPT and tetrahydrofolate in bacterial cells. The enzyme can be used to distinguish conditions were specific for H4MPT and does not react with tetrahydrofolate (16). Thus, the enzyme can be used to distinguish M. extorquens MtdB from other enzymes (21, 28). To test these hypotheses, the enzymatic assay was used to determine whether the His6-MtdB assay, no increase in absorbance was observed (Fig. 2, line 1). No increase in A340 was observed if any of the reaction components (formaldehyde, heated methanogen cell extract, His6-MtdB, and NAD+) were omitted (Fig. 2, lines 2 to 5). These results demonstrate that methylene-H4SPT is a substrate for His6-MtdB and that His6-MtdB can be used to detect H4MPT analogs in cell extracts.

The MtdB assay was then used to measure H4MPT levels in wild-type M. extorquens extracts. Initial attempts to measure H4MPT levels in M. extorquens extracts were unsuccessful due to the high background A340. To decrease the absorbance due to contaminating molecules, H4MPT was partially purified by DEAE-Sephadex and hydrophobic-interaction chromatography. By this procedure, H4MPT was detected at a concentration of 44 μM in wild-type M. extorquens cells grown on methanol (Fig. 3, line 1). When cells were grown on succinate, the H4MPT concentration was about half the level found in methanol-grown cells (Fig. 3, line 2). This result was expected based on the report that H4MPT-dependent cyclohydrolase activity in M. extorquens is lower during growth on succinate than during growth on methanol (30). This finding may indicate that the H4MPT-dependent pathway is inducible during growth on methanol.

Evidence for the role of two genes in bacterial H4MPT biosynthesis. The orf4 and dmrA genes of M. extorquens have previously been proposed to encode bacterial H4MPT biosynthetic enzymes (21, 28). To test these hypotheses, the enzymatic assay was used to determine whether the orf4 and dmrA deletion mutants grown on succinate were capable of producing H4MPT. When the orf4 mutant was tested by using the His6-MtdB assay, no increase in A340 was detected (Fig. 3, line 4), indicating the absence of H4MPT in orf4 mutant extracts. Similarly, no H4MPT was detected in extracts of the dmrA mutants.
RFAP synthase activity of the orf4 gene product. To provide biochemical evidence that the orf4 gene codes for RFAP synthase, we measured the RFAP synthase activity of M. extorquens wild-type and orf4 mutant cells. However, because of the low activity of the enzyme in M. extorquens cell extracts, it was necessary to incubate the assay solutions for an extended time period (16 h) to obtain reliable results. Extracts of wild-type M. extorquens cells contained a low level of RFAP synthase activity (0.49 nmol of RFAP produced in 16 h with 4 mg of protein) (Table 1). This value is about 100 times lower than the specific activity of RFAP synthase in methanogen cells (28). RFAP synthase activity was not observed when the substrate PRPP was omitted from the assay. Furthermore, the RFAP synthase activity of M. extorquens cells was inhibited by a known RFAP synthase inhibitor, p-methylaminobenzoic acid, under conditions that inhibit RFAP synthase from methanogens (26). In contrast, no RFAP synthase activity was detectable in extracts of the orf4 mutant (Table 1).

Attempts to purify RFAP synthase from M. extorquens cells were unsuccessful because of enzyme instability. Therefore, the orf4 gene was cloned into the pET15b vector for expression in E. coli. Initial attempts to express orf4 at 37°C with or without a His6 tag resulted in large amounts of insoluble protein. Both the soluble and the insoluble fractions from the cells lacked RFAP synthase activity (data not shown). A similar difficulty was previously encountered in expressing RFAP synthase from Methanothermobacter thermautotrophicus (2). This problem was overcome by coexpressing the RFAP synthase gene with a plasmid-encoded chaperone at 20°C. Under these same conditions, a small proportion of the His6-Orf4 protein was produced as soluble RFAP synthase. Over a period of 16 h, cell extract (1.5 mg of protein) produced 4.3 nmol of RFAP (Table 1). The His6-Orf4 protein was partially purified (23-fold) by nickel affinity chromatography; however, this proce-

![FIG. 3. Detection of dephospho-H4MPT in extracts of M. extorquens AM1. Cell extracts were prepared and concentrated as described in the text. The assay components were the same as those described in the legend to Fig. 2. Cell extracts were from wild-type AM1 grown on 0.5% methanol (line 1), wild-type AM-1 grown on 20 mM succinate (line 2), fae mutant cells grown on succinate (line 3), orf4 mutant cells grown on succinate (line 4), or dmRA mutant cells grown on succinate (line 5).](http://jb.asm.org/)
dure did not result in pure protein because of the low level of enzyme produced in the soluble form. RFAP synthase activity was undetectable in extracts of cells containing the pET15b vector without orf4. Taken together, these results demonstrate that M. extorquens cells contain RFAP synthase activity and that orf4 functions in H₄MPT biosynthesis as a bacterial RFAP synthase gene.

Discussion. M. extorquens contains several clusters of genes required for C₁ metabolism, including genes that encode homologs of archaeal H₄MPT-dependent and methanofuran-dependent enzymes (6, 7). The functions of many of the C₁ metabolism genes are known, but some have been proposed to play roles in H₄MPT and methanofuran biosynthesis (6). In this work, the production of a His₆-tagged form of MtbB enabled us to develop an enzymatic assay to measure H₄MPT levels in cell extracts and assign H₄MPT biosynthetic functions to two of the uncharacterized C₁ gene products. The orf4 mutant lacked RFAP synthase activity, while the recombinant His₆-Orf4 protein catalyzed the RFAP synthase reaction (Table 1). This is the first biochemical evidence for an RFAP synthase gene outside the archaea. The proposed role of dmra as a dihydromethanopterin reductase (21) is supported by the inability of the dmra mutant to produce H₄MPT (Fig. 3) and by additional evidence obtained in our laboratory that the Dmra protein catalyzes the NAD(P)H-dependent reduction of H₄MPT to H₂MPT (M. A. Caccamo, C. S. Malone, and M. E. Rasche, Abstr. 103rd Gen. Meet. Am. Soc. Microbiol., abstr. K-065, 2003). The His₆-MtbB assay described here will be used to identify additional genes of the H₄MPT biosynthesis pathway in methylo trophic bacteria.

The distribution of H₄MPT-dependent pathways among bacteria and archaea is becoming clearer in light of the many prokaryotic genomes being sequenced. H₄MPT-dependent enzymes have been found in autotrophic Xanthobacter strains, in methanotrophs, and in methylo trophic bacteria that use the serine pathway or the ribulose monophosphate (RuMP) pathway to assimilate formaldehyde (30). Genome sequencing indicates that the aerobic hyperthermophilic archaeon Aeropyrum pernix (182) and other diverse microorganisms contain RFAP synthase homologs (4, 11, 17, 18, 28). These organisms may contain previously unidentified forms of H₄MPT. At least six derivatives of H₄MPT have been characterized by structural analyses (7, 19, 20, 32), and the MtbB enzyme used in this work reacts with at least three of these analogs (H₄MPT from Methanothermobacter marburgensis [16], H₄SPT from Methanosarcina thermophila [Fig. 2], and dephospho-H₄MPT from M. extorquens [Fig. 3]). Thus, the enzymatic assay for H₄MPT may offer a convenient method for detecting previously uncharacterized forms of H₄MPT as well as for identifying the remaining H₄MPT biosynthetic genes of bacteria and archaea.

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