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

Madeline E. Rasche,* Stephanie A. Havemann,‡ and Mariana Rosenzvaig

Microbiology and Cell Science Department, University of Florida, Gainesville, Florida 32611-0700

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An enzymatic assay was developed to measure tetrahydromethanopterin (H\(_4\)MPT) levels in wild-type and mutant cells of *Methylobacterium extorquens* AM1. H\(_4\)MPT 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 H\(_4\)MPT biosynthesis. The protein encoded by *orf4* catalyzed the reaction of ribofuranosylaminobenzene 5'-phosphate synthase, the first committed step of H\(_4\)MPT biosynthesis. These results provide the first biochemical evidence for H\(_4\)MPT biosynthesis genes in bacteria.

*Methylobacterium extorquens* AM1 is a facultative methylotrophic bacterium capable of growth on succinate and one-carbon (C\(_1\)) compounds. Growth on C\(_1\) 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 (H\(_4\)MPT) 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 H\(_4\)MPT called dephospho-H\(_4\)MPT (7). Although it has been assumed that this bacterium produces dephospho-H\(_4\)MPT 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 H\(_4\)MPT 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 H\(_4\)MPT biosynthesis (26, 28). In *M. extorquens*, a gene encoding an RFAP synthase homolog (*orf4*, also called *mptG*) has been found clustered among several genes encoding H\(_4\)MPT-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 H\(_4\)MPT biosynthesis gene (*dmrA*) shows homology to bacterial dihydrofolate reductases and has been proposed by Marx et al. (21) to encode dihydrofolate reductase, which would catalyze the final step of H\(_4\)MPT biosynthesis. The *dmrA* mutant cannot grow on C\(_1\) compounds and exhibits a methanol- and formaldehyde-sensitive phenotype characteristic of mutants deficient in H\(_4\)MPT-dependent metabolism.

To test the hypothesis that *orf4* and *dmrA* encode H\(_4\)MPT biosynthesis enzymes, we have developed an enzymatic assay to measure H\(_4\)MPT levels in *M. extorquens* mutants. The assay is based on the NAD\(^+\)-reducing activity of methylene-H\(_4\)MPT dehydrogenase B (MtdB) (16) (Fig. 1). Here, we provide the initial biochemical evidence for two H\(_4\)MPT 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 CaCl\(_2\)·2H\(_2\)O was 2.5 mg per liter. *M. extorquens* AM1 was 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 (OD\(_{600}\)) of 0.6, either 10 ml of 1 M succinate (pH 7.0) or 5 ml of 100% methanol was added. At an OD\(_{600}\) 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...
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 GACGTCAATATGGCCGTCGATCTGCACA and 5'-GAAGGATCTCTATGGCGCATCTCGAC. 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, Beverly, Mass.) 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 dideoxy 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, St. Louis, Mo.) was added at a ratio of 1 ml of buffer per g of cells. Cells were disrupted anaerobically by two passages through a French pressure cell and centrifuged for 2 h at 27,000 × g for 60 min. The supernatant (cell extract) was stored in anaerobic vials at -80°C. Because H₄MPT is oxygen sensitive, His₆-MtdB was partially purified in an anaerobic chamber by using Ni-nitriloacetic acid (NTA) spin columns (QIAGEN). The protein was eluted with 250 mM imidazole (pH 8.0) according to the manufacturer’s instructions.

For determination of H₄SPT concentrations, M. extorquens cells (10 to 16 g) were thawed in an anaerobic chamber (Coy Products, Inc., Grass Lake, Mich.) containing 2% H₂ and 98% N₂. Breakage buffer (50 mM TES [pH 7.0], 10 mM MgCl₂, 20 mM 2-mercaptoethanol) with DNase I (Sigma Chemical Co., St. Louis, Mo.) was added at a ratio of 1 ml of buffer per g of cells. Cells were disrupted anaerobically by two passages through a French pressure cell and centrifuged for 2 h at 27,000 × g (4°C). The supernatant was filtered through a 0.45-μm-pore-size filter (Millipore, Bedford, Mass.). Proteins were removed by using a Centricron-3 filtration device (Millipore) in the absence of O₂. The filtrate (filtered cell extract) was stored anaerobically in a glass vial covered with foil to protect H₄MPT from light inactivation.

H₄MPT was partially purified from filtered 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 (A₃₄₀) 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 tetrahydroxysarcinapterin (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'-GATCCCATATGGACCGTGCGGCCGAGGTCCCGG and 5'-CATGGGATCCTAAACTTCCGCAA) introduced a 5' NdeI site and a 3' BamHI site for cloning into pET15b (Novagen), which provides an

![Diagram](image-url)
N-terminal His6 tag. The plasmid (pCL1) was transformed into chemically competent DH1 cells, and the sequence of the insert was verified. The plasmid was transformed into BL21(DE3) cells (Novagen) containing the pG-T2 plasmid for expression of a chaperone to assist in protein folding (HSP Research Institute, Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) (23). Expression of the His6-orf4 gene was induced as previously described for the RFAP synthase gene from *Methanothermobacter thermautotrophicus* (2) except that ampicillin (125 μg per ml) was used instead of kanamycin.

RFAP synthase activity was measured as previously described (28) except that the reaction mixtures were incubated for 16 h at 30°C in 50 mM TES (pH 7.0). Protein concentrations were measured by using the Bradford assay (Bio-Rad) (3) with bovine serum albumin as the standard. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue R-250 (Bio-Rad) (12). Phosphoribosylpyrophosphate (PRPP) was obtained from Sigma. All other chemicals were obtained from Fisher Scientific.

**Development of an enzymatic assay to measure H4MPT.** To facilitate the discovery of H4MPT biosynthetic genes, an enzymatic assay was developed to enable the rapid screening of mutants deficient in H4MPT production. In this assay, formaldehyde is added to protein-free cell extracts to chemically convert H4MPT to methylene-H4MPT (Fig. 1). The oxidation of methylene-H4MPT is coupled to the reduction of NAD\(^+\). When H4MPT-dependent cyclohydrolase activity is measured in the presence of NADH, an increase in absorbance at 340 nm is observed (Fig. 1, line 1). In the absence of NADH, no increase in absorbance is observed (Fig. 1, line 2). The MtdB assay was then used to measure H4MPT levels in wild-type *M. extorquens* extracts. Initial attempts to measure H4MPT levels in wild-type *M. extorquens* extracts were unsuccessful due to the high background absorbance in wild-type extracts (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 wild-type *M. extorquens* cells 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 absorbance at 340 nm 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* deletion mutant.
mutant (Fig. 3, line 5). This result is consistent with roles for orf4 and dmrA as H\textsubscript{4}MPT biosynthetic genes.

As an additional control, we measured the level of H\textsubscript{4}MPT in a mutant for a gene that is not involved in H\textsubscript{4}MPT biosynthesis. The fae gene codes for the formaldehyde-activating enzyme (31), which catalyzes the reaction between formaldehyde and H\textsubscript{4}MPT to produce methylene-H\textsubscript{4}MPT. This enzyme is not required for H\textsubscript{4}MPT biosynthesis. As predicted, H\textsubscript{4}MPT was detected in extracts of the fae mutant (Fig. 3, line 3) at about two-thirds the level found in wild-type cells grown on succinate. We suspect that this difference may be due to the inefficiency of the fae mutant in converting formaldehyde and H\textsubscript{4}MPT to methylene-H\textsubscript{4}MPT, the substrate for His\textsubscript{c}-MtbB. In support of this hypothesis, we found that the complete nonenzymatic conversion of formaldehyde and H\textsubscript{4}MPT to methylene-H\textsubscript{4}MPT required 10 min in wild-type cell extracts but 2 h in fae mutant extracts, suggesting that a smaller proportion of the H\textsubscript{4}MPT in fae mutants was originally present as methylene-H\textsubscript{4}MPT.

**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 methanogens (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 His\textsubscript{c} 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 *Methanothermobacterthermautotrophicus* (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 His\textsubscript{c}-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 His\textsubscript{c}-Orf4 protein was partially purified (23-fold) by nickel affinity chromatography; however, this proce-

![Graph](http://jb.asm.org/)

**FIG. 3.** Detection of dephospho-H\textsubscript{4}MPT 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).

<table>
<thead>
<tr>
<th>Cell extract</th>
<th>Amt of protein used in assay (mg)</th>
<th>Amt of RFAP produced (nmol)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. extorquens</em> wild type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ PABA\textsuperscript{a} (6.4 mM), + PRPP (8.8 mM)</td>
<td>4</td>
<td>0.49 ± 0.17</td>
</tr>
<tr>
<td>+ PABA (6.4 mM), without PRPP</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>+ PABA (85 \textmu M), + PRPP (8.8 mM)</td>
<td>6</td>
<td>0.38 ± 0.06</td>
</tr>
<tr>
<td>+ PABA (85 \textmu M), + PRPP (8.8 mM), + p-methylaminobenzoic acid (5 mM)</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td><em>M. extorquens</em> orf4 mutant</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td><em>E. coli</em> BL21(DE3) producing His\textsubscript{c}-Orf4</td>
<td>1.5</td>
<td>4.3 ± 1.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Average ± standard error for three readings. Cell extracts (140 to 180 \mu l) (1.5 to 6 mg of protein) were incubated for 16 h at 30°C. The product (RFAP) was converted to the pink azo-dye derivative, and RFAP synthase activity was measured as described in the text. Samples with activity showed a pink color, while samples with no detectable activity were clear. The spectrophotometric detection limit for the assay was 0.3 nmol of RFAP. ND, none detected.

\textsuperscript{b}PABA, p-aminobenzoic acid.

TABLE 1. RFAP synthase activity of *M. extorquens* AM1 strains and *E. coli* BL21(DE3) producing His\textsubscript{c}-Orf4

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Note: The table provides a summary of RFAP synthase activity in extracts of *M. extorquens* AM1 strains and *E. coli* BL21(DE3) producing His\textsubscript{c}-Orf4, with varying concentrations of PABA and PRPP. The activity is measured in nanomoles (nmol) of RFAP produced. The table includes data for wild-type and orf4 mutant strains, as well as conditions with and without an additional component, PABA, to test its effect on synthase activity. The data is presented as mean values ± standard error, with ND indicating no detectable activity. The assay's detection limit is 0.3 nmol of RFAP. PABA, p-aminobenzoic acid, is added to the reaction mixture to test its role in synthase activity.

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For a comprehensive understanding, please refer to the original text for additional details and context.
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 H2MPT-dependent and methanofuran-dependent enzymes (6, 7). The functions of many of the C1 metabolism genes are unknown, but some have been proposed to play roles in H2MPT and methanofuran biosynthesis (6). In this work, the production of a His6-tagged form of MtdB enabled us to develop an enzymatic assay to measure H2MPT levels in cell extracts and assign H4MPT biosynthetic functions in M. extorquens and the MtdB enzyme used in this work.

**References.**


