JOURNAL OF BACTERIOLOGY, Mar. 2004, p. 1565–1570 Vol. 186, No. 5
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.5.1565–1570.2004
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NOTES

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

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Received 7 July 2003/Accepted 12 November 2003

An enzymatic assay was developed to measure tetrahydromethanopterin (H_4MPT) levels in wild-type and mutant cells of *Methylobacterium extorquens* AM1. H_4MPT 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_4MPT biosynthesis. The protein encoded by *orf4* catalyzed the reaction of ribofuranosylaminobenzene 5′-phosphate synthase, the first committed step of H_4MPT biosynthesis. These results provide the first biochemical evidence for H_4MPT 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_4MPT) 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_4MPT called dephospho-H_4MPT (7). Although it has been assumed that this bacterium produces dephospho-H_4MPT 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_4MPT 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_4MPT 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_4MPT-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_4MPT biosynthesis gene (*dmrA*) shows homology to bacterial dihydrofolate reductases and has been proposed by Marx et al. (21) to encode dihydromethanopterin reductase, which would catalyze the final step of H_4MPT biosynthesis. The *dmrA* mutant cannot grow on C_1 compounds and exhibits a methanol- and formaldehyde-sensitive phenotype characteristic of mutants deficient in H_4MPT-dependent metabolism.

To test the hypotheses that *orf4* and *dmrA* encode H_4MPT biosynthesis enzymes, we have developed an enzymatic assay to measure H_4MPT levels in *M. extorquens* mutants. The assay is based on the NAD^+-reducing activity of methylene-H_4MPT dehydrogenase B (MtdB) (16) (Fig. 1). Here, we provide the initial biochemical evidence for two H_4MPT 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 $CaCl_2 \cdot 2H_2O$ 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 (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

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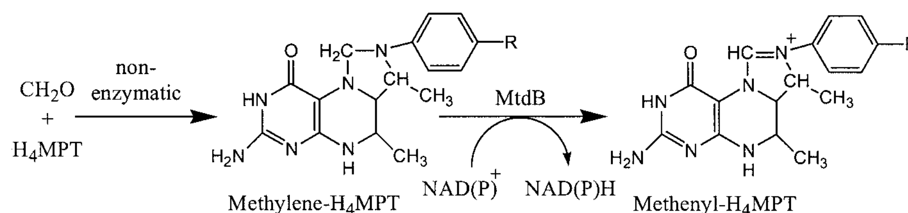

FIG. 1. Reaction of MtdB. The R group represents the side chain of H_4MPT, which consists of ribitol, ribofuranosyl phosphate, and hydroxglutaryl groups. H_4SPT from *Methanosarcina thermophila* contains an additional glutamate residue, while dephospho-H_4MPT from *M. extorquens* lacks the phosphate and hydroxyglutaryl groups of H_4MPT. Formaldehyde addition can occur nonenzymatically; however, in cells of *M. extorquens*, the reaction is catalyzed enzymatically by the formaldehyde-activating enzyme (31).

$MgCl_2$, and either 10 mM succinate (for cells grown on succinate) or 1% methanol. Cells were stored in liquid N_2.

For high-level expression of MtdB, the *mtdB* gene (16) was amplified by the PCR (27) for cloning into the *Nde*I and *Bam*HI sites of pET28b (Novagen, Inc., Madison, Wis.). This vector introduces an N-terminal six-histidine (His_6) tag. The template was plasmid pALS8 (7), and the primers were 5′-G GACGTCCATATGGCCCGCTCGATCCTGCACA and 5′-GAAGGATCCTCATCCGGCGATCTCGAC. After amplification with *Pfu* polymerase (Stratagene, La Jolla, Calif.), the PCR product was purified with a PCR purification kit (QIAGEN, Valencia, Calif.), cut with *Nde*I and *Bam*HI (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 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 His_6-MtdB, SW11 cells were grown in Luria-Bertani medium with kanamycin (50 μg per ml) at 37°C. When cells reached an OD_{600} of 0.8, expression was induced with isopropylthiogalactoside (IPTG; Inalco Pharmaceuticals, San Luis Obispo, Calif.) at 1 mM. Cells were harvested after 3 h, washed with 50 mM MOPS (morpholinepropanesulfonic acid) (pH 7.0) and 10 mM $MgCl_2$, suspended in the same buffer (2 ml of buffer per g of cells), disrupted by French pressure cell lysis at 20,000 lb/in^2, and centrifuged at 27,000 × *g* for 60 min. The supernatant (cell extract) was stored in 400-μl portions at −80°C. Because H_4MPT is oxygen sensitive, His_6-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 H_4MPT concentrations, *M. extorquens* cells (10 to 16 g) were thawed in an anaerobic chamber (Coy Products, Inc., Grass Lake, Mich.) containing 2% H_2 and 98% N_2. Breakage buffer (50 mM TES [pH 7.0], 10 mM $MgCl_2$, 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 Centricon-3 filtration device (Millipore) in the absence of O_2. The filtrate (filtered cell extract) was stored anaerobically in a glass vial covered with foil to protect H_4MPT from light inactivation.

H_4MPT 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 H_4MPT did not bind to the column, some contaminants bound to the column and were removed. H_4MPT 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 KH_2PO_4 [pH 6.8], 3 mM formaldehyde) and 20 μl of Ni-NTA-purified His_6-MtdB were added. The absorbance at 340 nm (A_{340}) 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 at 340 nm of 6.22 per mM NADH per cm (8).

To prepare samples containing tetrahydrosarcinapterin (H_4SPT) from *Methanosarcina thermophila* TM1, cells (5 g) were sealed in a stoppered serum vial and purged with H_2 gas for 5 min. H_2 treatment was required for the enzymatic reduction of the oxidized forms of sarcinapterin to H_4SPT. 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 H_4SPT was stored in anaerobic vials at −80°C. For the measurement of H_4SPT, the assay mixture contained 1.8 ml of assay buffer (120 mM KH_2PO_4 [pH 6.8], 3 mM formaldehyde), 20 μl of Ni-NTA-purified His_6-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′-GATCCATATGAGACCGTGGCCCGA GGTCCCG and 5′-CATGGGATCCCTAAACTTCCGCAA CCGAG; Genosys) introduced a 5′ *Nde*I site and a 3′ *Bam*HI site for cloning into pET15b (Novagen), which provides an

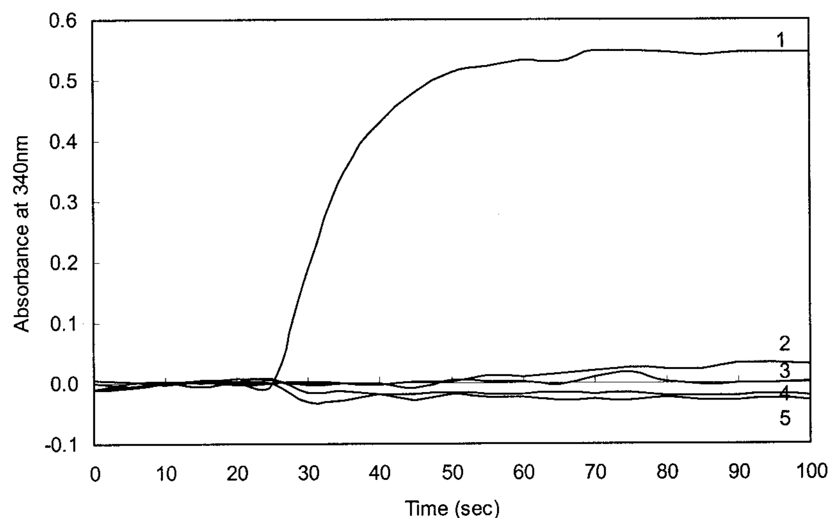

FIG. 2. Detection of H_4MPT in methanogen cell extracts by the MtdB assay. The complete reaction mixture (line 1) contained 120 mM KH_2PO_4 (pH 6.8), 3 mM formaldehyde, 20 μl of Ni-NTA-purified His_6-MtdB (15 μg of protein), and 100 μl of *Methanosarcina thermophila* heat-treated cell extract. After a stable baseline was established at 340 nm for 25 s, the reaction was initiated with 100 μl of 2 mM NAD^+. Control assays contained all of the components except His_6-MtdB (line 2), NAD^+ (line 3), heated cell extract (line 4), or formaldehyde (line 5).

N-terminal His_6 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-Tf2 plasmid for expression of a chaperone to assist in protein folding (HSP Research Institute, Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) (23). Expression of the His_6-*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 H_4MPT. To facilitate the discovery of H_4MPT biosynthetic genes, an enzymatic assay was developed to enable the rapid screening of mutants deficient in H_4MPT production. In this assay, formaldehyde is added to protein-free cell extracts to chemically convert H_4MPT to methylene-H_4MPT (Fig. 1). The oxidation of methylene-H_4MPT is coupled to the reduction of NAD^+ via MtdB from *M. extorquens*, producing an increase in A_{340}. MtdB is highly specific for H_4MPT and does not react with tetrahydrofolate (16). Thus, the enzyme can be used to distinguish between H_4MPT and tetrahydrofolate in bacterial cells. The production of a histidine-tagged version of the enzyme (His_6-MtdB) allowed for the rapid purification of large quantities of the enzyme by nickel affinity chromatography.

Because *M. extorquens* cells contain low concentrations of H_4MPT relative to those of methanogens (7, 13), the assay conditions were first optimized by using extracts of the methanogen *Methanosarcina thermophila*. This organism produces H_4SPT, an H_4MPT analog (20). When H_2-reduced *Methanosarcina thermophila* extracts were heated to remove proteins and combined with formaldehyde, NAD^+, and His_6-MtdB, an increase in A_{340}, corresponding to the production of NADH, was observed (Fig. 2, line 1). No increase in A_{340} was observed if any of the reaction components (formaldehyde, heated methanogen cell extract, His_6-MtdB, and NAD^+) were omitted (Fig. 2, lines 2 to 5). These results demonstrate that methylene-H_4SPT is a substrate for His_6-MtdB and that His_6-MtdB can be used to detect H_4MPT analogs in cell extracts.

The MtdB assay was then used to measure H_4MPT levels in wild-type *M. extorquens* extracts. Initial attempts to measure H_4MPT levels in *M. extorquens* extracts were unsuccessful due to the high background A_{340}. To decrease the absorbance due to contaminating molecules, H_4MPT was partially purified by DEAE-Sephadex and hydrophobic-interaction chromatography. By this procedure, H_4MPT 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 H_4MPT concentration was about half the level found in methanol-grown cells (Fig. 3, line 2). This result was expected based on the report that H_4MPT-dependent cyclohydrolase activity in *M. extorquens* is lower during growth on succinate than during growth on methanol (30). This finding may indicate that the H_4MPT-dependent pathway is inducible during growth on methanol.

Evidence for the role of two genes in bacterial H_4MPT biosynthesis. The *orf4* and *dmrA* genes of *M. extorquens* have previously been proposed to encode bacterial H_4MPT 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 H_4MPT. When the *orf4* mutant was tested by using the His_6-MtdB assay, no increase in A_{340} was detected (Fig. 3, line 4), indicating the absence of H_4MPT in *orf4* mutant extracts. Similarly, no H_4MPT was detected in extracts of the *dmrA*

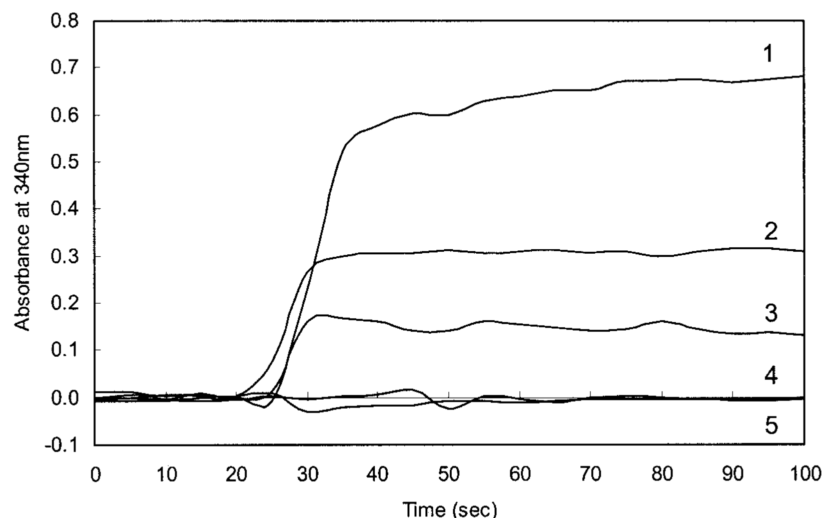

FIG. 3. Detection of dephospho-H_4MPT in extracts of *M. extorquens* AM1. Cell extracts were prepared and concentrated as described in the text. The assay components were the same as those described in the legend to Fig. 2. Cell extracts were from wild-type AM1 grown on 0.5% methanol (line 1), wild-type 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).

mutant (Fig. 3, line 5). This result is consistent with roles for *orf4* and *dmrA* as H_4MPT biosynthetic genes.

As an additional control, we measured the level of H_4MPT in a mutant for a gene that is not involved in H_4MPT biosynthesis. The *fae* gene codes for the formaldehyde-activating enzyme (31), which catalyzes the reaction between formaldehyde and H_4MPT to produce methylene-H_4MPT. This enzyme is not required for H_4MPT biosynthesis. As predicted, H_4MPT 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_4MPT to methylene-H_4MPT, the substrate for His_6-MtdB. In support of this hypothesis, we found that the complete nonenzymatic conversion of formaldehyde and H_4MPT to methylene-H_4MPT required 10 min in wild-type cell extracts but 2 h in *fae* mutant extracts, suggesting that a smaller proportion of the H_4MPT in *fae* mutants was originally present as methylene-H_4MPT.

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 His_6 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 His_6-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_6-Orf4 protein was partially purified (23-fold) by nickel affinity chromatography; however, this proce-

TABLE 1. RFAP synthase activity of *M. extorquens* AM1 strains and *E. coli* BL21(DE3) producing His_6-Orf4

Cell extract	Amt of protein used in assay (mg)	Amt of RFAP produced (nmol)[a]
M. extorquens wild type		
+ PABA[b] (6.4 mM), + PRPP (8.8 mM)	4	0.49 ± 0.17
+ PABA (6.4 mM), without PRPP	4	ND
+ PABA (85 μM), + PRPP (8.8 mM)	6	0.38 ± 0.06
+ PABA (85 μM), + PRPP (8.8 mM), + *p*-methylaminobenzoic acid (5 mM)	6	ND
M. extorquens orf4 mutant	6	ND
E. coli BL21(DE3) producing His_6-Orf4	1.5	4.3 ± 1.7

[a] Average ± standard error for three readings. Cell extracts (140 to 180 μl) (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.

[b] PABA, *p*-aminobenzoic acid.

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 H_4MPT biosynthesis as a bacterial RFAP synthase gene.

Discussion. *M. extorquens* contains several clusters of genes required for C_1 metabolism, including genes that encode homologs of archaeal H_4MPT-dependent and methanofuran-dependent enzymes (6, 7). The functions of many of the C_1 metabolism genes are unknown, but some have been proposed to play roles in H_4MPT and methanofuran biosynthesis (6). In this work, the production of a His_6-tagged form of MtdB enabled us to develop an enzymatic assay to measure H_4MPT levels in cell extracts and assign H_4MPT biosynthetic functions to two of the uncharacterized C_1 gene products. The *orf4* mutant lacked RFAP synthase activity, while the recombinant His_6-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 H_4MPT (Fig. 3) and by additional evidence obtained in our laboratory that the DmrA protein catalyzes the NAD(P)H-dependent reduction of H_2MPT to H_4MPT (M. A. Caccamo, C. S. Malone, and M. E. Rasche, Abstr. 103rd Gen. Meet. Am. Soc. Microbiol., abstr. K-065, 2003). The His_6-MtdB assay described here will be used to identify additional genes of the H_4MPT biosynthesis pathway in methylotrophic bacteria.

The distribution of H_4MPT-dependent pathways among bacteria and archaea is becoming clearer in light of the many prokaryotic genomes being sequenced. H_4MPT-dependent enzymes have been found in autotrophic *Xanthobacter* strains, in methanotrophs, and in methylotrophic 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* and other diverse microorganisms contain RFAP synthase homologs (4, 11, 17, 18, 28). These organisms may contain previously unidentified forms of H_4MPT. At least six derivatives of H_4MPT have been characterized by structural analyses (7, 19, 20, 32), and the MtdB enzyme used in this work reacts with at least three of these analogs (H_4MPT from *Methanothermobacter marburgensis* [16], H_4SPT from *Methanosarcina thermophila* [Fig. 2], and dephospho-H_4MPT from *M. extorquens* [Fig. 3]). Thus, the enzymatic assay for H_4MPT may offer a convenient method for detecting previously uncharacterized forms of H_4MPT as well as for identifying the remaining H_4MPT biosynthetic genes of bacteria and archaea.

We are grateful to Mary Lidstrom, Ludmila Chistoserdova, and Christopher Marx for their generosity in sharing the plasmid pALS8 and the *M. extorquens* wild-type, *orf4*, *dmrA*, and *fae* mutant strains. We thank Jack Shelton for sequencing the *mtdB* and *orf4* genes and Vicki Kopf and Chi Bissett for their research contributions.

This work was supported by National Science Foundation grant numbers MCB-9876212 and MCB-9815924 and the Florida Agricultural Experiment Station.

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