Elucidation of the Role of the Methylene-Tetrahydromethanopterin Dehydrogenase MtdA in the Tetrahydromethanopterin-Dependent Oxidation Pathway in *Methylobacterium extorquens* AM1

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The methylotroph *Methylobacterium extorquens* AM1 oxidizes methanol and methylamine to formaldehyde and subsequently to formate, an intermediate that serves as the branch point between assimilation (formation of biomass) and dissimilation (oxidation to CO₂). The oxidation of formaldehyde to formate is dephosphotetrahydromethanopterin (dH₄MPT) dependent, while the assimilation of carbon into biomass is tetrahydrofolate (H₄F) dependent. This bacterium contains two different enzymes, MtdA and MtdB, both of which are dehydrogenases able to use methylene-dH₄MPT, an intermediate in the oxidation of formaldehyde to formate. Unique to MtdA is a second enzymatic activity with methylene-H₄F. Since methylene-H₄F is the entry point into the biomass pathways, MtdA plays a key role in assimilatory metabolism. However, its role in oxidative metabolism via the dH₄MPT-dependent pathway and its apparent inability to replace MtdB in *in vivo* on methanol growth are not understood. Here, we have shown that an mtdB mutant is able to grow on methylamine, providing a system to study the role of MtdA. We demonstrate that the absence of MtdB results in the accumulation of methenyl-dH₄MPT. Methenyl-dH₄MPT is shown to be a competitive inhibitor of the reduction of methenyl-H₄F to methylene-H₄F catalyzed by MtdA, with an estimated Ki of 10 μM. Thus, methenyl-dH₄MPT accumulation inhibits H₄F-dependent assimilation. Overexpression of mch in the mtdB mutant strain, predicted to reduce methenyl-dH₄MPT accumulation, enhances growth on methylamine. Our model proposes that MtdA regulates carbon flux due to differences in its kinetic properties for methylene-dH₄MPT and for methenyl-H₄F during growth on single-carbon compounds.

Methylotrophy is the ability of microorganisms to utilize reduced compounds with no carbon-carbon bonds as a sole source of energy and carbon, a metabolism that has been studied in detail for over 50 years (1, 2). Examples of these carbon sources include methanol, methylamine, and methane, placing methylotrophs as key players in global cycling of carbon and nitrogen (3, 4, 5, 6). From a physiological and biochemical perspective, methylotrophy is an intriguing model of study, since methylotrophs must accommodate high flux through toxic metabolites such as formaldehyde and glyoxylate. The metabolism of one-carbon compounds in the facultative methylotroph *Methylobacterium extorquens* AM1 (Fig. 1) (7) involves the oxidation of methanol and methylamine to formaldehyde via methanol dehydrogenase or methylamine dehydrogenase (MaDH), respectively. Formaldehyde is incorporated into the cytoplasm and coupled with the carbon carrier dephosphotetrahydromethanopterin (dH₄MPT) via Fae (formaldehyde-activating enzyme) to generate methylene-dH₄MPT. MtdA (methylene-dH₄MPT/H₄F dehydrogenase) and MtdB (methenyl-dH₄MPT dehydrogenase) catalyze the oxidation of methylene-dH₄MPT to methylene-dH₄MPT with NAD(P)⁺ as a cosubstrate. Mch (methylene-H₄MPT cyclohydrolase) catalyzes the conversion of methenyl-dH₄MPT to formyl-dH₄MPT. Fhc (formyltransferase/hydrolase complex) catalyzes the conversion of formyl-dH₄MPT to formate via a methanofuran derivative. Together, these reactions constitute the dH₄MPT-dependent oxidative pathway. The partitioning of carbon between assimilatory and oxidative metabolism occurs at formyl (8). Oxidative metabolism involves CO₂ production via formate dehydrogenases (Fdh). Four different formate dehydrogenases are known, two NAD linked (Fdhd1 and Fdh2) and two non-NAD linked, for which the *in vivo* electron acceptors are not known (Fdh3 and Fdh4) (9).

Assimilatory metabolism starts with the tetrahydrofolate (H₄F) pathway. FtfL (formate-H₄F ligase) catalyzes the conversion of formate and H₄F to generate formyl-H₄F. Fch (methylene-H₄F cyclohydrolase) catalyzes the reversible dehydration to methenyl-H₄F. MtdA (methylene-H₄MPT/H₄F dehydrogenase) catalyzes the reversible reduction to methylene-H₄F, the intermediate that is incorporated into the assimilatory cycles (Fig. 1). The interlinked assimilatory cycles comprise 22 enzymes (10). An alternative pathway for methylamine oxidation, the N-methyl glutamate (NMG) pathway, has been described for several microorganisms (11). Although biochemical details of the pathway are not well understood, it is known that three enzymes are necessary for the conversion of methylamine to presumably methylene-H₄F (11): an NMG synthase, a gamma-glutamylmethylamide synthetase (GMA synthetase), and an NMG dehydrogenase (NMGDH). Low NMG dehydrogenase activity was detected in cell extracts of *M. extorquens*, suggesting that *M. extorquens* has the capacity to oxidize methylamine via the indirect N-methyl glutamate pathway (12). However, a mau mutant (lacking MaDH) is unable to grow on methylamine (12), questioning the functionality of the pathway.

Studies with purified MtdA have shown that it is NAD⁺ spe-
cific and is able to use methylene-$H_2$MPT, methylene-$dH_2$MPT, methenyl-$H_2$F, and methylene-$H_2$F as the substrates (13, 14, 15).

Characterization of the second methylene-$H_4$MPT dehydrogenase, MtdB, showed that it can use both NAD$^+$ and NADP$^+$ but is preferentially an NAD$^+$-dependent enzyme ($K_m = 20 \mu M$ for NADP$^+$ versus 200 $\mu M$ for NAD$^+$). Like MtdA, MtdB can use either methylene-$H_2$MPT or methylene-$dH_2$MPT (15). With NADP$^+$ as a cosubstrate, MtdA catalyzes the dehydrogenation with an approximately 3-fold-higher catalytic efficiency ($V_{\text{max}}/K_m$) than that of MtdB (15). In addition, in cell extracts, the $dH_2$MPT- and NADP$^+$-dependent activity of MtdA is 10-fold higher than that of MtdB (14, 15). Therefore, it seemed likely that MtdA should be able to replace MtdB in vivo. However, an mtdB mutant strain that contained wild-type mtdA was unable to grow on either methanol or succinate in the presence of methanol. This methanol-sensitive phenotype was attributed to formaldehyde accumulation (16), suggesting that MtdA was not able to allow sufficient carbon flux from formaldehyde to formate to avoid formaldehyde toxicity. This result was inconsistent with the in vitro data regarding catalytic efficiency activity in cell extract. Further, increasing levels of MtdA relieved the formaldehyde toxicity when the mutant strain was grown with succinate plus methanol but did not allow growth in methanol liquid medium, complicating the interpretation of the role of MtdA in the oxidative step (16). The current study was undertaken to address the contradiction between the phenotypic and biochemical results and to determine the role of MtdA in methylotrophic metabolism. The results suggest that MtdA functions in the distribution of the formate pool to maintain the balance between assimilation and oxidation.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** All *M. extorquens* AM1 strains were grown at 30°C on a minimal salts medium (18) containing carbon sources at the following concentrations: 35 mM methylamine, 125 mM methanol, and 15 mM succinate. *Escherichia coli* strains were grown on Luria-Bertani medium. For conjugation between the helper strain *E. coli* S17-1 (19) and *M. extorquens*, Difco nutrient broth supplemented with Difco Biotek agar (1.5% [wt/vol]) was used. Antibiotics were added when needed to the following final concentrations: 100 $\mu g$ of ampicillin/ml, 50 $\mu g$ of kanamycin/ml, 50 $\mu g$ of rifampicin/ml, and 10 $\mu g$ of tetracycline/ml. Chemicals were obtained from Sigma (St. Louis, MO).

**Generation of mutant strains.** *M. extorquens* deletion mutants lacking *mgsA* (NMG synthase) were generated with the allelic exchange suicide vector pCM184 (20). Approximately 0.3- to 0.5-kb regions upstream and downstream of *mgsA* were amplified by PCR and directly cloned into pCM184 (donor) with the primers nmgsyn AM1 Rev KpnI up, 5'-ACGG TACCCGACACGAGGTAAAGAAG-3' and nmgsyn AM1 For EcoRI down, 5'-GGCGAATTCGCGATCTCGACCGCAGAAGTCGT-3'; and nmgsyn AM1 For Hpal down, 5'-CAGTTAACGCAAGGTTCCCGCGAGAC-3'. Mutant strains of *M. extorquens* were generated by conjugation of the plasmid from an *E. coli* S17-1 donor as previously described (21). Unmarked deletion strains were generated with the cre-lox-expressing plasmid pCM157 (20). Mutant strains were confirmed by diagnostic PCR analysis.

**Phenotypic analyses of mutant strains.** Growth of *M. extorquens* strains was assessed for 2 biological replicates grown in liquid medium containing the carbon source described and with monitoring of the optical density at 600 nm. Strains were incubated for 12 to 16 h at 30°C with shaking. Strain growth was assessed by OD measurements.

**TABLE 1** *M. extorquens* strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>Strains</td>
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<tr>
<td>CM 253.1</td>
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<td>pCM80 with fghA, fhaA</td>
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<td><em>M. extorquens</em> expression vector (Pmet1, 2136)</td>
<td>E. Skovran, unpublished data</td>
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<td><em>M. extorquens</em> expression vector (Pmet1, 002)</td>
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<td>pAP774 with mtdA</td>
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<td>pNM125</td>
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cal density at 600 nm (OD$_{600}$). The strains were grown in minimal medium with succinate (15 mM) at 30°C to late exponential phase and subcultured into flasks containing 100 ml of minimal medium containing methyamine (35 mM) and the appropriate antibiotic if needed.

**Generation of plasmids overexpressing mtdA.** The coding region of mtdA was amplified by PCR and cloned into pCM62 (21) with the primers MtdASacIFor, 5'- GGCAAGCTTATGGAAAGGCTTCTTTCCAG TTCG3', and MtdA HindIIIRev, 5'- GGCAATTTCTCAGGCTATCTCG AGTGGCCAGC 3'. The resulting plasmid containing the promoter region: Meta_2136 for low expression, Meta_0002 for medium expression, and Meta_3616 for high expression. Relative expression was tested by published microarray results (18, 22).

**Purification and characterization of dH$_4$MPT species.** M. extorquens AM1 wild type was grown at 30°C on a minimal salts medium containing methanol (125 mM) until it reached an OD$_{600}$ of 2.2. Cells were harvested by centrifugation. The cell paste (30 g) was introduced into an anaerobic chamber (Coy, Grass Lake, MI) containing 95% N$_2$ and 5% H$_2$. All experiments were performed in the dark. Cells were resuspended in 1 ml of K$_2$HPO$_4$ buffer (5 mM potassium phosphate buffer, pH 4.8, 10 mM β-mercaptoethanol) and broken by boiling (15 min). Cell extracts were cleared by centrifugation in a scaled tube outside the anaerobic glove box (28,000 × g, 45 min, 4°C) and transferred back into the glove box. Forty milliliters of supernatant was applied to an Oasis weak anion exchange (WAX) extraction cartridge (6 ml, 500 mg) (Waters, Milford, MA) previously activated with 1% (vol/vol) formic acid and equilibrated with methanol. After loading, the column was washed with 1 column volume of distilled water. dH$_4$MPT was eluted with 1 column volume of elution solution I (5% (vol/vol) NH$_4$OH, 80% (vol/vol) methanol, 15% (vol/vol) H$_2$O). The elution fraction was analyzed under UV-visible light to confirm the characteristic maximal peaks of the species. The fraction was also analyzed using MALDI-TOF mass spectrometry (MALDI-TOF MS) corroborated the m/z typical of NH$_4$MPT and methenyl-dH$_4$MPT, respectively. The mass spectrometer was first operated in Q1MS mode to detect the parent ions of interest (targeted ions). It was then operated in MS/MS mode to look for the product ions for the selected parent. The collision energy was optimized to obtain a good signal-to-noise ratio of the product ions.

**Preparation of extracts.** M. extorquens strains, wild type and mutants, were grown on minimal medium with methyamine (100 ml) and harvested at an OD$_{600}$ of 0.4 to 0.5. Cell pellets were harvested by centrifugation, and the supernatant was removed and immediately transferred to the anaerobic chamber (Coy, Grass Lake, MI) containing 95% N$_2$ and 5% H$_2$. Further experiments were performed under strictly anoxic conditions and in the dark. Cell pellets were resuspended in anoxic buffer (100 mM potassium phosphate buffer, pH 6.0; 1 ml). Lysozyme was added and incubated on ice for 10 min. Cells were broken by sonication (9 cycles of intermittent pulses for 45 s each), monitoring of the temperature to ensure that it remained below 10°C. The cell extracts were centrifuged (10 min, 28,000 × g, 25°C), and the supernatant was set on ice until used for assays. When indicated, the extracts were desalted with a PD-10 gel filtration column (8.5-ml bed volume, 5-cm bed height) previously equilibrated with 100 mM anoxic potassium phosphate buffer, pH 6.0, and further concentrated with microconcentrators (Amicon-Ultra; Millipore, Billerica, MA).

**Purification of MtdA.** For high-level expression of MtdA, mtdA was amplified and cloned into pQE30Xa with chromosomal DNA (purified according to the MoBio protocol [Carlsbad, CA]) from wild-type M. extorquens AM1 as the template. The construct was transformed into M15/prep4 cells. This strain was grown at 30°C in Superbroth medium with kanamycin (50 μg/ml) and ampicillin (50 μg/ml). IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM) was added to induce expression of MtdA when the culture reached an OD$_{600}$ of 0.5. Cultures were grown after induction for 4 h at 30°C, and cells were harvested by centrifugation (4,800 × g, 10 min, 4°C). The cell paste (30 g) was resuspended in 30 ml of K$_2$HPO$_4$ buffer (50 mM Tris-HCl, pH 8.0, 5 mM imidazole, and 15% [vol/vol] glycerol), and cells were broken with a French press. Cell extracts were cleared by centrifugation (28,000 × g, 45 min, 4°C), and the supernatant was applied to a Ni$_2$-charged chelating Sepharose column (Qiagen, Germantown, MD) (8 ml) previously equilibrated with buffer A. After loading, the column was washed with 5 column volumes of buffer B (buffer A with 200 mM NaCl). MtdA-H$_4$ was eluted off the column by running an imidazole gradient (0 to 500 mM) over 50 ml in buffer A. Fractions (4 fractions of 3 ml each) were pooled and desalted with a PD-10 gel filtration column (8.5-ml bed volume, 5-cm bed height) equilibrated with buffer C (50 mM morpholinoethanesulfonic acid [MES]–NaOH buffer, pH 5.3). The protein was concentrated with centrifugal filter devices (Amicon-Ultra 10K; 4,000 × g, 15 min, 4°C). The concentrated protein sample (100 μM) was purified further with a HiTrap Sepharose S column (GE Healthcare, Pittsburgh, PA) (5 ml) previously equilibrated with buffer C. After loading, the column was washed with 5 column volumes of buffer B (buffer A with 200 mM NaCl). MtdA-H$_4$ was eluted off the column by running an imidazole gradient (0 to 0.2 M NaCl) over 25 ml in buffer C. Two fractions of 2 ml each were pooled and desalted with buffer D (120 mM potassium phosphate buffer, pH 6.0) and the gel filtration PD-10 column previously described. Proteins were concentrated to a final concentration of 100 μM and used as indicated. The protein was stable when stored at −80°C. Protein concentration was determined by the bicinchoninic acid method (Pierce).

**MtdA activity.** Activity of MtdA was measured with NADP$^+$ as a cosubstrate, and the dehydrogenation of methylene-dH$_4$MPT to methenyl-dH$_4$MPT was followed by monitoring production of NADPH as described previously (13) with the following differences: 200 mM potassium phosphate buffer, purified methylene-H$_4$MPT dehydrogenase MtdA (100 μM, 50 μl), and formaldehyde (2 mM). All assays were performed under anaerobic conditions and in the dark. Formaldehyde was prepared by autoclaving paraformaldehyde for 10 min (Sigma, St. Louis, MO) in distilled water (4.6 mg/ml). The reaction mixture was incubated at room temperature for 20 min. The total volume of the reaction was 420 μl. Activity was monitored at 340 nm (i.e., NADPH production) after addition of the
formate as it does when methanol is the carbon source (Fig. 1). In
metrically at 412 nm (11).
reagent. Accumulation of formaldehyde was recorded spectrophoto-
addition of NMG and, after incubation of the reaction mixture at room
mtdB
The NMG pathway contributes to methylamine oxidation in the
RESULTS
The NGM pathway contributes to methyleamine oxidation in the
mtdB mutant but not in the wild type. Previous work (14, 16)
demonstrated that the mtdB mutant strain was unable to grow in
methanol liquid medium. However, in this study, it was found that
the mtdB mutant strain is able to grow poorly on the C1 compound
methyleamine as a sole source of carbon and energy, with a doubling time of 9.5 h and a final OD600 of 0.7 compared to a
doubling time of 4 h and a final OD600 of 1.3 for the wild type
(Fig. 2A). It has been shown that M. extorquens uses methylene
dehydrogenase to grow on methyleamine, and this enzyme cata-
lyzes the conversion of methyleamine to formaldehyde (12). Once
formaldehyde is formed and transported into the cytoplasm, it
undergoes the same dH4MPT-dependent oxidation to generate
formate as it does when methanol is the carbon source (Fig. 1). In
order to understand the relative roles of MtdA and MtdB in C1
metabolism, it is important to understand why MtdB appears to
have different roles for growth on methanol and methyleamine.
Recently, Latypova et al. defined the genetics of the NMG pathway
(an alternative pathway for methyleamine oxidation) in another
methylo troph, Methyloversatilis universalis FAM5, and defined a
cluster of sox-like, glhB-like, and gshIII-like genes as encoding the
enzymes necessary for an operational NMG (11). Homologs of
this alternative pathway were identified in M. extorquens—
Metal_1545, Metal_1546, Metal_1547, and Metal_1548 for the
sox-like cluster; Metal_1550, Metal_1551, and Metal_1552 for the
glhB-like cluster; and Metal_1553 for the gamma-glutamylm-
eethylamide synthetase (GMA synthetase) homolog (Fig. 2B)—
suggesting that M. extorquens has the potential for a functional
NGM pathway. NMG dehydrogenase was detected in wild-type
cell extracts at 0.2 ± 0.1 nmol/min/mg protein, supporting the
existence of this pathway. However, its contribution is not suffi-
cient to support growth, as a mau mutant (missing MadH activity)
is not able to grow on methyleamine (12). It has been suggested
that the final product of the NGM pathway is not formaldehyde
but methylene-H4F (11). If the NGM pathway is functional in the
mtdB mutant strain, it could allow contribution of carbon flux to the
assimilation pathways bypassing the dH4MPT-dependent ox-
idation pathway and, consequently, MtdB activity. No equivalent
alternative is available for methanol oxidation, and so the differ-
ence in phenotypes of the mtdB mutant strain on methanol and
methyleamine may be due to this alternative pathway. In order
to test this possibility, NMG dehydrogenase activity was measured in
cell extracts from the mtdB mutant strain grown in methyleamine
medium. This activity was found to be 17.8 ± 2 nmol/min/mg protein, 89-fold higher than that in the wild-type strain, suggesting
that when MtdB is not present, the NGM pathway for meth-
eyleamine oxidation is upregulated. Phenotypic studies of mutants
were used to corroborate the involvement of this pathway for
methyleamine growth of the mtdB mutant strain. As shown in Fig.
2A, the mga mutant strain (mga encodes NMG synthase)
showed no growth defect in methyleamine medium, while the double
mtdB mga mutant strain showed a growth defect compared to the
mga mutant (final OD600 of 0.2 to 0.3 of 2 biological repli-
cates). Together, these data demonstrate the contribution of the
NGM pathway for methyleamine growth in a strain lacking mtdB.
Reduced growth of the mtdB mutant strain on methyleamine
is due in part to formaldehyde toxicity. Each of the mutants that
affect formaldehyde consumption has two possible components
to growth defects, the change in flux to later steps in metabolism
and the toxicity of formaldehyde, if it accumulates. Formaldehyde
toxicity is manifested as decreased growth in the presence of a
cosubstrate in combination with methanol (methanol sensitivity)
or formaldehyde compared to that of the wild type (16). The glu-
uthione (GSH)-dependent formaldehyde oxidation pathway
(FghA and FhLA [Fig. 1]) alleviates both methanol and formalde-
hyde sensitivity in M. extorquens mutants (including the mtdB
mutant), due to decreased formaldehyde accumulation (16, 24,
25), and it also likely provides increased flux to formate, which
might increase assimilation. In order to test the formaldehyde
toxicity component of growth defects in the mtdB mutant strain,
phenotypic studies were carried out comparing this strain to
mptG, mga, and mga mtdB mutant strains with and without the
GSH-dependent formaldehyde oxidation pathway provided in
trans. mptG is the gene that encodes the first enzyme in the

FIG 2 Contribution of the NMG pathway to methylamine growth. (A) Growth of wild-type M. extorquens (crosses), mtdB mutant strain (triangles), an mga (encoding N-methyl glutamate synthase) mutant strain (diamonds), and a double mtdB mga mutant strain (circles) pregrown on succinate and inoculated in medium containing methyleamine (35 mM). (B) Comparison of the gene clusters encoding the N-methyl glutamate pathway in Methylobacteri-
universals FAM5. and M. extorquens AM1 and Methyloversatilis

Mch activity. The activity of Mch was followed photometrically
by monitoring the decrease in absorbance at 335 nm (ε = 21.6 mM−1 cm−1) as described previously (23) with the following differences: the assay mix-
ture contained 50 mM potassium phosphate buffer (pH 8.0), 0.3 M NaCl, and 125 mM methylene-dH4MPT. The reaction was started with extract.
NMGDH activity. NMG dehydrogenase (NMGDH) activity included
(per 0.5 ml): 50 mM sodium phosphate buffer, pH 7.6, 5 mM NMG, 0.5
mM NAD+, and 0.5 mg protein extract. The reaction was initiated by
addition of NMG and, after incubation of the reaction mixture at room
temperature for 15 to 20 min, terminated by addition of 0.5 ml Nash
reagent. Accumulation of formaldehyde was recorded spectrophoto-
nometrically at 412 nm (11).
dH₄MPT biosynthesis pathway, β-ribofuranosylaminobenzene 5’-phosphate synthase (26). A null mutant in mptG generates no dH₄MPT (27) and therefore is unable to synthesize the substrate mutant lacking MptG was confirmed (Fig. 3A). It had previously been shown that an mptG mutant containing the heterologous GSH-dependent formaldehyde oxidation pathway was no longer methanol sensitive but grew only poorly on methanol (16). Thus, the GSH-dependent formaldehyde oxidation pathway alleviates formaldehyde toxicity and allows only slow growth.

When the GSH-dependent formaldehyde oxidation pathway was introduced into the mptG mutant, the strain grew better on methyamine than did the mptG strain carrying the empty plasmid but still not at the wild-type level (doubling time of 11.5 h; final OD₆₀₀ of 0.22) (Fig. 3), similar to the results reported previously with methanol (16). This amount of growth reflects the contributions of formate assimilation via the GSH-dependent formaldehyde oxidation pathway plus assimilation of methylene-H₄F via the NMG pathway. The difference in growth between the mgsA mtdB double mutant and the mtdB mutant reflects assimilation via the NMG pathway. Since that growth difference is similar to the growth of the mptG mutant containing the GSH-dependent formaldehyde oxidation pathway, it suggests that the main impact of the GSH-dependent formaldehyde oxidation pathway is in detoxification. Therefore, the difference in growth between the mptG mutant expressing FghA and FlhA and other mutants containing this pathway is an indicator of the relative contribution of formaldehyde toxicity to the mutant phenotype. However, when the GSH-dependent formaldehyde oxidation pathway was expressed in the mtdB mutant, the strain was able to grow in methyamine medium with a growth rate (4 h) and final optical density (OD₆₀₀ of 1.02) similar to those of the wild type (Fig. 3B). FghA and FlhA were also introduced in the double mtdB mgsA mutant, and the growth of the strain was measured. Significant increases in growth rate and final optical density were observed compared to those of the same double mutant without the path-

way (Fig. 3). However, the growth of the double mutant containing the GSH-dependent formaldehyde oxidation pathway was clearly defective compared to those of the wild-type strain and the mtdB mutant strain containing the same heterologous pathway. Together, these results suggest that part but not all of the methy-

amine growth defect of an mtdB mutant strain is due to formal-

dehyde toxicity.

Increasing MtdA levels inhibit growth on methyamine. Previous studies on methanol medium (16) have shown that increasing levels of MtdA in an mtdB mutant strain relieved methanol and formaldehyde sensitivity but did not allow growth on methanol. However, the effect of MtdA overexpression on methy-

amine growth was not tested. In this study, the region encompass-

ing mtdA was cloned and introduced into a set of plasmids with different promoters (pAP774, low expression; pAP775, medium expression; pAP776, high expression [Table 1]) of the expression vector pCM62 (21). These plasmids were used to overexpress mtdA at different levels in the mtdB mutant strain during growth on methyamine. Increasing levels of MtdA inhibited growth on methyamine (Fig. 4). One plausible explanation is that increased levels of MtdA resulted in the accumulation of a downstream metab-

olite affecting growth. Since the mtdB mutant strain is able to grow on formate (16), an intermediate downstream from formate would be an unlikely candidate for this inhibitor.

An inhibitor of MtdA accumulates in the mtdB mutant strain. A likely target for inhibition is the methylene-H₄F reduction activity by MtdA, since methylene-H₄F is the entry metabolite for the assimilatory pathways. To test the hypothesis that an inhibitor of the H₄F-dependent activity of MtdA accumulates in the mtdB mutant strain, methylene-H₄F oxidation activity by MtdA was measured in cell extracts from cells grown on methyamine of the wild type, the mtdB mutant strain, and the mtdB mutant strain overexpressing mtdA. The extracts were generated under strict anoxic conditions and in the dark to minimize degradation of dH₄MPT derivatives. As shown in Table 2, the activities of MtdA with limiting amounts of methylene-H₄F were similar for the wild-type extract and the mtdB extract and decreased slightly when MtdA was overexpressed. However, when the extracts were desalted to remove a potential small-molecule inhibitor, a 4-fold

FIG 3 Formaldehyde accumulation reduces growth of the mtdB mutant strain in methyamine medium. (A) Growth of wild-type M. extorquens (crosses), mtdB mutant (triangles), mgsA mutant (diamonds), mptG (encoding the first gene product necessary for dH₂MPT biosynthesis) mutant (asterisks), and mtdB mgsA double mutant (circles), all carrying the plasmid pCM80 as the vector control. All strains were pregrown on succinate and inoculated in medium containing methyamine (35 mM). (B) Growth of the same strains (represented by the same symbols but open instead of solid) under the same conditions, carrying the heterologous GSH-dependent formaldehyde oxidation system [pCM106 (fghA flhA)].
FIG 4 Increased levels of MtdA inhibit growth of the mtdB mutant strain. Growth of wild-type M. extorquens (crosses) in methylimine medium with empty plasmid (pCM62) and mtdB mutant strains overexpressing different levels of mtdA. Higher levels of mtdA are denoted by different symbols (triangles, empty vector; squares, pNM1; circles, pNM2; diamonds, pNM3).

increase of the MtdA activity catalyzing the oxidation of methylene-H$_4$F to methenyl-H$_4$F was found (from 0.3 mU/mg to 1.2 mU/mg enzyme) from the mtdB extract and a 10-fold increase was found in the mtdB extract overexpressing MtdA (from 0.13 mU/mg to 1.4 mU/mg), while the activity from the wild-type extract remained the same. These results confirmed the presence of an inhibitor of H$_4$F-dependent activity of MtdA in the mtdB mutant strain.

Methenyl-dH$_4$MPT accumulates when MtdB is absent. Since MtdA has dual specificity for H$_4$F and dH$_4$MPT intermediates, a plausible candidate for the inhibitor of H$_4$F-dependent activity is a dH$_4$MPT species. To test for accumulation of dH$_4$MPT species in the mtdB mutant strain compared to the wild type, dH$_4$MPT and its derivatives were purified from the wild-type and mtdB mutant strains grown on methylimine and analyzed as described in Materials and Methods. Two fractions showed higher absorbance in the extract derived from the mtdB mutant than in that from the wild type, and each fraction was further purified. UV-visible maximal peaks of 200 nm, 255 nm, and 301 nm were observed in the purified fractions, along with maximal absorbance peaks of 210 nm, 255 nm, and 352 nm, consistent with dH$_4$MPT and methenyl-dH$_4$MPT, respectively (14, 28) (Fig. 5A). Each of these fractions was then analyzed by electrospray ionization mass spectrometry along with standards. The $m/z$ for the two species (567 and 577 [Fig. 5A, inset]) corroborated the identification of dH$_4$MPT and methenyl-dH$_4$MPT, respectively, suggesting that these two species accumulated in the mtdB mutant strain when growing in methylimine medium. Quantification of the accumulation of both compounds with respect to the wild-type strain showed a 5-fold increase for dH$_4$MPT (by UV-visible analysis and mass spectrometry analysis) and a 2- to 3-fold increase for methenyl-dH$_4$MPT (Fig. 5B). In keeping with accumulation of methenyl-dH$_4$MPT, the methenyl-H$_4$MPT cyclohydrolase (Mch) activity decreased in an mtdB mutant strain compared to the wild type, with activities in extracts of $0.4 \pm 0.1$ µmol/min/mg protein and $1 \pm 0.2$ µmol/min/mg protein, respectively. Overexpression of mch should decrease the methenyl-dH$_4$MPT pool and thereby alleviate inhibition. mch in an overexpression plasmid was introduced into the mtdB mutant strain, and the cells were grown on methylimine. Increasing the levels of Mch (from 0.4 µmol/min/mg protein in the mtdB mutant to 1.2 µmol/min/mg protein with the overexpression plasmid) allowed the mutant to grow to higher densities (OD$_{600}$ of 1.2 versus 0.6 [Fig. 5C]) and partially rescued the defect in growth rate. This result is consistent with an inhibitory effect of methenyl-dH$_4$MPT on the assimilation step.

Methenyl-dH$_4$MPT inhibits MtdA H$_4$F-dependent activity. The methylene-H$_4$MPT dehydrogenase MtdA was purified to assess the inhibitory effect of methenyl-dH$_4$MPT on methenyl-H$_4$F reduction activity by MtdA. As shown in Table 3, when methenyl-dH$_4$MPT was present in the assay, the $K_m$ value of MtdA activity for methenyl-H$_4$F increased more than 3-fold and the $V_{max}$ changed slightly, suggesting a competitive inhibition. The estimated $K_i$ was 10 µM.

**DISCUSSION**

MtdA is a well-characterized methylene-dH$_4$MPT dehydrogenase (13, 15, 29) known to efficiently catalyze the oxidation of methylene-dH$_4$MPT to methenyl-dH$_4$MPT during methanol and methylimine metabolism and also to catalyze the reversible reaction with methenyl-H$_4$F. However, the significance of its activity under physiological conditions in this step has been unclear, considering that MtdA is unable to replace the alternative methylene-dH$_4$MPT dehydrogenase MtdB. One plausible explanation concerns differential pyridine nucleotide usage, since MtdA is able to use only NADP$^+$ as a cosubstrate, while MtdB can use both NAD$^+$ and NADP$^+$. However, the intracellular concentrations of both species are on the order of millimolar concentrations and should be saturating (30). Furthermore, deuterium experiments have shown that the majority of flux during methanol oxidation to formate generates NADPH (8).

In methanol medium, two problems are associated with the lack of MtdB in the cell: the accumulation of formaldehyde in the presence of methanol and the decreased production of formate for further metabolism (16). If the methylene-dH$_4$MPT-dependent activity of MtdA in vivo is not sufficient for the flux to methenyl-dH$_4$MPT, formaldehyde would accumulate in an mtdB mutant strain. However, when MtdA was overexpressed and its activity increased 7-fold, it was observed that MtdA was able to avoid the accumulation of formaldehyde but the mtdB mutant strain was still unable to grow on methanol (16). This result suggests that the normal level of MtdA is insufficient to handle the full formaldehyde flux through the dH$_4$F-dependent oxidative pathway. When MtdA is overexpressed in the mtdB mutant strain, formaldehyde flux is sufficient and should allow growth on methanol.

**TABLE 2** Specific activity of the methylene-H$_4$MPT dehydrogenase

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sp act (mU/mg) with 20 µM H$_4$F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal extracts</td>
</tr>
<tr>
<td>WT</td>
<td>0.23 ± 0.09</td>
</tr>
<tr>
<td>mtdB mutant</td>
<td>0.34 ± 0.1</td>
</tr>
<tr>
<td>mtdB mutant/pNM3</td>
<td>0.13 ± 0.12</td>
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</tbody>
</table>
The lack of growth under this condition suggests a block in assimilation.

We showed that the \textit{mtdB} mutant strain is able to grow on methylamine, although with a lower growth rate than that of the wild type. The growth of the mutant on methylamine is dependent on the presence of an alternative methylamine oxidation pathway (the NMG pathway), which is proposed to generate methylene-\(\text{H}_4\text{F}\) directly without involvement of the \(\text{dH}_4\text{MPT}\)-dependent oxidative pathway. The lower growth rate and lower final cell density of the \textit{mtdB} mutant strain than those of the wild type suggest that \textit{MaDH} provides a higher \textit{in vivo} flux of methylamine than does the \textit{NMG synthase/GMA synthetase}. Although this finding did not illuminate the reason why MtdA cannot substitute for MtdB, it did provide a system for studying the role of MtdA in one-carbon utilization. However, this finding does provide evidence of a functional NMG pathway for methylamine oxidation in \textit{M. extorquens} as previously hypothesized but not shown.

It is clear that MtdB alone at its normally expressed level is sufficient to accommodate the flux from formaldehyde, since an \textit{mtdA} mutant is not methanol sensitive (31). In this study, we demonstrate that the activity of MtdA alone allows growth on methylamine in the presence of alternate pathways such as the NMG pathway and the heterologous GSH-dependent formaldehyde oxidation pathway. Each of these pathways contributes to both the detoxification of formaldehyde and the contribution of carbon to assimilation in a \(\text{dH}_4\text{MPT}\)-independent manner, thus at least partially alleviating both problems that arise when MtdB is lacking. In addition, the difference in biochemistry between the

TABLE 3 Kinetic parameters of the methylene-\(\text{H}_4\text{MPT}\) dehydrogenase MtdA with NADPH as a cosubstrate in the presence and absence of methenyl-\(\text{dH}_4\text{MPT}\)\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m) ((\mu)M)</th>
<th>(V_{\text{max}}) (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methenyl-(\text{H}_4\text{F})</td>
<td>45</td>
<td>103</td>
</tr>
<tr>
<td>Methenyl-(\text{H}_4\text{F}) + methenyl-(\text{dH}_4\text{MPT})</td>
<td>187</td>
<td>135</td>
</tr>
</tbody>
</table>

\(^a\) Concentrations of methenyl-\(\text{H}_4\text{F}\) varied from 10 \(\mu\)M to 100 \(\mu\)M; the concentration of methenyl-\(\text{dH}_4\text{MPT}\) was 100 \(\mu\)M.
oxidative and assimilatory metabolism, based on the dual H4F/dH4MPT specificity of MtdA. A previous study had shown that when succinate-grown cells are exposed to methanol, one-carbon flux is directed to oxidative metabolism until the entire assimilatory machinery is induced and functional (22). This strategy is one mechanism to prevent formaldehyde from accumulating during shifts in formaldehyde flux. Our results suggest a possible model for a mechanism of this block in assimilatory flux (Fig. 6) that is consistent with our results, in which MtdB is the dehydrogenase involved in constitutively removing formaldehyde while MtdA is dynamically changed to increase or decrease levels of methenyl-dH4MPT. The levels of methenyl-dH4MPT modulate the assimilatory activity of MtdA. Thus, the dual H4F/dH4MPT specificity of MtdA couples oxidative flux to assimilatory flux and ensures that assimilation does not occur under conditions of imbalance in the oxidative flux. Interestingly, FtfL and Fch, the enzymes that complete the H4F pathway that generates the methylene H4F precursor for assimilatory metabolism, both catalyze reversible reactions. This would suggest that the entire H4F pathway would respond to MtdA activity inhibition, consistent with our hypothesis for flux regulation. In this model, the extra formate produced while assimilation is blocked is oxidized to CO2, as was shown to occur previously in metabolic imbalance in M. extorquens AM1 (22). It is likely that the one-step oxidation of formate to CO2 provides a relatively simple mechanism for dynamic adjustment to keep formaldehyde from accumulating during formaldehyde flux changes, as opposed to the alternative of maintaining and regulating activity of the 22 enzymes of the combined serine cycle and ethylmalonyl coenzyme A (CoA) pathways, the machinery of assimilatory metabolism.

Likewise, we suggest that the inhibitory effect on MtdA by accumulation of methenyl-dH4MPT when the strain lacks MtdB could also regulate distribution of carbon flux from the NMG pathway when methyamine is the carbon substrate. In this scenario, the inhibition of MtdA catalyzing the oxidation of methyl-
The inhibitory effect of methenyl-dH4MPT and MtdA might not be a molecule regulator for one-carbon compound metabolism is shown. Further, the identification of methenyl-dH4MPT as a small-molecule regulator for one-carbon compound metabolism is to regulate methylotrophy. This growing insight into how the metabolic network is controlled at the level of small molecules will facilitate the manipulation of methylotrophic metabolic networks for a variety of applications, including the production of valued-added chemicals from methanol.

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