Journal of Bacteriology, Jan. 2003, p. 669–673 Vol. 185, No. 2
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.2.669–673.2003
Copyright © 2003, American Society for Microbiology. All Rights Reserved.

Novel Methylotrophy Genes of *Methylobacterium extorquens* AM1 Identified by using Transposon Mutagenesis Including a Putative Dihydromethanopterin Reductase

Christopher J. Marx,[1] Brooke N. O'Brien,[1] Jennifer Breezee,[1] and Mary E. Lidstrom[1,2]*

Departments of Microbiology[1] and Chemical Engineering,[2] University of Washington, Seattle, Washington 98195-1750

Received 26 August 2002/Accepted 16 October 2002

Ten novel methylotrophy genes of the facultative methylotroph *Methylobacterium extorquens* AM1 were identified from a transposon mutagenesis screen. One of these genes encodes a product having identity with dihydrofolate reductase (DHFR). This mutant has a C_1-defective and methanol-sensitive phenotype that has previously only been observed for strains defective in tetrahydromethanopterin (H_4MPT)-dependent formaldehyde oxidation. These results suggest that this gene, *dmrA*, may encode dihydromethanopterin reductase, an activity analogous to that of DHFR that is required for the final step of H_4MPT biosynthesis.

Methylobacterium extorquens AM1 is a model organism for the study of methylotrophic metabolism and is one of the first methylotrophs for which genome sequence data are available (http://www.integratedgenomics.com/genomereleases.html#list6). *M. extorquens* AM1 is an α-proteobacterium and is a facultative methylotroph capable of growth on C_1, C_2, C_3, and C_4 compounds (4, 14). Methylotrophic metabolism in *M. extorquens* AM1 begins with the oxidation of C_1 substrates in the periplasm, producing formaldehyde. In the cytoplasm, formaldehyde condenses with either tetrahydrofolate (H_4F) or tetrahydromethanopterin (H_4MPT) to form the methylene derivatives of each C_1 carrier (3). Methylene-H_4F can either be assimilated into cell material via the serine cycle or oxidized to formate and then CO_2, whereas methylene-H_4MPT is solely oxidized to formate (19) and then CO_2.

Eighty-six genes involved in C_1 metabolism in *M. extorquens* AM1 have been identified elsewhere (4, 14). These include genes involved in methanol oxidation, methylamine oxidation, formaldehyde oxidation, formate oxidation, and the serine cycle, most of which are organized in a small number of large gene clusters. Since the known methylotrophy gene clusters have now been analyzed, a search for any remaining methylotrophy genes requires more global approaches. Past attempts to identify methylotrophy genes using transposon mutagenesis have been problematic and found only previously known genes (13, 25). Recently, however, mutagenesis with mini-Tn*5* identified novel genes involved in chloromethane utilization in the closely related strain *Methylobacterium chloromethanicum* CM4 (23). We have used another mini-Tn*5* derivative, Is*phoA*/hah-Tc (2, 6, 15), to perform a transposon mutagenesis screen in *M. extorquens* AM1 to search for previously unknown methylotrophy genes.

Transposon mutagenesis and mutant screen. Biparental matings were performed to introduce pCM639 (6) from *Escherichia coli* SM10 λ_{pir} (17) into wild-type *M. extorquens* AM1 (18) on nutrient agar (Difco, Detroit, Mich.) overnight at 30°C. Dilutions of the biparental mating mixtures were then plated onto minimal salt medium (1) containing succinate (15 mM) for growth with rifamycin (50 μg/ml) and tetracycline (10 μg/ml) for selection. Analogous experiments using the related transposons Tn*phoA* and mini-*Tn*5 Tc resulted in a significantly lower rate of transposition (C. J. Marx and M. E. Lidstrom, unpublished data). Fresh cultures of IS*phoA*/hah-Tc-containing mutants were then screened on plates containing methanol (125 mM) for growth defects. From the 24,000 transposon insertion colonies obtained on succinate, 55 methanol-defective mutant strains were isolated.

Phenotypic analysis. To further classify the metabolic defect in each strain, the methanol-defective mutants were tested for growth defects on methylamine (35 mM), formate (35 mM), or ethylamine (30 mM). Growth phenotypes were assessed after 3 to 5 days of growth based on colony size relative to wild-type *M. extorquens* AM1 and placed into three categories: wild type (++), intermediate (+), and trace to none (−). Defects in methanol oxidation will show a growth defect only on methanol, while defects in the serine cycle and H_4F or H_4MPT pathway will show a growth defect on all C_1 compounds. A portion of C_1 and C_2 metabolism overlaps in the conversion of acetyl-coenzyme A to glyoxylate, so defects in this part of metabolism will show growth defects on all C_1 and C_2 substrates (11, 12). Strains were also tested on medium containing both succinate and methanol (at 125 mM) to identify methanol-sensitive mutants, a phenotype specifically associated with defects in H_4MPT-dependent formaldehyde oxidation (8, 24). Of the 55 methanol-defective mutants, 22 were only defective on methanol, 11 were C_1 defective, 19 were C_1 and C_2 defective, and three were C_1 defective and methanol sensitive.

Identification of the transposon insertion sites. A semirandom two-step PCR procedure to amplify the junction at the site of transposon insertion into the chromosome was performed essentially as described elsewhere (2, 5, 15). Modifications included using boiled colony preparations or chromosomal DNA preparations as template, use of primer CEKG 2B

* Corresponding author. Mailing address: Department of Chemical Engineering, University of Washington, Box 351750, Seattle, WA 98195-1750. Phone: (206) 616-5282. Fax: (206) 616-5721. E-mail: lidstrom@u.washington.edu.

in the first amplification, and supplementation of the reaction mixtures with either 0.1 mg of bovine serum albumin/ml or 5% dimethyl sulfoxide. PCR products were purified using QIAquick PCR purification columns (Qiagen, Hilden, Germany) and sequenced (University of Washington Biochemistry Department DNA Sequencing Facility) using the following primer: 5′-AAACGGGAAAGGTTCCGTCCA-3′. Sequence analyses were performed using the ERGO website (www.ergo.integratedgenomics.com/ERGO/CGI; Integrated Genomics, Chicago, Ill.) and the analysis tools available through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

The 55 independent IS*phoA*/hah-Tc mutant strains with a methanol growth defect contained insertions into 31 different genes, 12 of which were represented more than once. In those cases in which multiple insertions were identified in the same gene, the insertions were located in different sites and the mutants exhibited a generally consistent mutant phenotype. Of the 86 previously characterized genes involved in methylotrophy, only 49 give clear growth defects on methanol. The others are either involved in the oxidation of methylamine (*mau* genes), are genes involved in redundant functions for which single mutants do not generate a growth phenotype, or carry out functions in both heterotrophic and methylotrophic metabolism so that null mutants cannot be isolated. The transposon mutagenesis identified 21 known methylotrophy genes (data not shown), which is about half of the methylotrophy genes for which methanol-defective mutants would be expected, indicating that the mutagenesis was not saturating. In addition to the 21 characterized genes, however, this screen also identified 10 previously uncharacterized methylotrophy genes (Table 1).

TABLE 1. Mutants containing IS*phoA*/hah-Tc insertions into novel methylotrophy genes[a]

Phenotype	Gene	Mutants	Growth on[b]:				
			M	Ma	F	Ea	S
Methanol defective	*mxdA*	S09-09	+	++	+	++	++
C_1 defective	*cbbR*	S90-71	+	+	+	++	++
		S203-07	+	+	+	++	++
		S235-75	+	++	++	++	++
		S252-39	+	+	+	++	++
	ftfL	S57-87	−	−	−	++	++
		S65-76	−	−	−	++	++
		S95-64	+	−	−	++	++
C_1 and C_2 defective	*meaC*	S06-17	+	−	−	−	+
	meaD	S33-90	−	−	+	+	++
		S203-14	+	−	+	+	++
		S235-22	+	+	+	++	++
	meaE	S109-30	−	+	+	+	++
	meaF	S151-59	+	+	+	+	++
	moaA	S06-42	+	+	−	+	++
	mobA	S165-21	−	+	−	++[c]	++
C_1 defective and methanol sensitive	*dmrA*	S64-67	−	−	+	+	+

[a] The interrupted gene, corresponding mutant strain(s), and growth phenotype are indicated.
[b] M, methanol; Ma, methylamine; F, formate; Ea, ethylamine; and S, succinate.
[c] Mutant S165-21 grew slowly on ethanol (20 mM).

Ten novel methylotrophy genes. The mutants containing insertions into the 10 novel methylotrophy genes fell into four different mutant classes (Table 1). Only one methanol-defective strain contained an insertion into a novel gene. This open reading frame (ORF), designated here *mxdA* (methanol oxidation, cluster D), is unlinked to previously known methylotrophy genes. The predicted amino acid sequence of MxdA has 60% identity and 77% similarity to a putative dehydrogenase of *Mesorhizobium loti* (GenBank accession no. BAB50156) and 52% identity and 68% similarity to the putative zinc-binding alcohol dehydrogenase encoded by *yhdH* of *E. coli* (GenBank accession no. P26646).

Seven of the C_1-defective mutants contained insertions into two previously uncharacterized genes that are unlinked to known methylotrophy genes (Table 1). Four independent mutants were obtained with insertions into an ORF with significant similarity to *cbbR* (LysR-type transcriptional regulator of autotrophy). The predicted amino acid sequence of the *M. extorquens* AM1 CbbR has 44% identity and 61% similarity to the CbbR in *Xanthobacter flavus* (GenBank accession no. P25545). Three mutants were also found with insertions into an ORF that is predicted to encode formate-tetrahydrofolate ligase (FtfL). The *M. extorquens* AM1 FtfL has a predicted amino acid sequence with 65% identity and 78% similarity to the FtfL of *M. loti* (GenBank accession no. BAB49812).

Eight C_1- and C_2-defective mutants were isolated with insertions into six genes previously uncharacterized in *M. extorquens* AM1 and unlinked to previously known C_1 genes. Two C_1- and C_2-defective mutants were found to contain insertions into genes apparently involved in molybdenum cofactor biosynthesis. Mutant S06-42 contains an insertion into an ORF with significant similarity to *moaA* (molybdenum cofactor biosynthesis, protein A), which encodes a protein with 73% identity and 83% similarity to the MoaA of *Brucella melitensis* (GenBank accession no. AAL52200). Mutant S165-21 contains an insertion into an ORF with significant similarity to *mobA* (molybdopterin-guanine dinucleotide biosynthesis protein A), which has a predicted amino acid sequence with 56% identity and 64% similarity to the MobA from *Rhodobacter sphaeroides* (GenBank accession no. CAA70754). Additionally, four C_1- and C_2-defective mutants were obtained in genes of uncertain function, here designated *mea* genes (methyl and ethyl assimilation defective). This designation has been used previously for genes required for C_1 and C_2 growth (22). Mutant S06-17 contains an insertion into an ORF, here designated *meaC*, which encodes a conserved hypothetical protein with 67% identity and 80% similarity to a MaoC family protein of *Caulobacter crescentus* (GenBank accession no. AAK25618). Three mutants were isolated with an insertion into an ORF, here designated *meaD*, encoding another conserved hypothetical protein. The predicted amino acid sequence of *meaD* has 71% identity and 82% similarity to an ortholog in *B. melitensis*

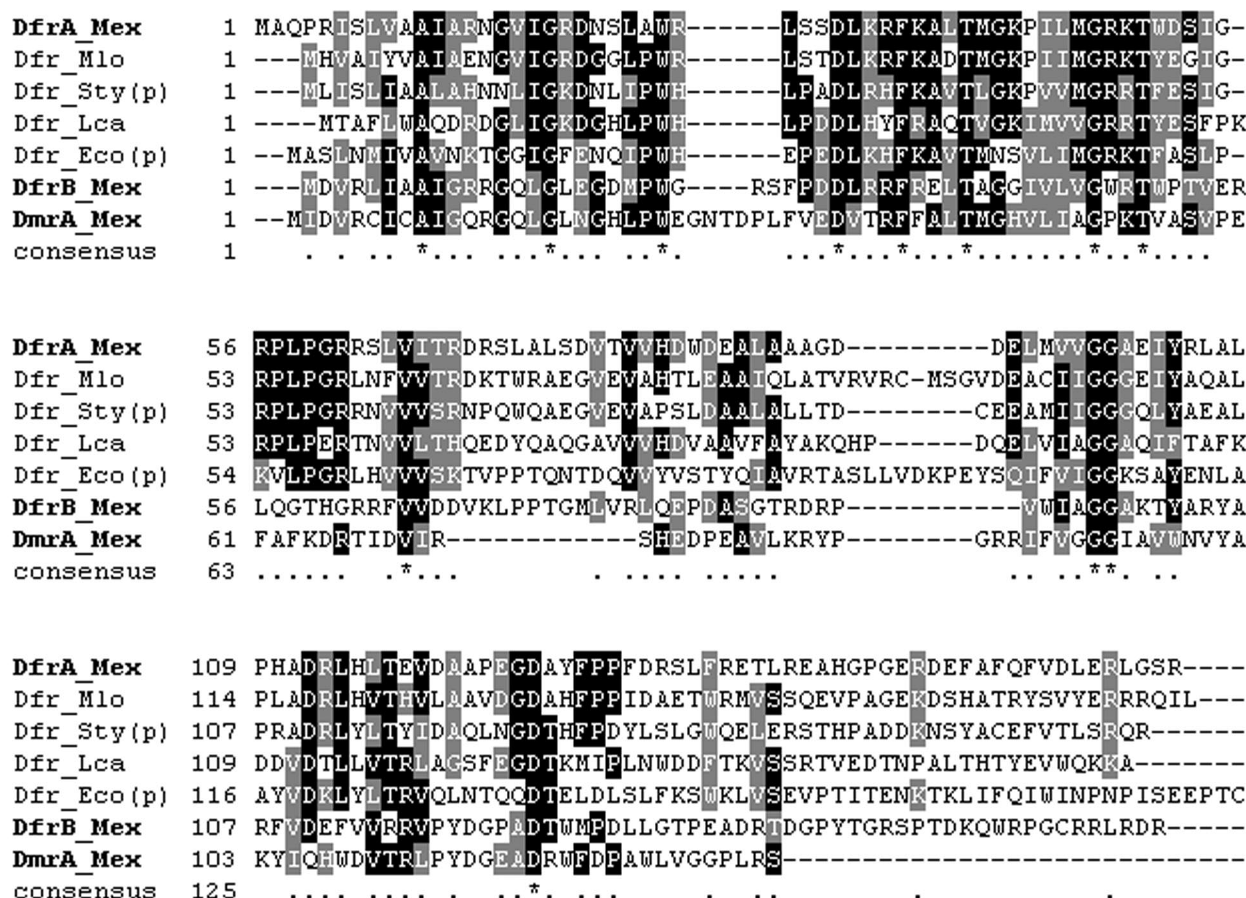

FIG. 1. Multiple-sequence alignment of DHFR amino acid sequences. DHFR homologs encoded by *M. extorquens* AM1 are indicated in boldface. Sequences used include DfrA_Mex (*M. extorquens* AM1, GenBank accession no. AY093432), Dfr_Mlo (*M. loti*, GenBank accession no. BAB48827), Dfr_Sty(p) (*S. enterica* serovar Typhimurium plasmid pAZ1, AAA25550), Dfr_Lca (*L. casei*, GenBank accession no. P00381), Dfr_Eco(p) (*E. coli* plasmid pCJ001, GenBank accession no. A49788), DfrB_Mex (*M. extorquens* AM1, GenBank accession no. AY093433), and DmrA_Mex (putative dihydromethanopterin reductase of *M. extorquens* AM1, GenBank accession no. AYO93431). Alignment was constructed using Clustal W (www.es.embnet.org/Doc/phylodendron/clustal-form.html) and Shadebox (www.ch.embnet.org/software/BOX_form.html).

(GenBank accession no. AAL51247) and 43% identity and 56% similarity to the PduO protein of *Salmonella enterica* serovar Typhimurium LT2 (GenBank accession no. AAD39014). Mutants S109-30 and S151-59 contain insertions into ORFs, here designated *meaE* and *meaF*, which encode small hypothetical proteins with no significant similarity to other known proteins.

Finally, one C_1-defective and methanol-sensitive mutant strain (S64-67) was identified in a previously uncharacterized ORF unlinked to all known methylotrophy genes. Previously, this phenotype had only been observed for *M. extorquens* AM1 mutants defective for the H_4MPT-dependent enzymes *fae* (encodes formaldehyde-activating enzyme) (24) and *mtdB* (encodes methylene-H_4MPT dehydrogenase) (8) and is believed to be caused by the inability of these mutant strains to detoxify formaldehyde. This ORF, here designated *dmrA* (putative dihydromethanopterin reductase, see below), encodes a protein (GenBank accession number AY093431) that has 26% identity and 44% similarity to the dihydrofolate reductase (DHFR) of *Lactobacillus casei* (GenBank accession no. P00381) (Fig. 1). In order to better understand functions required for formaldehyde oxidation, this mutant was chosen for further study.

Generation of a Δ*dmrA*::*kan* mutant strain and phenotypic analysis. In order to confirm that the IS*phoA*/hah-Tc insertion into the gene designated *dmrA* is responsible for the methanol-sensitive mutant phenotype of the S64-67 strain, a directed Δ*dmrA*::*kan* mutant was generated. The chromosomal regions directly upstream and downstream of *dmrA* were amplified by PCR, and the resulting products were cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.) to generate pCM207 and pCM208, respectively. The 0.6-kb *Bgl*II-*Not*I fragment from pCM207 was cloned between the *Bgl*II and *Not*I sites of the new broad-host-range allelic exchange vector pCM184 (16) to create pCM211. The 0.6-kb *Sac*II-*Sac*I fragment from pCM208 was then inserted between the corresponding sites of pCM211 to generate pCM212, which was then transferred into wild-type *M. extorquens* AM1 by conjugation using the helper strain *E. coli* S17-1 (20). Kanamycin-resistant transconjugants were screened for tetracycline sensitivity, indicating a double-crossover event resulting in a null mutant genotype. PCR analysis confirmed the generation of a Δ*dmrA*::*kan* strain, designated CM212K.1.

The Δ*dmrA*::*kan* strain, CM212K.1, was indistinguishable from the transposon mutant S64-67 in all phenotypic charac-

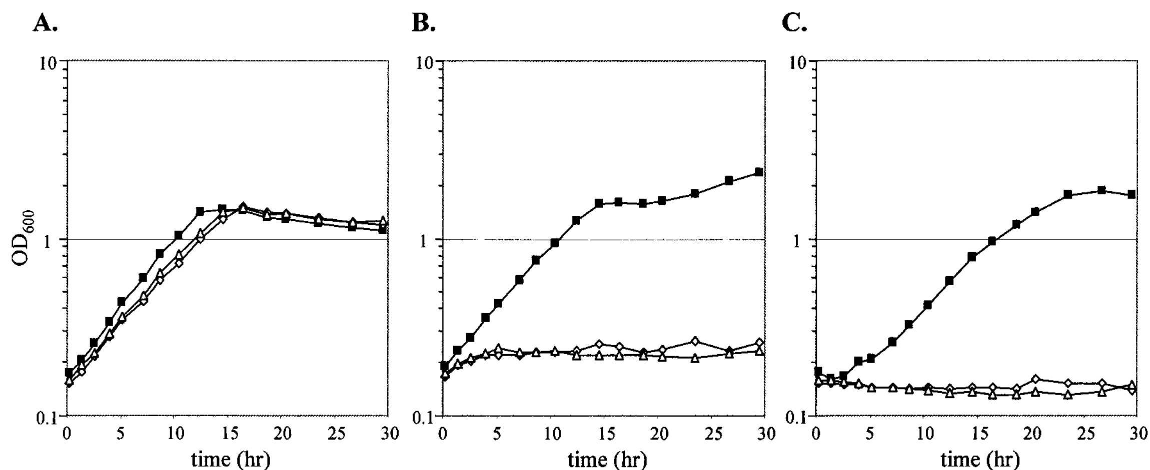

FIG. 2. Growth of *M. extorquens* AM1 strains pregrown in succinate, harvested, and resuspended in media containing succinate (A), succinate and methanol (B), or methanol (C). Strains examined are wild type (■), and the *dmrA* mutants are S64-67 (◇) and CM212K.1 (△).

terizations performed (Fig. 2, data not shown). The MIC of methanol for the *dmrA* mutants S64-67 and CM212K.1 was determined by comparing the rate and extent of colony formation of wild-type *M. extorquens* AM1. Methanol at final concentrations ranging from 125 mM to 50 nM was added to the plates containing succinate immediately prior to the addition of the molten agar. Because an undetermined fraction of the methanol will volatilize, the reported MIC of methanol is a maximum value. The MIC of methanol for S64-67 and CM212K.1 was 1 μM during growth on succinate plates. The MIC for the wild type is above 125 mM, and the MIC for mutants defective in *fae*, which encodes an enzyme catalyzing the first step of the H_4MPT-dependent formaldehyde oxidation pathway, is 50 μM methanol (24). This suggests that the insertion into *dmrA* results in a severe defect in formaldehyde oxidation.

The growth defect of *dmrA* mutant strains is also readily observed in liquid culture. Mid-exponential-phase cultures of wild-type *M. extorquens* AM1, S64-67, and CM212K.1 were centrifuged and resuspended to an optical density at 600 nm of 0.15 into fresh media containing either succinate, succinate plus methanol, or methanol alone (Fig. 2). S64-67 and CM212K.1 grew essentially like the wild type in succinate but did not grow in methanol. In flasks containing succinate and 125 mM methanol, growth arrested after about 5 h and did not increase further. Even after 48 h, no additional increase in optical density was observed for the *dmrA* mutant strains (data not shown).

Complementation analysis. As a final test that the C_1-defective and methanol-senstive phenotype is due to loss of *dmrA* function, a plasmid expressing *dmrA* from *lacZp* was constructed for complementation analysis. The coding region of *dmrA* was amplified by PCR and cloned into pCR2.1 (Invitrogen) to produce pCM185. The 0.4-kb *Hin*dIII-*Bgl*II fragment of pCM185 was cloned between the *Hin*dIII and *Bam*HI sites of pCM62 to produce pCM186. Introduction of pCM186 into either S64-67 or CM212K.1 using the helper plasmid pRK2073 (7) resulted in wild-type growth on methanol or succinate plus methanol. These results confirm that the loss of *dmrA* is responsible for the methanol-sensitive mutant phenotype observed in both S64-67 and CM212K.1.

Multiple DHFR homologs present in *M. extorquens* AM1. If *dmrA* encoded DHFR, interruption by mutation should be lethal due to the central role of DHFR in biosynthetic reactions involving C_1 units. However, the genome sequence contains three ORFs with identity to DHFR. One of these ORFs, here designated *dfrA*, has a predicted amino acid sequence significantly more similar to other DHFRs than are the other two (Fig. 1). The DfrA sequence (GenBank accession no. AY093432) has 50% identity and 63% similarity to the DHFR present in the closely related α-proteobacterium *M. loti* (GenBank accession no. BAB48827). Additionally, *dfrA* is located directly downstream of an apparent thymidylate synthase, a gene organization that is conserved across a wide variety of bacteria. These sequence analyses suggest that *dfrA* encodes the general DHFR activity.

Antibiotics such as trimethoprim and methotrexate inhibit bacteria through the inhibition of DHFR activity. Resistance to trimethoprim is most often accomplished by expression of an alternate antibiotic-resistant DHFR (21). Because *Methylobacterium* strains generally exhibit resistance to this class of antibiotics (9), we determined whether *dmrA* was involved. Wild-type *M. extorquens* AM1, S64-67, and CM212K.1 were grown on plates containing up to 400 μg of trimethoprim/ml. No differences in growth were observed, indicating that the *dmrA* gene product is not required for trimethoprim resistance. The third ORF with identity to DHFR, here designated *dfrB*, has a predicted amino acid sequence (GenBank accession number AY093433) with 25% identity and 47% similarity to the trimethoprim-resistant DHFR found on the *E. coli* plasmid pCJ001 (10) (Fig. 1). Mutational and biochemical analyses will be required to assess the role of *dfrA* and *dfrB* in *M. extorquens* AM1.

Suggested role for DmrA in methylotrophy. The *dmrA* mutants have a C_1-defective and methanol-sensitive phenotype that has been associated with defects in H_4MPT-dependent formaldehyde oxidation (8, 24). Given the similarity of the predicted DmrA protein sequence to that of DHFR, we sug-

gest that *dmrA* encodes dihydromethanopterin reductase. If so, this would represent a novel enzymatic activity analogous to DHFR but functioning with H_4MPT rather than with H_4F:

$$H_2MPT + NADPH \rightleftharpoons H_4MPT + NADP^+$$

If this is the function of DmrA, it would explain the severe methanol sensitivity of the mutant, since the entire H_4MPT pathway would be inoperable with no H_4MPT in the cell. Consistent with this hypothesis, an apparent *dmrA* ortholog was found in the genome sequence of *Methylococcus capsulatus* Bath (www.jgi.doe.gov/JGI_microbial/html/index.html), a methanotrophic bacterium that contains the H_4MPT pathway, but not in any other available genome sequences (L. Chistoserdova, C. J. Marx, and M. E. Lidstrom, unpublished data). Furthermore, it has recently been determined that *M. extorquens* AM1 *dmrA* mutants fail to synthesize H_4MPT (S. Wyles and M. Rasche, personal communication). Detailed biochemical characterization will be required to test our hypothesis that *dmrA* encodes a dihydromethanopterin reductase.

Conclusions. The results presented here represent the first successful application of transposon mutagenesis to the study of *M. extorquens* AM1 and bring the total number of known methylotrophy genes in this organism to 96. In addition to the putative dihydromethanopterin reductase, nine other novel methylotrophy genes have been identified and are presently under investigation in our laboratory. Since only about half of the known methylotrophy genes were identified in the mutagenesis screen in each of the four mutant categories, it is expected that several more methylotrophy genes are as yet undiscovered. New approaches involving expression microarrays and proteomics may uncover new methylotrophy genes, and these experiments are presently in progress. It is clear, however, that the ability to grow on C_1 compounds requires a substantial genetic investment by the organism, involving many dozens of genes.

We thank S. Wyles and M. Rasche for communicating their unpublished data, D. D'Argenio, L. Gallagher, and C. Manoil for providing the IS*phoA*/hah-Tc delivery strain, C. Speake for her help with the mutagenesis, H. Rothfuss for computer support, Y. Okubo for her assistance with the growth experiments, and L. Chistoserdova and N. Korotkova for useful discussions.

This work was supported by a grant from the NIH (GM58933).

REFERENCES

1. **Attwood, M. M., and W. Harder.** 1972. A rapid and specific enrichment procedure for *Hyphomicrobium* spp. Antonie Leeuwenhoek **38:**369–377.
2. **Bailey, J., and C. Manoil.** 2002. Genome-wide internal tagging of bacterial exported proteins. Nat. Biotechnol. **20:**839–842.
3. **Chistoserdova, L., J. A. Vorholt, R. K. Thauer, and M. E. Lidstrom.** 1998. C_1 transfer enzymes and coenzymes linking methylotrophic bacteria and methanogenic Archaea. Science **281:**99–102.
4. **Chistoserdova, L. V.** 1996. Metabolism of formaldehyde in *M. extorquens* AM1. Molecular genetic analysis and mutant characterization, p. 16–24. *In* M. E. Lidstrom and F. R. Tabita (ed.), Microbial growth on C_1 compounds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
5. **Chun, K. T., H. J. Edenberg, M. R. Kelley, and M. G. Goebl.** 1997. Rapid amplification of uncharacterized transposon-tagged DNA sequences from genomic DNA. Yeast **13:**233–240.
6. **D'Argenio, D. A., L. A. Gallagher, C. A. Berg, and C. Manoil.** 2001. *Drosophila* as a model host for *Pseudomonas aeruginosa* infection. J. Bacteriol. **183:**1466–1471.
7. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
8. **Hagemeier, C. H., L. Chistoserdova, M. E. Lidstrom, R. K. Thauer, and J. A. Vorholt.** 2000. Characterization of a second methylene tetrahydromethanopterin dehydrogenase from *Methylobacterium extorquens* AM1. Eur. J. Biochem. **267:**3762–3769.
9. **Holloway, B. W.** 1984. Genetics of methylotrophs, p. 87–106. *In* C. Hou (ed.), Methylotrophs: microbiology, biochemistry and genetics. CRC Press, Boca Raton, Fla.
10. **Jansson, C., and O. Sköld.** 1991. Appearance of a new trimethoprim resistance gene, *dhfrIX*, in *Escherichia coli* from swine. Antimicrob. Agents Chemother. **35:**1891–1899.
11. **Korotkova, N., L. Chistoserdova, V. Kuksa, and M. E. Lidstrom.** 2002. Glyoxylate regeneration pathway in the methylotroph *Methylobacterium extorquens* AM1. J. Bacteriol. **184:**1750–1758.
12. **Korotkova, N., and M. E. Lidstrom.** 2001. Connection between poly-β-hydroxybutyrate biosynthesis and growth on C_1 and C_2 compounds in the methylotroph *Methylobacterium extorquens* AM1. J. Bacteriol. **183:**1038–1046.
13. **Lee, K. E., S. Stone, P. M. Goodwin, and B. W. Holloway.** 1991. Characterization of transposon insertion mutants of *Methylobacterium extorquens* AM1 (*Methylobacterium* strain AM1) which are defective in methanol oxidation. J. Gen. Microbiol. **137:**895–904.
14. **Lidstrom, M. E.** 2 November 2001, posting date. Aerobic methylotrophic prokaryotes. *In* M. Dworkin (ed.), The prokaryotes. link.springer.de/link/service/books/10125/. [Online.]
15. **Manoil, C.** 2000. Tagging exported proteins using Escherichia coli alkaline phosphatase gene fusions. Methods Enzymol. **326:**35–47.
16. **Marx, C. J., and M. E. Lidstrom.** 2002. A broad-host-range *cre-lox* system for antibiotic marker recycling in Gram-negative bacteria. BioTechniques **33:**1062–1067.
17. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
18. **Nunn, D. N., and M. E. Lidstrom.** 1986. Isolation and complementation analysis of 10 methanol oxidation mutant classes and identification of the methanol dehydrogenase structural gene of *Methylobacterium* sp. strain AM1. J. Bacteriol. **166:**581–590.
19. **Pomper, B. K., O. Saurel, A. Milon, and J. A. Vorholt.** 2002. Generation of formate by the formyltransferase/hydrolase complex (Fhc) from *Methylobacterium extorquens* AM1. FEBS Lett. **523:**133–137.
20. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784–791.
21. **Skold, O., and A. Widh.** 1974. A new dihydrofolate reductase with low trimethoprim sensitivity induced by an R factor mediating high resistance to trimethoprim. J. Biol. Chem. **249:**4324–4325.
22. **Smith, L. M., W. G. Meijer, L. Dijkhuizen, and P. M. Goodwin.** 1996. A protein having similarity with methylmalonyl-CoA mutase is required for the assimilation of methanol and ethanol by *Methylobacterium extorquens* AM1. Microbiology **142:**675–684.
23. **Vannelli, T., A. Studer, M. Kertesz, and T. Leisinger.** 1998. Chloromethane metabolism by *Methylobacterium* sp. strain CM4. Appl. Environ. Microbiol. **64:**1933–1936.
24. **Vorholt, J. A., C. J. Marx, M. E. Lidstrom, and R. K. Thauer.** 2000. Novel formaldehyde-activating enzyme in *Methylobacterium extorquens* AM1 required for growth on methanol. J. Bacteriol. **182:**6645–6650.
25. **Whitta, S., M. I. Sinclair, and B. W. Holloway.** 1985. Transposon mutagenesis in *Methylobacterium* AM1 (*Pseudomonas* AM1). J. Gen. Microbiol. **131:**1547–1549.