

Development of an insertional expression vector system for *Methylobacterium extorquens* AM1 and generation of null mutants lacking *mtdA* and/or *fch*

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Over the past few years, the genetic 'toolkit' available for use with *Methylobacterium extorquens* AM1 has expanded significantly. Here a further advance is presented and demonstrated, an insertional expression system that allows expression of genes from a stable, unmarked chromosomal locus. This system has been used to better understand the role of the tetrahydrofolate (H₄F) pathway in methylotrophy. Previously, it has not been possible to generate null mutants lacking either *mtdA* (encoding an NADP-dependent methylene-H₄F/methylene-tetrahydromethanopterin dehydrogenase) or *fch* (encoding methenyl-H₄F cyclohydrolase). An unmarked strain was generated that expressed the analogous *foID* gene (encoding a bifunctional NADP-dependent methylene-H₄F dehydrogenase/methenyl-H₄F cyclohydrolase) from *Methylobacterium chloromethanicum* CM4^T. In this strain, null mutants could be obtained that grew normally on multicarbon substrates but were defective for growth on C₁ substrates. Additionally, null mutants of *mtdA* and/or *fch* could also be generated in the wild-type by supplementing the succinate medium with formate. These strains were unable to grow on C₁ compounds but were not methanol-sensitive. These approaches have demonstrated that the apparent essentiality of *mtdA* and *fch* is due to the need for formyl-H₄F for biosynthesis of purines and other compounds, and have provided clear genetic evidence that the H₄F pathway is required for methylotrophy.

Received 22 June 2003

Revised 8 September 2003

Accepted 9 September 2003

INTRODUCTION

As genome sequence data become available for an ever-increasing number of microbial species, the need for sophisticated genetic tools to experimentally validate new hypotheses also grows. Therefore, improved genetic tools are needed, especially for the many bacteria for which genome sequence data are now available but which do not have well-established genetic systems. One such organism that has lacked facile genetic tools is the facultative methylotroph *Methylobacterium extorquens* AM1. The genome sequence of this organism is currently being completed (http://www.integratedgenomics.com/genome_releases.html#list6); already the available sequence data have facilitated many key discoveries concerning the central metabolism of this organism (Chistoserdova *et al.*, 2003). Recently, the breadth of genetic tools available for *M. extorquens* AM1 has expanded significantly, including

the development of small broad-host-range plasmids for cloning, expression and promoter-probing (Marx & Lidstrom, 2001), the generation of a *cre-lox*-based allelic exchange system for the generation of unmarked mutant strains (Marx & Lidstrom, 2002) and the successful application of transposon mutagenesis (Marx *et al.*, 2003b).

One problem of central metabolism in methylotrophy that requires a new genetic tool involves formaldehyde utilization. In *M. extorquens* AM1, the formaldehyde produced from the primary oxidation of C₁ substrates condenses with either tetrahydrofolate (H₄F) or tetrahydromethanopterin (H₄MPT) to form the respective methylene derivatives (Fig. 1; reviewed by Vorholt, 2002). The reaction with H₄MPT, a folate analogue long thought unique to methanogenic archaea (Chistoserdova *et al.*, 1998), is catalysed by the formaldehyde-activating enzyme, Fae, or can occur spontaneously (Vorholt *et al.*, 2000). Methylene-H₄MPT is converted to methenyl-H₄MPT and then formyl-H₄MPT through the action of the NAD(P)-dependent methylene-H₄MPT dehydrogenases MtdA (Vorholt *et al.*, 1998) and MtdB (Hagemeier *et al.*, 2000), and methenyl-H₄MPT cyclohydrolase, Mch (Pomper *et al.*, 1999). The C₁ unit is then hydrolysed by the formyltransferase-hydrolase complex, Fhc (Pomper & Vorholt, 2001), to produce formate and free H₄MPT

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Abbreviations: *gfp*/GFP, green fluorescent protein; H₄F, tetrahydrofolate; H₄MPT, tetrahydromethanopterin; MCS, multiple-cloning site; *P* prefix, promoter; *t* prefix, terminator of transcription.

The GenBank accession numbers for the vector sequences reported in this article are AY307999 (pCM168) and AY308000 (pCM172).

(Pomper *et al.*, 2002). Mutants defective for the H₄MPT pathway fail to grow on C₁ substrates and are sensitive to the presence of compounds that lead to the production of formaldehyde (Hagemeyer *et al.*, 2000; Marx *et al.*, 2003b; Vorholt *et al.*, 2000), leading to the suggestion that the H₄MPT-linked pathway serves as the primary formaldehyde oxidation and detoxification pathway in *M. extorquens* AM1.

The formaldehyde that condenses with H₄F to form methylene-H₄F serves as the C₁ donor for assimilation through the serine cycle (reviewed by Lidstrom, 2001). Methylene-H₄F may also be converted to methenyl-H₄F, formyl-H₄F and, ultimately, free formate and H₄F through the action of an NADP-dependent methylene-H₄F dehydrogenase, MtdA (Chistoserdova & Lidstrom, 1994b; Vorholt *et al.*, 1998), methenyl-H₄F cyclohydrolase, Fch (Chistoserdova & Lidstrom, 1994a; Pomper *et al.*, 1999), and formyl-H₄F ligase, FtlL (Marx *et al.*, 2003b), respectively (Fig. 1). The formate produced through either the H₄F or the H₄MPT C₁ transfer pathway may then be oxidized to CO₂ by formate dehydrogenases (Laukel *et al.*, 2003).

The enzymes of the H₄F pathway are found at high specific activities during heterotrophic growth and are generally present at three- to fourfold higher levels during growth on C₁ compounds (Chistoserdova & Lidstrom, 1994b; Marison & Attwood, 1982; Pomper *et al.*, 1999; Vorholt *et al.*, 1998). This finding led to the original suggestion that the H₄F pathway functions as the primary formaldehyde oxidation route during methylo trophy (Marison & Attwood, 1982). The discovery of the H₄MPT pathway in the methylo trophic bacteria and archaea (Chistoserdova *et al.*, 1998) and the elucidation of its critical role in formaldehyde oxidation (Chistoserdova *et al.*, 1998; Hagemeyer *et al.*, 2000; Marx *et al.*, 2003b; Vorholt *et al.*, 2000) have brought this suggestion into question. It has also been suggested that the H₄F pathway potentially could function in the reductive direction to produce methylene-H₄F from formate during methylo trophic growth (Pomper *et al.*, 2002; Vorholt, 2002). Recently, a C₁-defective mutant with a transposon insertion into an *ftfL* homologue has been obtained (Marx *et al.*, 2003b). Unfortunately, a complete understanding of the role of the H₄F pathway has been complicated by the inability to obtain null mutants of *mtdA* or *fch* even during growth on succinate (Chistoserdova & Lidstrom, 1994a, b; Vorholt *et al.*, 1998), suggesting a role for these gene products in heterotrophic metabolism. Although mutants with a reduced activity of MtdA or Fch were obtained and these strains were found to be defective for growth on C₁ compounds, the presence of significant activities of the two gene products in these mutants makes the role of the H₄F pathway in methylo trophy and heterotrophy uncertain. It is possible that the requirement for this pathway during heterotrophic growth is due to the need for formyl-H₄F for biosynthesis. However, this does not explain the lack of growth on methanol, as formyl-H₄F can be synthesized from formate via the FtlL reaction during methylo trophy (Fig. 1).

In addition, MtdA differs from a standard methylene-H₄F dehydrogenase, in that it also has significant activity with methylene-H₄MPT (Fig. 1; Vorholt *et al.*, 1998). Therefore, it was not possible to rule out a role for MtdA in the H₄MPT pathway during heterotrophic and/or methylo trophic growth.

To define the role of the H₄F pathway in heterotrophy and methylo trophy, an approach was taken that required a new genetic tool, an insertional expression system that allows expression of genes from a stable, unmarked chromosomal locus. This system has been utilized to express *foldD*, which encodes a bifunctional NADP-dependent methylene-H₄F dehydrogenase/methenyl-H₄F cyclohydrolase that does not have activity with H₄MPT derivatives, from *Methylobacterium chloromethanicum* CM4^T in an unmarked *M. extorquens* AM1 strain. This allowed the generation of null mutants lacking *mtdA* and/or *fch*. Additionally, we found that null mutants of *mtdA* and/or *fch* could be generated in the wild-type by supplementing the medium with formate. These approaches have demonstrated that the apparent essentiality of *mtdA* and *fch* during growth on succinate is due to the need for formyl-H₄F and have clearly demonstrated the requirement for MtdA and Fch during methylo trophy.

METHODS

Bacterial strains and growth conditions. *M. extorquens* AM1 (Nunn & Lidstrom, 1986) strains were grown at 30 °C on a minimal salts medium (Attwood & Harder, 1972) containing carbon sources at the following concentrations: 35 mM formate; 125 mM methanol; 35 mM methylamine; 15 mM oxalate; or 15 mM succinate. *Escherichia coli* strains were grown on LB medium (Sambrook *et al.*, 1989). All strains and plasmids used in this study are described in Table 1. Antibiotics were added to the following final concentrations: 50 µg ampicillin ml⁻¹; 50 µg kanamycin ml⁻¹; 50 µg rifamycin ml⁻¹; 35 µg streptomycin ml⁻¹; and 10 µg tetracycline ml⁻¹. Chemicals were obtained from Sigma. Nutrient agar and Bacto-agar were obtained from Difco.

Construction of *M. extorquens* AM1 insertional expression vector. A 1.2 kb *NruI*-*NcoI* fragment containing the erythromycin cassette from pMTL23E (Purdy *et al.*, 2002) was blunted using T4 DNA polymerase and inserted into the *katA* gene present in pLC11.28 (Chistoserdova & Lidstrom, 1996) which had been cut with *NruI* and *PstI* and also blunted, to generate pCM116. Attempts to use erythromycin as a selective marker in *M. extorquens* AM1 were unsuccessful (C. J. Marx & M. E. Lidstrom, unpublished data). To preserve useful cloning sites in the final insertion vectors, pCM116 was cut with *EcoRI* and *NdeI*, blunted and self-ligated to produce pCM117, which was subsequently cut with *HindIII*, blunted and re-ligated to produce pCM118. A construct containing *tetAR* flanked by *loxP* sites was constructed by inserting the 2.3 kb *XmnI*-*StuI* fragment from pCM50 (Marx & Lidstrom, 2001) into the blunted *XbaI* site of pLox1 (Palmeros *et al.*, 2000) to produce pCM159. Sequencing of the pCM159 construct revealed that an intact *XbaI* site remained on the *tetR* side of the cassette. Fortunately, the resulting sequence is also recognized by Dam methylase, leading to methylation that blocks *XbaI* cleavage. The 2.6 kb *NcoI*-*ScaI* fragment from pCM159 was ligated between the *NcoI* and *HincII* sites of pCM118 to produce pCM165. To remove further useful restriction sites from the insertional vector, pCM165

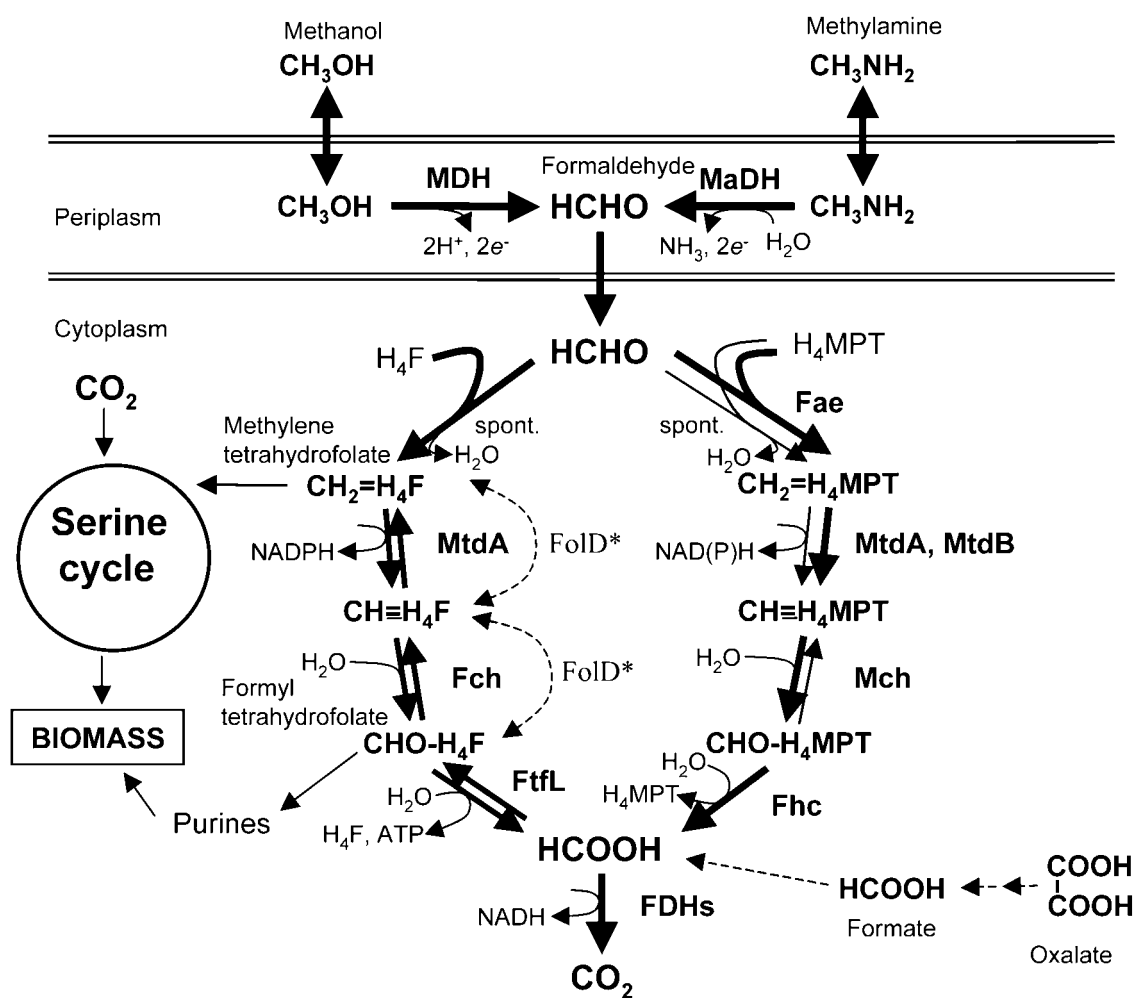


Fig. 1. Methylotrophic metabolism in *M. extorquens* AM1. The activities performed by FoID of *M. chloromethanicum* CM4^T are indicated with the dashed arrows. Those reactions that can occur spontaneously (spont.) or are catalysed by two enzymes are indicated. The thin arrows leading from methylene-H₄F and formyl-H₄F to biomass represent biosynthetic reactions directly involving these two C₁-H₄F derivatives. The entry point for formate and oxalate in C₁ metabolism is also depicted. MDH, methanol dehydrogenase; MaDH, methylamine dehydrogenase; MtdA, NADP-dependent methylene-H₄F/methylene-H₄MPT dehydrogenase; Fch, methenyl-H₄F cyclohydrolase; FtfL, formate-H₄F ligase; FDHs, formate dehydrogenases; Fae, formaldehyde-activating enzyme; MtdB, NAD(P)-dependent methylene-H₄MPT dehydrogenase; Mch, methenyl-H₄MPT cyclohydrolase; Fhc, formyltransferase-hydrolase complex.

was digested with *Nco*I and *Nde*I, blunted and self-ligated to produce pCM166, which was cut with *Nsi*I, blunted and self-ligated to produce pCM167.

The *E. coli* *rrnB* terminator (*t_{rrnB}*) from pCM130 (Marx & Lidstrom, 2001) and the T7 terminator (*t_{T7}*) from pET-3a (Novagen) were amplified by PCR and cloned into pCR2.1 (Invitrogen) to generate pCM119 and pCM120, respectively. The 0.5 kb *Bam*HI-*Hinc*II fragment from pCM119 containing *t_{rrnB}* was ligated into the same sites of pMTL23 to generate pCM123. The 0.4 kb *Nru*I-*Xho*I fragment from pCM120 was inserted into the same sites of pCM123 to generate pCM124. A terminator-flanked cassette bearing *P_{mxoF}* was generated by inserting the 0.3 kb *Nru*I-*Hind*III fragment from pCM80 (Marx & Lidstrom, 2001) between the *Hinc*II and *Hind*III sites of pCM124 to generate pCM126.

The insertional vector backbone pCM167 contains unique *Bgl*II and

*Sma*I sites between which terminator-flanked cassettes were inserted as *Bam*HI-*Nru*I fragments. The insertional cloning vector pCM168 contains the 0.9 kb fragment from pCM124, whereas the insertional expression vector pCM172 contains the 1.2 kb pCM126 fragment. Additionally, a construct was made to generate a *katA*:*kan* strain that allows the identification of those recombinants with a complete allelic exchange at the *katA* locus. The 3.4 kb *Eco*RI-*Sph*I fragment from pLC1128.Km (Chistoserdova & Lidstrom, 1996) was blunted and cloned into the *Sma*I site of pAYC61 (Chistoserdov *et al.*, 1994) to generate pCM82.

Construction of plasmids to test the utility of the insertional systems. To test the efficiency of transcription termination afforded by *t_{rrnB}* or *t_{T7}* in *M. extorquens* AM1, *P_{mxoF}* present in the 0.4 kb *Bam*HI-*Eco*RI fragment from pCM27 (Marx & Lidstrom, 2001) was introduced between the same sites upstream of the reporter gene *xylE* in pCM76 (Marx & Lidstrom, 2001) to generate pCM77. The

Table 1. *M. extorquens* AM1 strains and plasmids used in this study

Strain	Description	Source or reference
CM82.1	<i>katA::kan</i>	This study
CM168.1	<i>katA::(loxP-t_{rrnB}-MCS-t_{T7})</i>	This study
CM168T.1	<i>katA::(loxP-tetAR-loxP-t_{rrnB}-MCS-t_{T7})</i>	This study
CM172.1	<i>katA::(loxP-t_{rrnB}-P_{mxoF}-MCS-t_{T7})</i>	This study
CM172T.1	<i>katA::(loxP-tetAR-loxP-t_{rrnB}-P_{mxoF}-MCS-t_{T7})</i>	This study
CM174.1	<i>katA::(loxP-t_{rrnB}-P_{mxoF}-gfp-t_{T7})</i>	This study
CM174T.1	<i>katA::(loxP-tetAR-loxP-t_{rrnB}-P_{mxoF}-gfp-t_{T7})</i>	This study
CM216.1	Δ <i>fffL</i>	Marx <i>et al.</i> (2003a)
CM219.1	<i>katA::(loxP-t_{rrnB}-P_{mxoF}-fold-t_{T7})</i>	This study
CM219T.1	<i>katA::(loxP-tetAR-loxP-t_{rrnB}-P_{mxoF}-fold-t_{T7})</i>	This study
CM219-275K.1	<i>katA::(loxP-t_{rrnB}-P_{mxoF}-fold-t_{T7}), Δ<i>mtdA::kan</i></i>	This study
CM219-279K.1	<i>katA::(loxP-t_{rrnB}-P_{mxoF}-fold-t_{T7}), Δ<i>fch::kan</i></i>	This study
CM219-280K.1	<i>katA::(loxP-t_{rrnB}-P_{mxoF}-fold-t_{T7}), Δ<i>mtdA-fch::kan</i></i>	This study
CM275K.1	Δ <i>mtdA::kan</i>	This study
CM279K.1	Δ <i>fch::kan</i>	This study
CM280K.1	Δ <i>mtdA-fch::kan</i>	This study
AM1	Rif ^R derivative	Nunn & Lidstrom (1986)
Plasmid		
pAYC61	Allelic exchange vector	Chistoserdov <i>et al.</i> (1994)
pCM21	pCR2.1 with <i>gfp</i>	Marx & Lidstrom (2001)
pCM27	pCR2.1 with <i>P_{mxoF}</i>	Marx & Lidstrom (2001)
pCM50	Small IncP replicon	Marx & Lidstrom (2001)
pCM76	IncP replicon with <i>xylE</i>	Marx & Lidstrom (2001)
pCM77	pCM76 with <i>P_{mxoF}</i> upstream of <i>xylE</i>	This study
pCM80	<i>M. extorquens</i> AM1 expression vector (<i>P_{mxoF}</i>)	Marx & Lidstrom (2001)
pCM82	pAYC61 with <i>katA::kan</i> fragment from pLC1128.Km	This study
pCM116	pLC11.28 with <i>katA::erm</i>	This study
pCM117	pCM116 with restriction sites removed	This study
pCM118	pCM117 with a restriction site filled-in	This study
pCM119	pCR2.1 with <i>t_{rrnB}</i>	This study
pCM120	pCR2.1 with <i>t_{T7}</i>	This study
pCM121	pCM77 with <i>P_{mxoF}-t_{rrnB}-xylE</i>	This study
pCM122	pCM77 with <i>P_{mxoF}-t_{T7}-xylE</i>	This study
pCM123	pMTL23 with <i>t_{rrnB}</i>	This study
pCM124	pMTL23 with <i>t_{rrnB}-MCS-t_{T7}</i> cassette	This study
pCM126	pMTL23 with <i>t_{rrnB}-P_{mxoF}-MCS-t_{T7}</i> cassette	This study
pCM130	Broad-host-range promoter probe vector with <i>xylE</i>	Marx & Lidstrom (2001)
pCM158	Broad-host-range <i>cre</i> expression vector	Marx & Lidstrom (2002)
pCM159	pLox1 with <i>tetAR</i>	This study
pCM165	pCM118 with <i>loxP-tetAR-loxP</i>	This study
pCM166	pCM165 with restriction sites removed	This study
pCM167	pCM166 with a restriction site filled-in	This study
pCM168	pCM167 with <i>t_{rrnB}-MCS-t_{T7}</i> (insertional cloning vector)	This study
pCM172	pCM167 with <i>t_{rrnB}-P_{mxoF}-MCS-t_{T7}</i> (insertional expression vector)	This study
pCM174	pCM172 with <i>gfp</i>	This study
pCM184	Broad-host-range allelic exchange vector	Marx & Lidstrom (2002)
pCM201	pCR2.1 with <i>purU</i> from <i>M. chloromethanicum</i> CM4 ^T	This study
pCM202	pCR2.1 with <i>fold</i> from <i>M. chloromethanicum</i> CM4 ^T	This study
pCM203	pCM80 with <i>purU</i>	This study
pCM205	pCM80 with <i>purU-fold</i>	This study
pCM206	pCM172 with <i>purU-fold</i>	This study
pCM219	pCM172 with <i>fold</i>	This study
pCM272	pCR2.1 with <i>mtdA</i> upstream flank	This study

Table 1. cont.

Strain	Description	Source or reference
pCM273	pCR2.1 with <i>mtdA</i> downstream flank	This study
pCM274	pCM184 with <i>mtdA</i> downstream flank	This study
pCM275	pCM274 with <i>mtdA</i> upstream flank	This study
pCM276	pCR2.1 with <i>fch</i> upstream flank	This study
pCM277	pCR2.1 with <i>fch</i> downstream flank	This study
pCM278	pCM184 with <i>fch</i> downstream flank	This study
pCM279	pCM278 with <i>fch</i> upstream flank	This study
pCM280	pCM278 with <i>mtdA</i> upstream flank	This study
pCR2.1	PCR cloning vector	Invitrogen
pET-3a	<i>E. coli</i> overexpression vector	Novagen
pLC11.28	pUC19 with <i>kata</i> region	Chistoserdova & Lidstrom (1996)
pLC1128.Km	pLC11.28 with <i>kan</i> inserted into <i>kata</i>	Chistoserdova & Lidstrom (1996)
pLC410a	Large IncP plasmid containing <i>mtdA</i> and <i>fch</i>	Chistoserdova & Lidstrom (1994b)
pLox1	Construct with a pair of <i>loxP</i> sites	Palmeros <i>et al.</i> (2000)
pMTL23	ColE1 replicon with large MCS	Chambers <i>et al.</i> (1988)
pMTL23E	pMTL23 with erythromycin-resistance cassette	Purdy <i>et al.</i> (2002)
pRK2073	Helper plasmid expressing IncP <i>tra</i> functions	Figurski & Helinski (1979)

0.6 kb *Bam*HI–*Sph*I fragment from pCM119 and the 0.4 kb *Bam*HI–*Sph*I fragment from pCM120 were then ligated into the same sites of pCM77 between *P_{msaF}* and *xylE* to generate pCM121 and pCM122, respectively. The expression level afforded by pCM172 was examined by inserting the 0.8 kb *Hind*III–*Nsi*I fragment from pCM21 with *gfp* (green fluorescent protein) into the same sites of pCM172 to generate pCM174.

Construction of plasmids containing *folD*. As a functional test of the insertional expression vector, *folD* and *purU* from *M. chloromethanicum* CM4^T (Vannelli *et al.*, 1999) were cloned and introduced into pCM172. The coding regions of *purU* and *folD* were amplified from a chromosomal DNA preparation of *M. chloromethanicum* CM4^T by PCR and cloned into pCR2.1 (Invitrogen) to produce pCM201 and pCM202, respectively. Both constructs were sequenced to confirm no errors had been introduced. The 0.9 kb *Xba*I–*Kpn*I fragment from pCM201 was cloned into the same sites of pCM80 (Marx & Lidstrom, 2001) to generate pCM203; subsequently, the 1.0 kb *Kpn*I–*Sac*I fragment from pCM202 was introduced between the same sites of pCM203 to generate pCM205. The 1.9 kb *Xba*I–*Nsi*I fragment from pCM205 containing *purU*–*folD* was then inserted into the same sites of pCM172 to generate pCM206. A construct for expression of *folD* alone was made by self-ligating the 9.3 kb blunted pCM206 *Xba*I–*Asp*718I fragment to produce pCM219. The *purU* constructs were not used in this study.

Construction of donor plasmids to generate mutants defective for *mtdA* and/or *fch*. *M. extorquens* AM1 deletion mutants lacking *mtdA* and/or *fch* were generated using the allelic exchange vector pCM184 (Marx & Lidstrom, 2002). Approximately 0.5 kb regions upstream and downstream of each of these genes were amplified by PCR. The resulting *mtdA* flanks were introduced into pCR2.1 (Invitrogen) to generate pCM272 and pCM273; the *fch* flanks are contained in pCM276 and pCM277. The construct to generate Δ *mtdA*::*kan* mutants was made by introducing the 0.5 kb *Sac*I–*Age*I fragment from pCM273 between the corresponding sites of pCM184 to produce pCM274; subsequently, the 0.5 kb *Bgl*II–*Nde*I fragment from pCM272 was ligated into the same sites of pCM274 to produce pCM275. The construct to generate Δ *fch*::*kan* mutants was made by introducing the 0.6 kb *Apa*I–*Sac*I

fragment from pCM277 into the same sites of pCM184 to produce pCM278; subsequently, the 0.5 kb *Eco*RI–*Nde*I fragment from pCM276 was ligated into the same sites of pCM278 to produce pCM279. Finally, a construct to make Δ *mtdA*–*fch*::*kan* mutants was generated by introducing the 0.5 kb *Bgl*II–*Nde*I fragment from pCM272 into the same sites of pCM278 to produce pCM280.

Generation of mutant strains. Strains carrying insertion vectors were generated by electroporating the appropriate constructs into the *kata*::*kan* strain CM82.1 as described previously (Toyama *et al.*, 1998). Tetracycline-resistant transformants were then screened for kanamycin sensitivity. Unmarked (tetracycline-sensitive) insertion strains were generated using the *cre*-expressing plasmid pCM158 as described previously (Marx & Lidstrom, 2002). Mutants were generated in the various strain backgrounds by introducing the appropriate donor constructs by conjugation from *E. coli* S17-1 (Simon *et al.*, 1983) as described previously (Chistoserdov *et al.*, 1994). All deletion mutants and insertion strains were confirmed by diagnostic PCR analysis. Plasmids were introduced into the appropriate strains via triparental matings using the helper plasmid pRK2073 (Figurski & Helinski, 1979).

Phenotypic analyses of mutant strains. To compare the growth of wild-type *M. extorquens* AM1 with mutants in liquid medium, cultures were grown to mid-exponential phase, centrifuged and then resuspended in fresh medium containing the carbon source described. To test for sensitivity to methanol, methanol was added to one set of succinate flasks to the reported final concentration. Mutant phenotypes were also assessed on solid medium by comparing the relative rate of colony formation. All phenotypic analyses were performed at least twice.

Enzymic assays. NADP-dependent methylene-H₄F dehydrogenase (Chistoserdova & Lidstrom, 1994b), methenyl-H₄F cyclohydrolase (Pomper *et al.*, 1999), formyl-H₄F hydrolase (Nagy *et al.*, 1995) and catechol 2,3-dioxygenase (Kataeva & Golovleva, 1990) activities were assayed as described with extracts prepared from cell material that was harvested from exponential-phase cultures. The H₄MPT-dependent activity of MtdA was not assayed. *FolD* activity was determined in a strain lacking MtdA (CM219-275K.1) or by the difference between the *folD*-expressing strain and the wild-type.

Activities are reported in mU [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$] unless otherwise noted. Between culture variability in enzyme activities was less than 20%. Total protein content of the extracts was determined spectrophotometrically (Kalb & Bernlohr, 1977; Whitaker & Granum, 1980) using a Beckmann DU 640B spectrophotometer. GFP expression was assayed in whole cells by measuring the relative fluorescence per OD_{600} unit using a Shimadzu RF-5301 PC spectrofluorophotometer with excitation and emission wavelengths of 410 and 509 nm, respectively.

RESULTS

Development of an insertional expression vector system for use in *M. extorquens* AM1

An insertional expression system for *M. extorquens* AM1 was developed in the following manner. First, the insertional vector backbone was generated through a series of cloning steps that were performed in order to remove selected restriction sites for later use in the final vectors. The resulting plasmid, pCM167, has a ColE1 replicon and a *loxP*-flanked tetracycline-resistance cassette (*tetAR*) inserted into *M. extorquens* AM1 *kata* (which encodes a catalase). This chromosomal locus was chosen for insertion because *M. extorquens* AM1 contains multiple active catalases and *kata* mutants grow like the wild-type under the conditions tested (Chistoserdova & Lidstrom, 1996). Second, a construct bearing a terminator-bounded multiple-cloning site (MCS) cassette, pCM124, was generated by introducing the *E. coli* *t_{rrnB}* and the *t_{T7}* on opposite ends, into which the strong

P_{mxoF} from *M. extorquens* AM1 was cloned to generate pCM126. Third, the terminator-bounded cassettes from pCM124 and pCM126 were introduced into pCM167 to generate the insertional cloning vector pCM168 and the insertional expression vector pCM172 (Fig. 2). Finally, a *kata::kan* *M. extorquens* AM1 strain, CM82.1, was generated to facilitate the screening of transformants for the desired insertion events. As had been reported previously for *M. extorquens* AM1 *kata* mutants (Chistoserdova & Lidstrom, 1996), strain CM82.1 exhibits no growth defects under all conditions tested (data not shown).

Demonstrations of the utility of the insertional expression vector system

Insertional vector constructs were introduced into the *kata::kan* *M. extorquens* AM1 strain CM82.1 by electroporation and colonies were selected on plates containing tetracycline. Tests of the vector were performed with the empty vectors pCM168 and pCM172, and tetracycline-resistant, kanamycin-sensitive colonies were shown, respectively, to have generated the *kata::(loxP-tetAR-loxP-t_{rrnB}-MCS-t_{T7})* strain CM168T.1 and the *kata::(loxP-tetAR-loxP-t_{rrnB}-P_{mxoF}-MCS-t_{T7})* strain CM172T.1. Additionally, in order to test the expression level afforded by this system, *gfp* was cloned behind the *P_{mxoF}* in pCM172, resulting in pCM174, and this construct was introduced into CM82.1 to generate CM174T.1 [*kata::(loxP-tetAR-loxP-t_{rrnB}-P_{mxoF}-gfp-t_{T7})*]. Subsequently, the *cre*-expression plasmid pCM158 (Marx & Lidstrom, 2002) was introduced into each of these

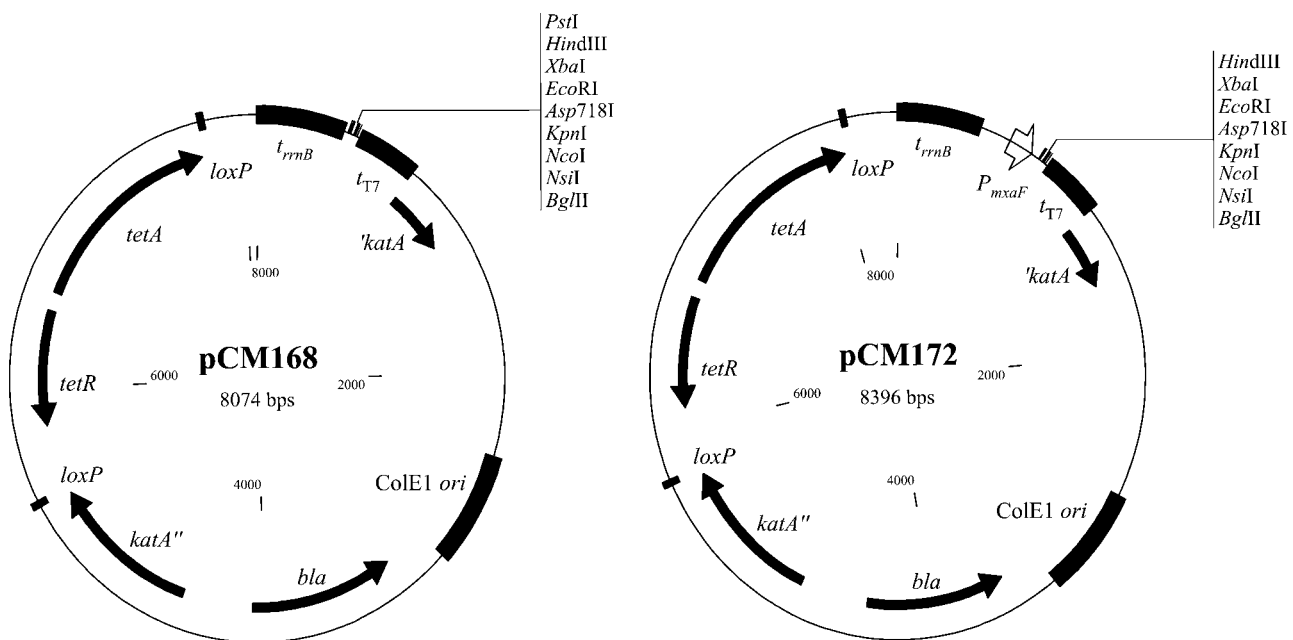


Fig. 2. Plasmid maps depicting the relevant features of the insertional cloning and expression vectors for use in *M. extorquens* AM1. GenBank accession numbers for these plasmids are AY307999 (pCM168) and AY308000 (pCM172). *ori*, origin of replication.

strains to excise the tetracycline-resistance cassette. This resulted in the unmarked (tetracycline-sensitive) strains CM168.1 [*katA*::(*loxP*-*t_{rrnB}*-MCS-*t_{T7}*)], CM172.1 [*katA*::(*loxP*-*t_{rrnB}*-*P_{mxaf}*-MCS-*t_{T7}*)] and CM174.1 [*katA*::(*loxP*-*t_{rrnB}*-*P_{mxaf}*-*gfp*-*t_{T7}*)]. Diagnostic PCR indicated that all desired recombination events occurred as predicted (data not shown). Finally, as with CM82.1, all of these strains grew the same as wild-type *M. extorquens* AM1 (data not shown), confirming that the introduction of the insertional system at the *katA* locus does not result in measurably altered growth under standard laboratory conditions.

The expression level afforded by the insertional expression vector was determined by comparing GFP fluorescence in CM174.1 to wild-type *M. extorquens* AM1 carrying pCM88 (Marx & Lidstrom, 2001), in which *gfp* is transcribed by *P_{mxaf}* of the plasmid expression vector pCM80 (Marx & Lidstrom, 2001). Succinate-grown wild-type with pCM88 had a fluorescence/OD₆₀₀ of 320 or 130 for methanol- and succinate-grown cells, respectively, compared to a relative fluorescence/OD₆₀₀ of 680 versus 240 for CM174.1 grown under the same conditions. Thus, for the case of GFP, the insertional expression vector provided twofold higher expression than the plasmid system but exhibited the same regulation pattern (2.6-fold induction on methanol) as that obtained with pCM88 (2.4-fold induction). Additionally, the termination efficiency of *t_{rrnB}* and *t_{T7}* in *M. extorquens* AM1 was examined by inserting each of the terminators between *P_{mxaf}* and *xylE* (which encodes catechol 2,3-dioxygenase). The XylE activities of cells containing the parental plasmid, pCM77 (*P_{mxaf}*-*xylE*), were 800 and 190 mU in extracts prepared from methanol- and succinate-grown cultures, respectively. These values dropped to 5 and 2 mU for pCM121 (*P_{mxaf}*-*t_{rrnB}*-*xylE*) and 290 and 95 mU for pCM122 (*P_{mxaf}*-*t_{T7}*-*xylE*). Therefore, the *E. coli* *t_{rrnB}* terminator provided a 99% reduction in activity, compared to only a 50–64% reduction by *t_{T7}*. Collectively, these data indicate that the insertional expression vector pCM172 provides significant expression from a chromosomal locus that is largely transcriptionally isolated from the surrounding genes.

***M. extorquens* AM1 mutants lacking *mtdA* and/or *fch* can be generated in a strain expressing *folD* from *M. chloromethanicum* CM4^T**

To better understand the role of the *M. extorquens* AM1 H₄F pathway in methylotrophy and the apparent essentiality of *mtdA* and *fch* during heterotrophic growth, mutants defective for these H₄F pathway activities were generated in strains expressing an analogous but non-orthologous enzyme from the related methylotroph *M. chloromethanicum* CM4^T. The *folD* gene, which encodes a bifunctional NADP⁺-dependent methylene-H₄F dehydrogenase/methenyl-H₄F cyclohydrolase from *M. chloromethanicum* CM4^T (Studer *et al.*, 2002; Vannelli *et al.*, 1999), was cloned and introduced into the insertional expression vector pCM172. This construct was introduced into CM82.1 and

tetracycline-resistant, kanamycin-sensitive transformants were isolated and confirmed to contain the *folD* chromosomal insertion, transcribed by *P_{mxaf}*. Enzymic assays confirmed that FolD was expressed in an active form, with 81 and 39 mU of NADP-dependent methylene-H₄F dehydrogenase activity in extracts of cells grown on methanol and succinate, respectively. The *folD*-expressing strain CM219T.1 was unmarked using a *cre*-expression vector (Marx & Lidstrom, 2002) to generate the antibiotic-resistance-free strain CM219.1 [*katA*::(*loxP*-*t_{rrnB}*-*P_{mxaf}*-*folD*-*t_{T7}*)] for further experiments.

Constructs based on the allelic-exchange vector pCM184 (Marx & Lidstrom, 2002) were generated to delete *mtdA*, *fch* or both, and these were introduced into both wild-type *M. extorquens* AM1 and the *folD*-expressing strain CM219.1. As had been reported previously, null mutants were not obtained in the wild-type on succinate medium (Chistoserdova & Lidstrom, 1994b; Pomper *et al.*, 1999), but were readily obtained in CM219.1. The resulting strains CM219-275K.1 [*katA*::(*loxP*-*t_{rrnB}*-*P_{mxaf}*-*folD*-*t_{T7}*), Δ *mtdA*::*kan*], CM219-279K.1 [*katA*::(*loxP*-*t_{rrnB}*-*P_{mxaf}*-*folD*-*t_{T7}*), Δ *fch*::*kan*], CM219-280K.1 [*katA*::(*loxP*-*t_{rrnB}*-*P_{mxaf}*-*folD*-*t_{T7}*), Δ *mtdA*-*fch*::*kan*] and CM219.1 grew like the wild-type in medium containing succinate (Fig. 3). The addition of methanol to the medium did not inhibit growth (Fig. 3), unlike the phenotype of mutants defective for the H₄MPT pathway (Hagemeier *et al.*, 2000; Marx *et al.*, 2003b; Vorholt *et al.*, 2000). The *folD*-expressing strain CM219.1 grew more slowly on methanol than the wild-type, however, and the *mtdA* and *fch* mutants generated in this strain failed to grow at all on methanol (Fig. 3). Similar results were obtained on solid medium; additionally, CM219-275K.1, CM219-279K.1 and CM219-280K.1 failed to grow on methylamine, formate or oxalate.

Null mutants in *mtdA* and/or *fch* can be obtained in wild-type *M. extorquens* AM1 by conjugation and selection upon succinate medium supplemented with formate or another C₁ compound

The ability of an alternative enzyme (FolD) that converts methylene-H₄F to formyl-H₄F to eliminate the need for MtdA and Fch under standard heterotrophic conditions supported the hypothesis that the H₄F pathway is required during heterotrophic growth for generating formyl-H₄F for biosynthetic purposes. Since formyl-H₄F can also be supplied via FtfL activity in the presence of formate, null mutants should also be obtained in succinate medium supplemented with formate or compounds that generate formate. To test this hypothesis, formate or compounds that are metabolized to formate (methanol, methylamine or oxalate) were added to the medium throughout the conjugation procedure and to the medium used to select transconjugants. Under these conditions, the mutant strains CM275K.1 (Δ *mtdA*::*kan*), CM279K.1 (Δ *fch*::*kan*) and CM280K.1 (Δ *mtdA*-*fch*::*kan*) lacking *mtdA*, *fch* or both were obtained. As expected, MtdA and/or Fch activity was

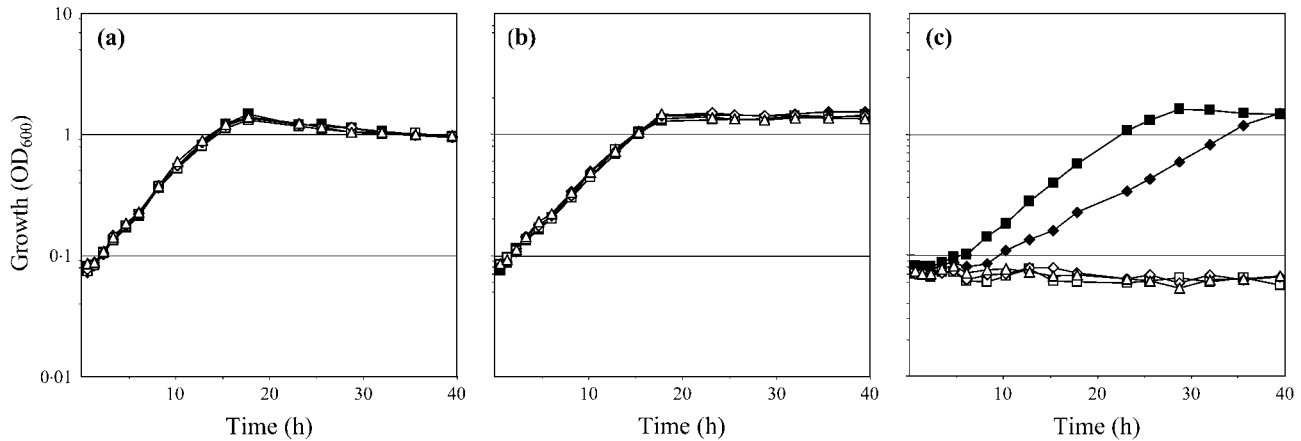


Fig. 3. Growth of wild-type *M. extorquens* AM1 and mutant strains pre-grown on succinate, harvested and resuspended in medium containing succinate (a), succinate plus methanol added to 125 mM at 2 h (b) or methanol (c). The strains represented are wild-type (■), the *folD*-expressing strain CM219.1 (◆) and three mutants generated in the CM219.1 background – the *mtdA* mutant CM219-275K.1 (□), the *fch* mutant CM219-279K.1 (◇) and the *mtdA fch* mutant CM219-280K.1 (△).

undetectable in these strains (< 1 mU), whereas the wild-type exhibited activities of 270 and 70 mU MtdA activity, and 250 and 240 mU Fch activity on methanol and succinate, respectively. Growth of these mutants was nearly equal to that of the wild-type in succinate medium supplemented with 7 mM methylamine, but they failed to grow when transferred to succinate medium without methylamine (Fig. 4). Similarly, no growth was observed on succinate plates unless they contained formate (7 mM), oxalate (4 mM), methanol (1 or 10 mM) or methylamine (7 mM), with the latter supplement supporting the most vigorous growth. Attempts to generate *mtdA* and/or *fch* mutants in the $\Delta ftfL$ strain CM216.1 (Marx *et al.*, 2003a) produced no null mutants, confirming that FtfL activity is required to provide formyl-H₄F from formate.

Mutants lacking *mtdA* and/or *fch* are unable to grow on C₁ compounds including formate but are not methanol-sensitive

To examine the role of MtdA and Fch in methylotrophy, cultures of the wild-type and strains CM275K.1, CM279K.1 and CM280K.1 grown on succinate plus methylamine were transferred to media containing succinate plus methylamine (7 mM), succinate plus methanol (added to 125 mM after 2 h) and methanol alone (Fig. 5). No growth was observed in liquid medium containing methanol, nor on plates containing methanol, methylamine, formate or oxalate. Introduction of pLC410a, which contains *mtdA* and *fch*, completely restored wild-type growth to all mutant strains. Unlike mutants defective for the H₄MPT pathway (Hagemeier *et al.*, 2000; Marx *et al.*, 2003b; Vorholt *et al.*, 2000), however, the addition of methanol did not inhibit growth (Fig. 5).

DISCUSSION

In this report, we describe the development of a system for the insertion and expression of genes from a stable, unmarked chromosomal locus in *M. extorquens* AM1. The

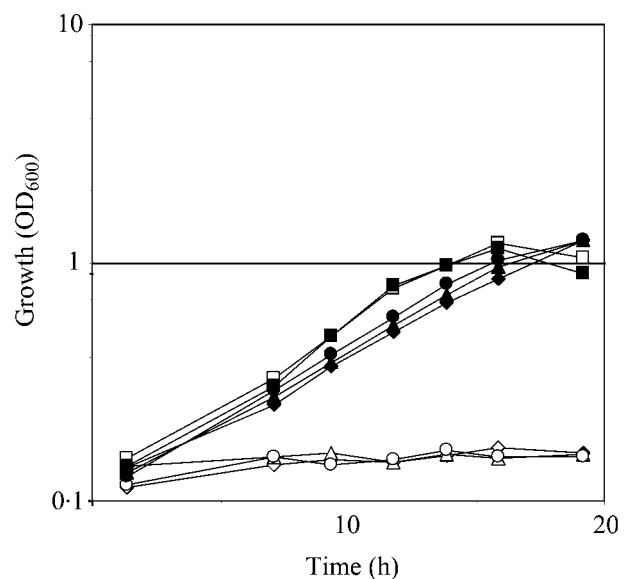


Fig. 4. Growth of wild-type *M. extorquens* AM1 and mutant strains pre-grown on succinate plus 7 mM methylamine, harvested and resuspended in medium containing only succinate (open symbols) or succinate plus 7 mM methylamine (solid symbols). The strains represented are wild-type (squares), the *mtdA* mutant CM275K.1 (diamonds), the *fch* mutant CM279K.1 (triangles) and the *mtdA fch* mutant CM280K.1 (circles).

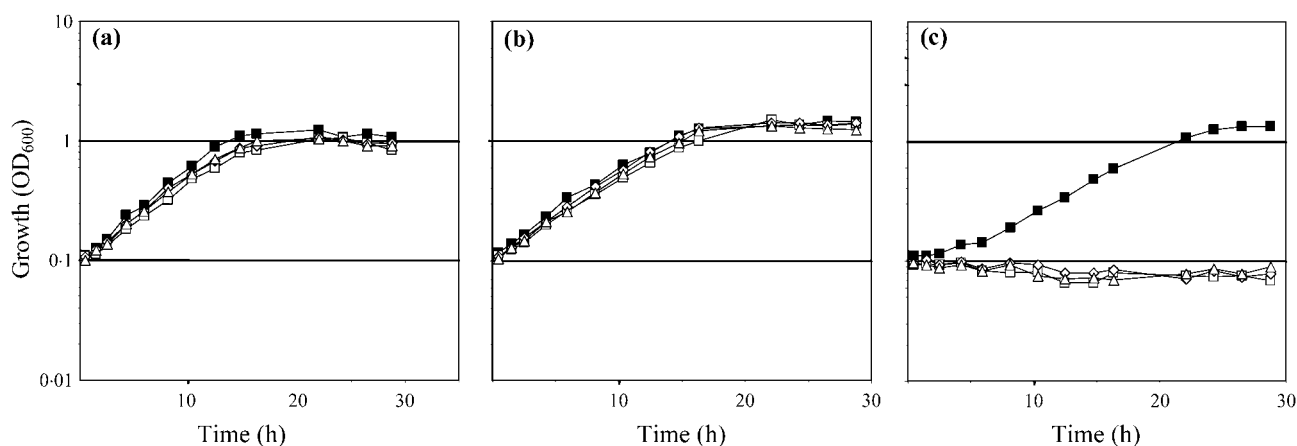


Fig. 5. Growth of wild-type *M. extorquens* AM1 and mutant strains pre-grown on succinate plus 7 mM methylamine, harvested and resuspended in medium containing succinate plus 7 mM methylamine (a), succinate plus 125 mM methanol added at 2 h (b) or methanol (c). The strains represented are wild-type (■), the *mtdA* mutant CM275K.1 (◇), the *fch* mutant CM279K.1 (△) and the *mtdA fch* mutant CM280K.1 (□).

P_{mxatF}-based insertional expression vector pCM172 provided significant expression of *folD* during growth on either methanol or succinate. Additionally, pCM168 can provide a starting point for the development of either other expression systems based on different endogenous or exogenous promoters, or insertional promoter-probe vectors generated through the introduction of reporter genes. Finally, the terminator-bounded MCS cassettes and the *loxP*-flanked antibiotic-resistance cassette used to generate this system could easily be inserted into a different backbone to generate insertional systems that incorporate into other loci of *M. extorquens* AM1, or other bacteria. Insertional systems such as the one described here have a significant advantage over plasmid-borne systems in that antibiotic selection is not required for maintenance and they provide single-copy expression.

The availability of the insertional expression vector pCM172 enabled experiments aimed at determining the role of the H₄F pathway in methylotrophy in *M. extorquens* AM1. Using this system to generate a FolD-expressing insertion strain, we showed that null mutants in *mtdA* and/or *fch* could be obtained on succinate in a background containing *folD*. These results suggested that the role of these genes during growth on succinate is to generate formyl-H₄F for biosynthetic purposes and showed that the H₄MPT-dependent activity of MtdA is not required during heterotrophic growth. This hypothesis was confirmed by the demonstration that the requirement for *mtdA* and *fch* could be alleviated by the FtfL reaction, provided that formate or other compounds generating formate were supplied in the medium.

Both sets of null mutants in *mtdA* and *fch* failed to grow on C₁ compounds, confirming a specific role in methylotrophy for the products of these genes. However, it was surprising

that the mutants expressing FolD failed to grow on C₁ compounds. FolD carries out the same reactions as MtdA/Fch together, except that MtdA has significant activity with methylene-H₄MPT (Vorholt *et al.*, 1998). However, if the methylene-H₄MPT dehydrogenase activity of MtdA was important during methylotrophic growth, the Fch mutant should still be complemented by FolD. Fch does not show detectable activity with methenyl-H₄MPT, and a different enzyme (Mch) carries out this reaction in *M. extorquens* AM1 (Pomper *et al.*, 1999). FolD from *M. chloromethanicum* CM4^T is required for growth on chloromethane, a C₁ substrate that is not oxidized to formaldehyde, but rather is catabolized through C₁-H₄F pathway intermediates by MetF (methylene-H₄F reductase), FolD and PurU (formyl-H₄F hydrolase) (Studer *et al.*, 2002; Vannelli *et al.*, 1999). It has been suggested (Pomper *et al.*, 2002; Vorholt, 2002) that the H₄F pathway of *M. extorquens* AM1 (MtdA, Fch and FtfL) may function in the assimilatory direction during growth on methanol to supply methylene-H₄F for the serine cycle from some fraction of the formate that is produced from formaldehyde by the H₄MPT pathway. Therefore, it is possible that the net fluxes through these two H₄F pathways are in opposite directions. This may be reflected, for example, in different affinities for substrates and/or the effect and identity of potential effector molecules that may modulate flow through C₁-H₄F intermediates. This hypothesis is supported by the growth inhibition in methanol medium observed in the wild-type expressing FolD, which could be explained by a futile cycle involving the methylene-H₄F/formyl-H₄F interconversions. Alternatively, it is possible that the *in vivo* activity of FolD is not sufficient for growth on C₁ compounds, as the *in vitro* FolD activity in methanol-grown cells was about one-third the *in vitro* activity of MtdA in the wild-type (Vorholt *et al.*, 1998). Regardless of which explanation is correct, the results we

present here clearly demonstrate that MtdA and Fch are necessary for growth on C₁ compounds.

The insensitivity to methanol during growth on succinate of *mtdA* and/or *fch* mutants provides additional support for the hypothesis that the H₄F pathway does not contribute significantly to formaldehyde oxidation to formate, and ultimately CO₂. So far, all mutants in the H₄MPT pathway are sensitive to methanol and other formaldehyde-producing substrates (Hagemeyer *et al.*, 2000; Marx *et al.*, 2003b; Vorholt *et al.*, 2000), underscoring the important role of this pathway in formaldehyde oxidation. In the case of *mtdA*, in particular, the lack of sensitivity to formaldehyde-producing substrates suggests that MtdB activity alone is sufficient for the detoxification of formaldehyde. This confirms the previous suggestion (Hagemeyer *et al.*, 2000) that MtdB is the primary methylene-H₄MPT dehydrogenase *in vivo*.

With the exception of the requirement for supplementation with formate to grow on succinate, or a compound that can be converted into formate (methanol, methylamine or oxalate), the growth of the *mtdA* and/or *fch* mutants is consistent with that reported for *ftfL* mutants (Marx *et al.*, 2003b). Therefore, there is a consistent phenotype associated with a defective H₄F pathway: no growth on C₁ compounds including formate, but lack of inhibition by methanol during growth on succinate. The inability to grow on formate or oxalate confirms that the H₄F pathway is required to convert formate into methylene-H₄F for assimilation of these substrates (Large *et al.*, 1961). Finally, the insensitivity to methanol during growth on succinate by mutants defective for the H₄F pathway contrasts with the distinct growth inhibition observed for H₄MPT pathway mutants. This difference in mutant phenotype suggests that these two C₁-transfer pathways may play distinct roles. Experiments designed to directly test the direction of carbon flow through the H₄F pathway are in progress.

ACKNOWLEDGEMENTS

We thank L. Chistoserdova, H. M. Rothfuss, S. Stolyar, R. K. Thauer and J. A. Vorholt for their helpful comments and suggestions, and S. Vuilleumier for providing a chromosomal preparation of *M. chloromethanicum* CM4^T. This work was supported by a grant from the NIH (GM 36296).

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