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 tetrahydrofolic acid (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 formyldehydrogenase–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 cyclohydrodrolase, Mch (Pomper et al., 1999). The C1 unit is then hydrolysed by the formyltransferase–hydrodrolase complex, Fhc (Pomper & Vorholt, 2001), to produce formate and free H4MPT.
The formaldehyde that condenses with H$_4$F to form methylene-H$_4$F serves as the C$_1$ donor for assimilation through the serine cycle (reviewed by Lidstrom, 2001). Methylene-H$_4$F may also be converted to methenyl-H$_4$F, formyl-H$_4$F and, ultimately, free formate and H$_4$F through the action of an NADP-dependent methylene-H$_4$F dehydrogenase, MtdA (Chistoserdova & Lidstrom, 1994b; Vorholt et al., 1998), methenyl-H$_4$F cyclohydrolase, Fch (Chistoserdova & Lidstrom, 1994a; Pomper et al., 1999), and formyl-H$_4$F ligase, FtfL (Marx et al., 2003b), respectively (Fig. 1). The formate produced through either the H$_4$F or the H$_4$MPT C$_1$ transfer pathway may then be oxidized to CO$_2$ by formate dehydrogenases (Laikel et al., 2003).

The enzymes of the H$_4$F pathway are found at high specific activities during heterotrophic growth and are generally present at three- to fourfold higher levels during growth on C$_1$ compounds (Chistoserdova & Lidstrom, 1994b; Marison & Attwood, 1982; Pomper et al., 1999; Vorholt et al., 1998). This finding led to the original suggestion that the H$_4$F pathway functions as the primary formaldehyde oxidation route during methylotrophy (Marison & Attwood, 1982). The discovery of the H$_4$MPT pathway in the methylotrophic bacteria and archaea (Chistoserdova et al., 1998) and the elucidation of its critical role in formaldehyde oxidation (Chistoserdova et al., 1998; Hagemier et al., 2000; Marx et al., 2003b; Vorholt et al., 2000) have brought this suggestion into question. It has also been suggested that the H$_4$F pathway potentially could function in the reductive oxidation into question. It has also been suggested that the role of the H$_4$F pathway has been complicated by the insertion into an intact XbaI fragment from pCM50 (Marx & Lidstrom, 2001) into the _M. chloromethanicum_ locus. This system has been utilized to express folD, which encodes a bifunctional NADP-dependent methylene-H$_4$F dehydrogenase/methenyl-H$_4$F cyclohydrolase that does not have activity with H$_4$MPT derivatives, from _Methylobacterium chloromethanicum_ CM4T 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-H$_4$F and have clearly demonstrated the requirement for MtdA and Fch during methylotrophy.

**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 rifampycin 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 _NrdI–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 _NrdI_ and _BsiI_ 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 _EcoRl_ and _Ndel_ and blunt-ligated to produce pCM117, which was subsequently cut with _HindIII_ and blunt-ligated and re-ligated to produce pCM118. A construct containing tetAR flanked by _loxP_ sites was constructed by inserting the 2·3 kb _Xmrnl–Stul_ fragment from pCM50 (Marx & Lidstrom, 2001) into the blunt-ended _XbaI_ site of pLoxI (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–SalI_ 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

(Pomper et al., 2002). Mutants defective for the H$_4$MPT pathway fail to grow on C$_1$ substrates and are sensitive to the presence of compounds that lead to the production of formaldehyde (Hagemier et al., 2000; Marx et al., 2003b; Vorholt et al., 2000), leading to the suggestion that the H$_4$MPT-linked pathway serves as the primary formaldehyde oxidation and detoxification pathway in _M. extorquens_ AM1.

In addition, MtdA differs from a standard methylene-H$_4$F dehydrogenase, in that it also has significant activity with methylene-H$_4$MPT (Fig. 1; Vorholt et al., 1998). Therefore, it was not possible to rule out a role for MtdA in the H$_4$MPT pathway during heterotrophic and/or methylotrophic growth.

To define the role of the H$_4$F pathway in heterotrophy and methylotrophy, 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 _folD_, which encodes a bifunctional NADP-dependent methylene-H$_4$F dehydrogenase/methenyl-H$_4$F cyclohydrolase that does not have activity with H$_4$MPT derivatives, from _Methylobacterium chloromethanicum_ CM4T 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-H$_4$F and have clearly demonstrated the requirement for MtdA and Fch during methylotrophy.
was digested with NcoI and NdeI, blunted and self-ligated to produce pCM166, which was cut with NsiI, blunted and self-ligated to produce pCM167.

The E. coli rrnB terminator (trrnB) from pCM130 (Marx & Lidstrom, 2001) and the T7 terminator (tT7) from pET-3a (Novagen) were amplified by PCR and cloned into pCR2.1 (Invitrogen) to generate pCM119 and pCM120, respectively. The 0.5 kb BamHI–HindIII fragment from pCM119 containing trrnB was ligated into the same sites of pMTL23 to generate pCM123. The 0.4 kb NruI–XhoI fragment from pCM120 was inserted into the same sites of pCM123 to generate pCM124. A terminator-flanked cassette bearing PmxaF was generated by inserting the 0.3 kb NruI–HindIII fragment from pCM80 (Marx & Lidstrom, 2001) between the HindII and HindIII sites of pCM124 to generate pCM126.

The insertional vector backbone pCM167 contains unique BglII and StuI sites between which terminator-flanked cassettes were inserted as BamHI–NruI 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 EcoRI–SphI fragment from pLC1128.Km (Chistoserdova & Lidstrom, 1996) was blunted and cloned into the Smal 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 trrnB or tT7 in M. extorquens AM1, PmxaF present in the 0.4 kb BamHI–EcoRI 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.
Table 1. *M. extorquens* AM1 strains and plasmids used in this study

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<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
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<td>CM82.1</td>
<td>katA::kan</td>
<td>This study</td>
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<tr>
<td>CM168.1</td>
<td>katA::(loxP-t&lt;sub&gt;mrna&lt;/sub&gt;−MCS−t&lt;sub&gt;T7&lt;/sub&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>CM168T.1</td>
<td>katA::(loxP-tetAR−loxP-t&lt;sub&gt;mrna&lt;/sub&gt;−MCS−t&lt;sub&gt;T7&lt;/sub&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>CM172.1</td>
<td>katA::(loxP-t&lt;sub&gt;mrna&lt;/sub&gt;−P&lt;sub&gt;maxif&lt;/sub&gt;−MCS−t&lt;sub&gt;T7&lt;/sub&gt;)</td>
<td>This study</td>
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<tr>
<td>CM172T.1</td>
<td>katA::(loxP-tetAR−loxP-t&lt;sub&gt;mrna&lt;/sub&gt;−P&lt;sub&gt;maxif&lt;/sub&gt;−MCS−t&lt;sub&gt;T7&lt;/sub&gt;)</td>
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<tr>
<td>CM174.1</td>
<td>katA::(loxP-t&lt;sub&gt;mrna&lt;/sub&gt;−P&lt;sub&gt;maxif&lt;/sub&gt;−gfp−t&lt;sub&gt;T7&lt;/sub&gt;)</td>
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<tr>
<td>CM174T.1</td>
<td>katA::(loxP-tetAR−loxP-t&lt;sub&gt;mrna&lt;/sub&gt;−P&lt;sub&gt;maxif&lt;/sub&gt;−gfp−t&lt;sub&gt;T7&lt;/sub&gt;)</td>
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<td>CM216.1</td>
<td>ΔfliF</td>
<td>Marx et al. (2003a)</td>
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<td>CM219.1</td>
<td>katA::(loxP-t&lt;sub&gt;mrna&lt;/sub&gt;−P&lt;sub&gt;maxif&lt;/sub&gt;−folD−t&lt;sub&gt;T7&lt;/sub&gt;)</td>
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<td>katA::(loxP-tetAR−loxP-t&lt;sub&gt;mrna&lt;/sub&gt;−P&lt;sub&gt;maxif&lt;/sub&gt;−folD−t&lt;sub&gt;T7&lt;/sub&gt;)</td>
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<td>CM219-275K.1</td>
<td>katA::(loxP-t&lt;sub&gt;mrna&lt;/sub&gt;−P&lt;sub&gt;maxif&lt;/sub&gt;−folD−t&lt;sub&gt;T7&lt;/sub&gt;)</td>
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<tr>
<td>CM219-279K.1</td>
<td>katA::(loxP-t&lt;sub&gt;mrna&lt;/sub&gt;−P&lt;sub&gt;maxif&lt;/sub&gt;−folD−t&lt;sub&gt;T7&lt;/sub&gt;)</td>
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<tr>
<td>CM219-280K.1</td>
<td>katA::(loxP-t&lt;sub&gt;mrna&lt;/sub&gt;−P&lt;sub&gt;maxif&lt;/sub&gt;−folD−t&lt;sub&gt;T7&lt;/sub&gt;)</td>
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<td>CM279K.1</td>
<td>Δfch::kan</td>
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<td>AM1</td>
<td>Rif&lt;sup&gt;R&lt;/sup&gt; derivative</td>
<td>Nunn &amp; Lidstrom (1986)</td>
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**Plasmid**

- pAYC61: Allelic exchange vector
- pCM21: pCR2.1 with *gfp*
- pCM27: pCR2.1 with *P<sub>maxif</sub>*
- pCM50: Small IncP replicon
- pCM76: IncP replicon with *xylE*
- pCM77: pCM76 with *P<sub>maxif</sub>* upstream of *xylE*
- pCM80: *M. extorquens* AM1 expression vector (*P<sub>maxif</sub>*)
- pCM82: pAYC61 with *katA::kan* fragment from pLC1128.Km
- pCM116: pLC11.28 with *katA::erm*
- pCM117: pCM116 with restriction sites removed
- pCM118: pCM117 with a restriction site filled-in
- pCM119: pCR2.1 with *t<sub>mrna</sub>*
- pCM120: pCR2.1 with *t<sub>T7</sub>*
- pCM121: pCM77 with *P<sub>maxif</sub>−t<sub>mrna</sub>−*xylE*
- pCM122: pCM77 with *P<sub>maxif</sub>−t<sub>T7</sub>−*xylE*
- pCM123: pMTL23 with *t<sub>mrna</sub>*
- pCM124: pMTL23 with *t<sub>mrna</sub>−MCS−t<sub>T7</sub> cassette
- pCM126: pMTL23 with *t<sub>mrna</sub>−P<sub>maxif</sub>−MCS−t<sub>T7</sub> cassette
- pCM130: Broad-host-range promoter probe vector with *xylE*
- pCM158: Broad-host-range *cre* expression vector
- pCM159: pLox1 with *tetAR*
- pCM165: pCM118 with *loxP−tetAR−loxP*
- pCM166: pCM165 with restriction sites removed
- pCM167: pCM166 with a restriction site filled-in
- pCM168: pCM167 with *t<sub>mrna</sub>−MCS−t<sub>T7</sub>* (insertional cloning vector)
- pCM172: pCM167 with *t<sub>mrna</sub>−P<sub>maxif</sub>−MCS−t<sub>T7</sub>* (insertional expression vector)
- pCM174: pCM172 with *gfp*
- pCM184: Broad-host-range allelic exchange vector
- pCM201: pCR2.1 with *purU* from *M. chloromethanicum* CM4<sup>T</sup>
- pCM202: pCR2.1 with *folD* from *M. chloromethanicum* CM4<sup>T</sup>
- pCM203: pCM80 with *purU*
- pCM205: pCM80 with *purU−folD*
- pCM206: pCM172 with *purU−folD*
- pCM210: pCM172 with *folD*
- pCM272: pCR2.1 with *mtdA* upstream flank
0.6 kb BamHI–SphI fragment from pCM119 and the 0.4 kb BamHI–SphI fragment from pCM120 were then ligated into the same sites of pCM77 between PurM and xylE to generate pCM212 and pCM212, respectively. The expression level afforded by pCM172 was examined by inserting the 0.8 kb HindIII–NsiI 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 CM4T (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 CM4T 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 XbaI–KpnI fragment from pCM201 was cloned into the same sites of pCM80 (Marx & Lidstrom, 2001) to generate pCM203; subsequently, the 1.0 kb KpnI–SacI fragment from pCM202 was introduced between the same sites of pCM203 to generate pCM205. The 1.9 kb XbaI–NsiI 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 0.3 kb blunt ended pCM206 XbaI–Asp718I 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 constructs to generate ΔmtdA::kan mutants were made by introducing the 0.5 kb SacI–AgeI fragment from pCM273 between the corresponding sites of pCM184 to produce pCM274; subsequently, the 0.5 kb BglII–NdeI fragment from pCM272 was ligated into the same sites of pCM274 to produce pCM275. The construct to generate Δfch::kan mutants was made by introducing the 0.6 kb ApaI–SacI fragment from pCM277 into the same sites of pCM184 to produce pCM278; subsequently, the 0.5 kb EcoRI–NdeI fragment from pCM276 was ligated into the same sites of pCM278 to produce pCM279. Finally, a construct to make ΔmtdA–fch::kan mutants was generated by introducing the 0.5 kb BglII–NdeI 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-H\textsubscript{4}F dehydrogenase (Chistoserdova & Lidstrom, 1994b), methyln-H\textsubscript{4}F cyclohydrodrolase (Pomper et al., 1999), formyl-H\textsubscript{4}F 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 H\textsubscript{4}MPT-dependent activity of MtdA was not assayed. FedA activity was determined in a strain lacking MtdA (CM219-275K.1) or by the difference between the folD-expressing strain and the wild-type.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>pCM273</td>
<td>pCR2.1 with mtdA downstream flank</td>
<td>This study</td>
</tr>
<tr>
<td>pCM274</td>
<td>pCM184 with mtdA downstream flank</td>
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<tr>
<td>pCM275</td>
<td>pCM274 with mtdA upstream flank</td>
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<td>pCM276</td>
<td>pCR2.1 with fch upstream flank</td>
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<td>pCM277</td>
<td>pCR2.1 with fch downstream flank</td>
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</tr>
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<td>pCM278</td>
<td>pCM184 with fch downstream flank</td>
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<td>pCM279</td>
<td>pCM278 with fch upstream flank</td>
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<tr>
<td>pCM280</td>
<td>pCM278 with mtdA upstream flank</td>
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<td>pCR2.1</td>
<td>PCR cloning vector</td>
<td>Invitrogen</td>
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<td>E. coli overexpression vector</td>
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<td>pLC1128</td>
<td>pUC19 with katA region</td>
<td>Chistoserdova &amp; Lidstrom (1996)</td>
</tr>
<tr>
<td>pLC410a</td>
<td>Large IncP plasmid containing mtdA and fch</td>
<td>Chistoserdova &amp; Lidstrom (1994b)</td>
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<td>pLxi</td>
<td>Construct with a pair of loxP sites</td>
<td>Palmeros et al. (2000)</td>
</tr>
<tr>
<td>pMTL23</td>
<td>ColE1 replicon with large MCS</td>
<td>Chambers et al. (1988)</td>
</tr>
<tr>
<td>pMTL23E</td>
<td>pMTL23 with erythromycin-resistance cassette</td>
<td>Purdy et al. (2002)</td>
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<td>pRR2073</td>
<td>Helper plasmid expressing IncP tra functions</td>
<td>Figurski &amp; Helinski (1979)</td>
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Activities are reported in mU [nmol min\(^{-1}\) (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* trrnB and the t\(T7\) on opposite ends, into which the strong *PmxaF* 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–trrnB–MCS–t\(T7\))* strain CM168T.1 and the *katA::(loxP–tetAR–loxP–trrnB–PmxaF–MCS–t\(T7\))* strain CM172T.1. Additionally, in order to test the expression level afforded by this system, gfp was cloned behind the *PmxaF* in pCM172, resulting in pCM174, and this construct was introduced into CM82.1 to generate CM174T.1 [*katA::(loxP–tetAR–loxP–trrnB–PmxaF–gfp–t\(T7\))*]. Subsequently, the cre-expression plasmid pCM158 (Marx & Lidstrom, 2002) was introduced into each of these constructs.

![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.](image-url)
strains to excise the tetracycline-resistance cassette. This resulted in the unmarked (tetracycline-sensitive) strains CM168.1 [katA:: (loxP–trnNB–MCS–t7)], CM172.1 [katA:: (loxP–trnNB–PmxaF–MCS–t7)] and CM174.1 [katA:: (loxP–trnNB–PmxaF–gfp–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 inserational system at the katA locus does not result in measurably altered growth under standard laboratory conditions.

The expression level afforded by the inserational 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 PmxaF 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 inserional 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 trnNB and t77 in M. extorquens AM1 was examined by inserting each of the terminators between PmxaF and xylE (which encodes catechol 2,3-dioxygenase). The XylE activities of cells containing the parental plasmid, pCM77 (PmxaF–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 (PmxaF–trnNB–xylE) and 290 and 95 mU for pCM122 (PmxaF–t77–xylE). Therefore, the E. coli trnNB terminator provided a 99% reduction in activity, compared to only a 50–64% reduction by t77. Collectively, these data indicate that the inserational 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 CM4T

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 CM4T. The folD gene, which encodes a bifunctional NADP+—dependent methylene-H4F dehydrogenase/methenyl-H4F cyclohydrolase from M. chloromethanicum CM4 (Studer et al., 2002; Vannelli et al., 1999), was cloned and introduced into the inserational 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 PmxaF. 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–trnNB–PmxaF–folD–t77)] 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–trnNB–PmxaF–folD–t77), ΔmtdA:: kan], CM219-279K.1 [katA:: (loxP–trnNB–PmxaF–folD–t77), Δfch:: kan], CM219-280K.1 [katA:: (loxP–trnNB–PmxaF–folD–t77), Δfch:: 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 (ΔmtdA:: kan), CM279K.1 (Δfch:: kan) and CM280K.1 (ΔmtdA–fch:: kan) lacking mtdA, fch or both were obtained. As expected, MtdA and/or Fch activity was
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 DftfL 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

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**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 (△).

**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).
$P_{mcaF}$-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 \textit{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 H$_4$F pathway in methylotrophy in \textit{M. extorquens} AM1. Using this system to generate a FolD-expressing insertion strain, we showed that null mutants in \textit{mtdA} and/or \textit{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-H$_4$F for biosynthetic purposes and showed that the H$_4$MPT-dependent activity of MtdA is not required during heterotrophic growth. This hypothesis was confirmed by the demonstration that the requirement for \textit{mtdA} and \textit{fch} could be alleviated by the FtlL reaction, provided that formate or other compounds generating formate were supplied in the medium.

Both sets of null mutants in \textit{mtdA} and \textit{fch} failed to grow on C$_1$ 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 C$_1$ compounds. FoLD carries out the same reactions as MtdA/Fch together, except that MtdA has significant activity with methylene-H$_4$MPT (Vorholt et al., 1998). However, if the methylene-H$_4$MPT 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-H$_4$MPT, and a different enzyme (Mch) carries out this reaction in \textit{M. extorquens} AM1 (Pomper et al., 1999). FoLD from \textit{M. chloromethanicum} CM4$^4$ is required for growth on chloromethane, a C$_1$ substrate that is not oxidized to formaldehyde, but rather is catabolized through C$_1$-H$_4$F pathway intermediates by MetF (methylene-H$_4$F reductase), FolD and PurU (formyl-H$_4$F hydrolase) (Studer et al., 2002; Vannelli et al., 1999). It has been suggested (Pomper et al., 2002; Vorholt, 2002) that the H$_4$F pathway of \textit{M. extorquens} AM1 (MtdA, Fch and FtlL) may function in the assimilatory direction during growth on methanol to supply methylene-H$_4$F for the serine cycle from some fraction of the formate that is produced from formaldehyde by the H$_4$MPT pathway. Therefore, it is possible that the net fluxes through these two H$_4$F 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 C$_1$-H$_4$F 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-H$_4$F/formyl-H$_4$F interconversions. Alternatively, it is possible that the \textit{in vivo} activity of FoLD is not sufficient for growth on C$_1$ compounds, as the \textit{in vitro} FoLD activity in methanol-grown cells was about one-third the \textit{in vitro} activity of MtdA in the wild-type (Vorholt et al., 1998). Regardless of which explanation is correct, the results we

![Fig. 5. Growth of wild-type \textit{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 \textit{mtdA} mutant CM275K.1 (◇), the \textit{fch} mutant CM279K.1 (△) and the \textit{mtdA fch} mutant CM280K.1 (□).](http://mic.sgmjournals.org)
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 (Hagemeier 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 (Hagemeier 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, methyamine or oxalate), the growth of the mtdA and/or fch mutants is consistent with that reported for ffl 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.

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