Molecular and mutational analysis of a DNA region separating two methylotrophy gene clusters in Methylobacterium extorquens AM1

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A region of 14.2 kb has been analysed that is a part of a locus on the Methylobacterium extorquens AM1 chromosome containing a number of genes involved in one-carbon (C₁) metabolism, including serine cycle genes, ppq genes, regulatory methanol oxidation genes and the gene for N5,N10-methylene tetrahydrofolate dehydrogenase (mtdA). Fifteen new ORFs have been identified within the new region, and their sequences suggest that they encode the following polypeptides: the C-terminal part of phosphoenolpyruvate carboxylase, malyl-CoA lyase, polypeptides of 94 and 31 kDa of unknown function, three putative subunits of an ABC-type transporter, two polypeptides similar to the products of mxAF and mxAJ from M. extorquens AM1 and other methylotrophs, a cytochrome c, three enzymes of folate metabolism, and polypeptides of 13 and 20.5 kDa with no homologues in the protein database. Ten insertion mutations have been generated in the region to determine if the newly identified genes are associated with C₁ metabolism. A mutation in mclA, encoding malyl-CoA lyase, resulted in a C₁-minus phenotype, while mutations in the other genes all showed a C₁-plus phenotype. It was not possible to obtain null mutants in a putative folate metabolism gene, folC, implying the necessity of these folate synthesis genes for metabolism of C₁ and multicarbon compounds. Mutations in the putative ABC transporter genes, the genes similar to maG and mxAl, and other unidentified ORFs produced double-crossover recombinants with a C₁-positive phenotype. Promoter regions have been investigated upstream of orf3 and orf4 using the promoter probe vector pHX200. Transcription from these promoters was weak in wild-type M. extorquens AM1 but increased in regulatory mox mutants.

Keywords: Methylobacterium extorquens AM1, methylotrophy

INTRODUCTION

Methylobacterium extorquens AM1 is a pink-pigmented facultative methylotroph of the α-proteobacteria class (Peel & Quayle, 1961; Tsuji et al., 1990). During growth of M. extorquens AM1 on C₁ compounds such as methanol or methylamine, specific enzyme systems involved in the oxidation and assimilation of C₁ compounds are induced (Anthony, 1982). More than 20 genes have been shown to be specifically involved in methanol oxidation to formaldehyde (Lidstrom et al., 1994), and at least 11 genes have been identified to be involved in methylamine oxidation (Chistoserdov et al., 1994). The formaldehyde produced by methanol dehydrogenase (MDH) or methylamine dehydrogenase (MADH) is assimilated via the serine cycle, and the levels of the assimilatory enzymes are found to be increased on C₁ substrates as compared to multicarbon substrates (Large & Quayle, 1962). Recently, a number of chromosomal regions of M. extorquens AM1 have been characterized which are involved in C₁ metabolism, showing that many C₁ genes are clustered. Genes

Abbreviations: MADH, methylamine dehydrogenase; MCL, malyl-CoA lyase; MDH, methanol dehydrogenase.

The GenBank accession numbers for the 12312 nt (EcoRI-PstI fragment) and 1849 nt (PstI-BglII fragment) sequences reported in this paper are U72662 and U60993, respectively.
required for the oxidation of methanol to formaldehyde have been found in four as-yet-unlinked clusters (Lidstrom et al., 1994), genes required for methyamine oxidation have been found within a separate DNA region (Chistoserdov et al., 1994), and serine cycle genes have been located in four unlinked regions (Chistoserdova, 1996). One well-characterized C1 region containing a serine cycle gene cluster has been linked with another C1 region, containing pqq genes and the regulatory methanol oxidation genes mxbdM (Fig. 1). To determine if more C1-essential genes are located between these two regions, a fragment of 14-2 kb separating them was characterized.

METHODS

Bacterial strains, plasmids and growth conditions. Escherichia coli strains DH5α (Bethesda Research Laboratories) and S17-1 (Simon et al., 1983) were used in this study. They were grown in LB medium in the presence of appropriate antibiotics as described by Maniatis et al. (1982). M. extorquens AM1 was grown in the minimal medium described previously (Harder et al., 1973). Succinate (20 mM), methanol (100 mM), methylamine (20 mM), or ethylamine (20 mM) were used as substrates. Methanol induction of mutants was carried out as described by Danstan et al. (1972). The following antibiotic concentrations were used for M. extorquens AM1: 10 μg tetracycline ml−1, 100 μg kanamycin ml−1, 100 mM methanol, ethanol, isopropanol, glycerol, formaldehyde, acetaldehyde, propionaldehyde, glycolaldehyde at 1–10 mM. Gels were stained in both presence and absence of 1 mM (NH4)2SO4.

Matings. Triparental or biparental matings between E. coli and M. extorquens AM1 were performed overnight on nutrient agar (Springer et al., 1995). Cells were then washed with sterile medium and plated on selective medium at appropriate dilutions. In triparental matings, pRK2013 (Ditta et al., 1985) was used as a helper plasmid. Rifampicin was used for E. coli counter selection.

RESULTS

Nucleotide sequence analysis

The physical map of the region of interest is shown in Fig. 1. Details of the start and finish points, overlap and coding potential of the 15 ORFs are shown in Table 1. Analysis of the nucleotide sequence of the region has revealed the presence of fifteen new ORFs, one of which is partial. The first ten are transcribed from left to right as shown in Fig. 1, and the other five are transcribed in the opposite orientation. The first, partial, ORF starts beyond the described fragment. The 641 nt between the third and fourth ORFs do not contain potential ORFs. The sixth and seventh ORFs have very weak potential Shine–Dalgarno sequences upstream of the start methionines. The next 689 nt seem to be a non-coding region. The fifteenth ORF is separated by 168 nt from pqqE, which is described elsewhere (Toyama et al., 1997).

Amino acid sequence comparisons

Amino acid sequences deduced from the potential ORFs were compared against known protein sequences and sequences deduced from DNA sequences in GenBank. The first (partial) ORF showed similarity with the C-terminal region of phosphoenolpyruvate carboxylases from various sources, with identities varying between 30 and 39% (Katagiri et al., 1985; Hudspeeth & Grula, 1989). In earlier studies, the gene for PEPC (pcaA) was localized to this region (Arps et al., 1993), thus the first ORF must encode the C-terminal part of phosphoenolpyruvate carboxylase. The product of the second ORF showed high homology with the citE genes from Haemophilus influenzae (Fleischmann et al., 1995) and Klebsiella pneumoniae encoding the β subunit (lyase component) of citrate lyase (Bott & Dimroth, 1994). In earlier studies, mutations leading to the disappearance
of malyl-CoA lyase (MCL) activity were mapped to this site (Arps et al., 1993), thus the ORF must be the gene for MCL (mclA). The translated products of the two ORFs downstream of mclA failed to show identity with the α- and γ subunits of citrate lyase. These genes were designated orf1 and orf2. A motif characteristic of ATP-binding proteins was found in the amino acid sequence derived from orf2 (amino acids 246-253). While the putative product derived from orf1 did not reveal any similarity with known proteins, the amino acid sequence derived from orf2 revealed similarity with dienolactone hydrolase from Pseudomonas strains (about 28.5 % identity; Frantz & Chakrabarty, 1987; Frantz et al., 1987) and an unidentified product of a putative ORF from Azospirillum brasilense Sp7 (41.6 % identity; Zimmer & Hundeshagen, 1994). Higher identities were found with putative polypeptides translated from the E. coli K-12 chromosome. One of these is from the region at about 86° on the chromosome, which had been previously identified as a non-coding region (Daniels et al., 1992) downstream of metE and divergently transcribed (45 % identity). Another is from a gene at about 85-3° of the E. coli map (Kobayashi et al., 1985), downstream of pldB and divergently transcribed (42 % identity). Neither dienolactone hydrolase or the putative polypeptides translated from the E. coli chromosome show the presence of the ATP-binding site. Based on amino acid sequence, Orf2 has one potential membrane-
spanning domain (Klein et al., 1985; Rao & Argos, 1986). It also has a potential leader peptide cleavage site between amino acids 34 and 35.

The fifth ORF did not reveal similarity with any known proteins. However, amino acid sequences deduced from the next two ORFs showed identity with components of the ABC-type transporters, the first one to a transmembrane protein and the second one to the ATP-binding component (Angerer et al., 1990; Omata et al., 1993). The ATP-binding site was found between amino acids 62 and 69 in this polypeptide. By analogy with known ABC transporters in which the specific periplasmic-binding protein is not conserved (Saier, 1994), the upstream fifth ORF probably encodes such a protein. The three ORFs were tentatively designated abcABC. The products of the first two ORFs have potential leader peptide cleavage sites. AbcA is predicted to have one to two membrane-spanning domains, while AbcB is predicted to have four to six. One potential membrane-spanning domain is present in AbcC (Klein et al., 1985; Rao & Argos, 1986).

The product of the next ORF was found to be highly similar (73% identity) to that translated from xoxF from Paracoccus denitrificans (Harms et al., 1996). XoxF shows identity to MxaF, the large subunit of MDH, and is thought to encode the large subunit of a different PQQ-linked dehydrogenase of unknown function (Harms et al., 1996). The eighth ORF from M. extorquens AM1 also showed identity with the sequences of the large subunit of MDH (MxaF) from M. extorquens AM1 (49% identity, Anderson et al., 1990) and P. denitrificans (48%, Harms et al., 1987) and with other PQQ-containing dehydrogenases (Inoue et al., 1989; Tamaki et al., 1991). The eighth ORF was designated orf3. The amino acid sequence deduced from the ninth ORF is 33% identical to the sequence of the P. denitrificans CycB, a methanol-inducible cytochrome c, linked to XoxF (Ras et al., 1991); its sequence bears no resemblance to the cytochrome c₅₅₃ (encoded by mxag) from M. extorquens AM1 or other methylotrophs. This gene was designated orf4. Another c-type cytochrome, cytochrome c₅₅₅₃, has been identified in M. extorquens AM1, which is heavily overexpressed in regulatory methanol oxidation mutants (Day et al., 1990). Therefore, Orf4 might be this cytochrome c₅₅₅₃. However, a comparison of the amino acid composition of the deduced mature polypeptide of Orf4 with the amino acid content of c₅₅₅₃ determined experimentally by Day et al. (1990) showed significant differences. For example, Orf4 contains only one serine, while nine serines were chemically determined in c₅₅₅₃. The threonine content also differs considerably (four and nine, respectively). In addition, the predicted molecular mass for mature Orf4 is 18150 Da while c₅₅₅₃ runs on SDS gels as a 23 kDa band (Day et al., 1990). The product of the next ORF showed similarity with XoxF from P. denitrificans (41%; Harms et al., 1996) and lower similarity with MxaF from M. extorquens AM1 (31% identity; Anderson et al., 1990) or P. denitrificans (29% identity; van Spanning et al., 1991). The corresponding gene was designated orf5. In contrast to P. denitrificans (Harms et al., 1996), a counterpart of mxaF was missing in M. extorquens AM1. The products of orf345 are all predicted to be periplasmic proteins.

The three upstream ORFs are separated from orf5 by only 47 nt and are divergently transcribed. The products of these ORFs showed high identity with three enzymes of folate metabolism from various sources: 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (40–48%), dihydropterin aldolase (30–40%), and dihydroxypteroyl synthase (40–47%), respectively (Talarico et al., 1992; Dallas et al., 1993; Lacks et al., 1995; Fleischmann et al., 1995). These three genes were designated folABC. folA and folC are homologues of folK and folP in E. coli, respectively (Talarico et al., 1992; Dallas et al., 1993). Polypeptides deduced from the two last ORFs, transcribed in the same direction as the fol genes, did not reveal identities with protein sequences in the database, and these genes were designated orf6 and orf7. The product of orf6 might be a periplasmic protein, based on a potential leader cleavage site between amino acids 25 and 26.

Insertion mutations in the mcl region

To obtain additional proof of the identity of mclA and to determine whether the downstream ORFs might encode subunits of MCL, insertion mutations were introduced into the chromosomal copies of mclA and orf2, by recombinational insertion as described for other C₄ genes (Chistoserdova & Lidstrom, 1992, 1996). The validity of all insertion mutations was verified by probing digests of chromosomal DNA from wild-type and mutant strains (data not shown). Double-crossover mutants were sensitive for vector-encoded antibiotic resistance and did not contain vector sequences, while single-crossover mutants showed antibiotic resistance and contained the vector, the mutated gene, and a second, functional copy of the target gene in the chromosome. The Sphl site in the second half of mclA (at nt 1525) was used for inserting a kanamycin (Km)-resistance gene, and two Aval sites (at nts 2783 and 3286) were used to generate a deletion of 503 nt from the beginning of orf2, and for inserting a Km-resistance gene. The corresponding mutants were obtained by homologous recombination, selecting on sucinate and then testing for their ability to grow on C₄ and C₅ compounds. Double-crossover mutants in mclA showed the expected phenotype, being unable to grow on C₄ compounds, while double-crossover mutants in orf2 were able to grow on C₅ compounds. Thus, orf2 is most probably not involved in the synthesis of active MCL or any other essential C₄ function. Mutants in mclA were further investigated. MCL activity was measured in two representative insertion mutants MCL6 and MCL50, a chemically induced mutant PCT57 (Dunstan et al., 1972) and mutants complemented by pLC19.10 (Fig. 1) containing the cloned mclA. The activity was absent in the mutants and was restored in the presence of cloned mclA (data not shown). Cloned mclA was able to complement mutants for growth on C₄ compounds in
either orientation with respect to the lac promoter (data not shown), indicating the presence of a promoter upstream of mclA. This was further confirmed by cloning the 1.4 kb BglII–BamHI fragment containing the 3' terminus of pccA, 187 bp separating the two genes, and the 5' terminus of mclA into a promoter probe vector pAYC67, using cat as a reporter gene to generate plasmid pLC7.11 (Fig. 1). Promoter activity was measured when the fragment was present in the correct orientation (pLC7.11A) with respect to the reporter gene [120 nmol min⁻¹ (mg protein)⁻¹]. Activity was not detectable when the fragment was oriented diversely with respect to the reporter gene (pLC7.11B).

**Mutations in the putative ABC transporter genes**

It was of interest to determine if the three genes abcABC, similar to various ABC-type transporter genes, were involved in C₄ metabolism. Insertion mutations with a Km-resistance gene were generated in all three genes, using the EcoRV site in the middle of abcA (at nt 4472), the Smal site in the middle of abcB (at nt 5490) and the BamHI site in the middle of abcC (at nt 6231), respectively (Fig. 1). Double-crossover mutants were readily obtained on succinate, and all of them were able to grow normally on C₄ compounds, indicating that the putative ABC-type transporter system is not essential for growth on C₄ compounds, at least under the conditions used.

**Mutations in the orf345 region**

orf345 most probably encode, respectively, a PQD-linked dehydrogenase, an associated cytochrome and a polypeptide analogous to MxaJ (MxaJ has been postulated to be involved in the assembly of MDH). Two mutations were obtained in the region. The first is an insertion into the BamHI site in the beginning of orf3 (at nt 7839). The second mutation involves a 188 nt BspMI deletion (nt 9686–10156) involving both orf4 and orf5, combined with the Km-resistance gene insertion. Double-crossover mutants in both loci were able to grow normally on C4 and C₃ compounds, indicating that these genes are not required for C₄ or C₃ growth of M. extorquens AM1. To explore the possibility that the phenotype of the mutants was obscured by the presence of MDH, double mutants were generated. Previously characterized chemically induced mutants carrying null mutations in either mxaF or one of the pqq genes (Nunn & Lidstrom, 1986a, b) were used to generate Km-resistance insertion mutations into orf3. These double mutants grew normally on methyamine.

**xyfE fusion studies employing putative promoter regions for orf3 and orf4**

DNA regions upstream of orf3 and orf4 were cloned into the xyfE transcription fusion vector pHX200 (Xu et al., 1993) to assess promoter activity. Two fragments upstream of orf3 were cloned, resulting in plasmids pLC86A (600 bp HindIII–BamHI fragment containing the 5' terminus of orf3 and 180 bp upstream of it, cloned in the correct orientation with respect to the xyfE reporter gene; Fig. 1); pLC92B-A (1.6 kb BamHI fragment containing the 5' terminus of orf3, 3' terminus of abcC and the region of 700 bp separating the two genes, cloned in the correct orientation with respect to xyfE; Fig. 1), and pLC92B-B (the 1.6 kb BamHI fragment cloned in the opposite orientation with respect to xyfE). One fragment upstream of orf4 was cloned (1.6 kb SauI–BamHI fragment containing the 3' terminus of orf3, 155 bp separating orf3 and orf4, the entire orf4 and the 5' terminus of orf5), resulting in pLC200G (Fig. 1). The plasmids were transferred into M. extorquens AM1 and into a number of mutants, namely MxbD, MxbM, MxcE and MxcQ, involved in the regulation of transcription of methanol oxidation genes (Springer et al., 1995). Activity of catechol 2,3-dioxygenase was measured in transconjugants containing pLC66A, pLC92B-A, pLC92B-B and pLC200G grown on succinate, methyamine, or grown on succinate, washed and then induced with methanol for at least 20 h (Dunstan et al., 1972). In addition, the activity of three C₄ enzymes, hydroxypyruvate reductase, MDH and MADH was also measured in all strains to confirm proper induction by C₄ substrates. All three enzymes were found at the expected levels in all of the strains (data not shown). In all strains, the activities of XyfE were similar in cells grown on succinate, methyamine or induced with methanol, and so only the activities measured in succinate-grown cells are shown in Table 2. The activity was low in constructs carrying pLC66A in wild-type M. extorquens AM1, as well as in the regulatory mutants. XyfE activity remained low when pLC92B-A, carrying a larger promoter fragment was present in wild-type cells, but it was increased about 50-fold in the MxbD mutant, and about 15-fold in the MxbM and the Mxc mutants. Similar results were obtained for pLC200G, indicating the presence of the repressible promoters upstream of both orf3 and orf4. The absolute level of transcription from these putative promoters, however, was low compared to transcription from mxaF (up to 4 pmol min⁻¹ (mg protein)⁻¹; C. Morris & M. E. Lidstrom, unpublished) or mauB, a MADH promoter (up to 6 µmol min⁻¹ (mg protein)⁻¹; unpublished).

**Search for activity of a dye-linked alcohol dehydrogenase**

A search was undertaken for a dye-linked alcohol dehydrogenase activity for which orf3 could be responsible, employing wild-type M. extorquens AM1, MxbD and MxbM mutants in which this putative activity should be increased, insertion mutants in orf3, in which it should be missing, and insertion mutants carrying orf3 cloned into a broad-host-range vector (pRK310) in which the activity should be increased. Cell-free extracts of the strains listed above grown on succinate, methanol or methylamine were isoelectrofocused in gels, and the gels stained in various conditions. A pH range from 7.0 to 9.0 was tried, ammonium ion was either added to or omitted from the buffers, and the
Table 2. Activity of catechol 2,3-dioxygenase in wild-type M. extorquens AM1 and regulatory methanol regulation mutants carrying promoter regions upstream of orf3 and orf4 grown on succinate

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Putative promoter</th>
<th>Activity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>AM1</td>
</tr>
<tr>
<td>pXH200</td>
<td>None</td>
<td>4</td>
</tr>
<tr>
<td>pLCB6-A</td>
<td>orf3</td>
<td>6</td>
</tr>
<tr>
<td>pLC92B-A</td>
<td>orf3</td>
<td>6</td>
</tr>
<tr>
<td>pLC92B-B</td>
<td>None</td>
<td>4</td>
</tr>
<tr>
<td>pLC200G</td>
<td>orf4</td>
<td>10</td>
</tr>
</tbody>
</table>

Activity is in nmol min⁻¹ (mg protein)⁻¹. Data were obtained from two to three experiments and agreed within 25%.

The substrate range included methanol, ethanol, glycerol, isopropanol, formaldehyde, acetaldehyde, propionaldehyde and glycolaldehyde. The artificial electron acceptor normally used for PQQ-linked dehydrogenases, phenazine methosulphate was used. A few aldehyde-oxidizing bands were detected in gels, but none was absent in orf3 mutants, and none was overexpressed in MxbD and M mutants, or in strains carrying cloned orf3. Therefore, the function of the putative enzyme encoded by orf345 remains unknown.

Study of fol region

One of the fol genes (folC) was mutated by insertion of a Km-resistance gene into the PstI site in the middle of the gene (at nt 12311). The two unidentified ORFs upstream of folC were also mutagenized by insertions in order to establish their possible involvement in C₄ metabolism. The SalI site at nt 12870 was used to mutate orf6, and orf7 was mutated by deleting the 340 nt BspMI fragment (nt 13366 and 13706), followed by the Km-resistance gene insertion. While double-crossover mutants were readily selected in orf6 and orf7, only single-crossovers were obtained in folC, even when mutants were selected in the presence of folate. All these mutants were able to grow on C₄ compounds, indicating that orf6 and orf7 are most probably not required for C₄ metabolism. folC must be essential for growth on C₄ as well as on multicarbon compounds, since only single-crossovers were selected, in which a complete copy of folC is restored as a result of recombination. The lack of recovery of null folC mutants in the presence of folate may reflect an inability of the cells to take up folate under these conditions.

A region of 381 bp (EcoRV-HincII fragment) upstream of folC was cloned into pHX200 to test whether fol genes have their own promoter. The correct orientation of the promoter region with respect to the xylE gene (pLC205.2) resulted in low levels of XylE activity [20 nmol min⁻¹ (mg protein)⁻¹] which did not increase considerably during growth on methanol or methylamine (data not shown). The opposite orientation of the cloned fragment with respect to xylE (pLC205.1) resulted in background XylE activity [3 nmol min⁻¹ (mg protein)⁻¹].

DISCUSSION

In this study, we have characterized a region of 14.2 kb separating two well-characterized loci on the chromosome of M. extorquens AM1, one encoding a number of serine cycle genes, another encoding four pqq genes along with two regulatory methanol oxidation genes. Fifteen new ORFs were identified by sequencing. Analysis of the locus previously shown to be responsible for the synthesis of MCL has shown that only one gene in this locus, mclA, is essential for growth on C₄ compounds. This indicates that MCL is composed of one kind of subunit, unlike citrate lyase which is functionally similar. The approximate size of MCL purified from M. extorquens AM1 was determined to be 190 kDa (Hacking & Quayle, 1974), which suggests it is a hexamer of 35 kDa subunits. It was interesting to find a cluster of genes encoding a transporter system in close proximity to C₄ genes, since it seems likely that strain AM1 might contain a specific transporter to regulate the flow of C₄ compounds into the cell or to export PQP to the periplasm for MDH assembly. However, our mutant studies showed that this putative transporter is not required for C₄ metabolism. The role of the gene cluster composed of genes similar to mxaF,G and J (orf345) also remains elusive. We were not able to demonstrate a dehydrogenase activity for which these genes could be responsible. Transcription from these genes seems to be very low under the tested conditions in the wild-type. However, it increases to moderate levels in regulatory methanol oxidation mutants, suggesting that expression of those genes is negatively controlled by the methanol oxidation regulatory system. The product of orf4 seems to be different from the cytochrome c₅₅₅ characterized earlier, which is subject to a similar regulation pattern and whose function is also not known (Day et al., 1990). In P. denitrificans, a similar gene cluster that is also not required for C₄ metabolism is located in close proximity to a C₄ gene, βhA, encoding methylotrophic formaldehyde dehydrogenase (Ras et al., 1995). In this case,
however, the genes seem to be methanol-inducible and mutants in these genes decrease the growth rate on methanol somewhat (Harms et al., 1996).

Three genes, apparently encoding enzymes of folate metabolism, were also found in the region, and are probably co-transcribed from their own promoter. The inability to obtain null mutants in folC demonstrates that these genes must not be C4-specific. It is expected that folate synthesis will be required during growth on multicarbon substrates. During growth on C4 compounds, folates also play a special role in transformations of C4 compounds via methylene tetrahydrofolate, a central intermediate for the serine cycle. Our data suggest both roles are fulfilled by a single set of genes, which may be the reason for these genes being located in a C4-gene cluster. Four new ORFs in the region remain unidentified, and they seem not to be required for C4 metabolism.

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