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, *pqq* 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 9.4 and 31 kDa of unknown function, three putative subunits of an ABC-type transporter, two polypeptides similar to the products of *mxoF* and *mxoJ* 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 *mxoG* and *mxoJ*, 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 methylamine 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 C_1 region containing a serine cycle gene cluster has been linked with another C_1 region, containing *pqq* genes and the regulatory methanol oxidation genes *mxhDM* (Fig. 1). To determine if more C_1 -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 DH5 α (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 Dunstan *et al.* (1972). The following antibiotic concentrations were used for *M. extorquens* AM1: 10 μ g tetracycline ml⁻¹, 100 μ g kanamycin ml⁻¹, 50 μ g rifamycin ml⁻¹. The growth responses of mutants were tested on plates containing the substrates listed above. The following cloning vectors were used: pUC19 (Pharmacia) for cloning and subcloning, pAYC61 (Chistoserdov *et al.*, 1994) as a suicide vector, pAYC67 (gift of A. Chistoserdov, Marine Science Research Center, SUNY at Stony Brook, NY, USA) and pHX200 (Xu *et al.*, 1993) as promoter cloning vectors, pRK310 (Ditta *et al.*, 1985) as an expression vector, and pRK2013 (Ditta *et al.*, 1985) as a helper plasmid.

DNA-DNA hybridization. DNA-DNA hybridizations were carried out with dried agarose gels as described by Meinkoth & Wahl (1984) at 68 °C. For hybridizations, 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate) was used, and 0.5 $\times$ SSC was used for washes.

DNA manipulations. Plasmid isolation, *E. coli* transformation, restriction enzyme digestion, ligation, blunting ends with T4 DNA polymerase, or filling in ends with Klenow enzyme were carried out as described by Maniatis *et al.* (1982). The chromosomal DNA of *M. extorquens* AM1 was isolated by the procedure of Saito & Miura (1963).

DNA sequencing. DNA sequencing was carried out with an Applied Biosystems automated sequencer by the Caltech Sequencing Facility, from both strands.

Computer analysis. Translation and analyses of DNA and DNA-derived polypeptide sequences were carried out using Genetic Computer Group (Wisconsin) and PC/GENE (Genofit SA, Geneva, Switzerland) programs. All putative ORFs showed high correlation to the known codon usage preferences of *M. extorquens* AM1.

Enzyme assays. Enzyme activities were determined in *M. extorquens* AM1 crude extracts prepared as described earlier (Chistoserdova & Lidstrom, 1992). All measurements were done at room temperature in a total volume of 1 ml. Hydroxypyruvate reductase was assayed as described by Chistoserdova & Lidstrom (1991). Methyl-CoA lyase activity was followed by measurement of acetyl-CoA disappearance (Goodwin, 1990). Methanol dehydrogenase, methylamine

dehydrogenase, catechol dioxygenase and chloramphenicol acetyltransferase were assayed, respectively, as described by Anthony & Zatman (1964), Eady & Large (1968), Kataeva & Golovleva (1990) and Shaw (1975). Spectrophotometric methods (Kalb & Bernlohr, 1977; Whitaker & Granum, 1980) were used for protein determination.

IEF. Crude cell extracts were isoelectrofocussed in a pH range of 3–9 using the PhastSystem as described by the manufacturer (Pharmacia Biotech). For alcohol dehydrogenase assays, gels were stained using a reaction mixture containing one of the following buffers: potassium phosphate pH 7.0 or Tris/HCl pH 7.5–8.0, or glucosamine pH 9.0 (100 mM), 1 mM nitro blue tetrazolium, 0.5 mM phenazine methosulphate and one of the following substrates: 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. Rifamycin 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; Hudspeth & Grula, 1989). In earlier studies, the gene for PEPC (*pccA*) 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

Fig. 1. Physical map of the chromosomal region analysed in this work. Only restriction sites discussed in this study are shown. Arrows indicate the direction of transcription. Genes encoding putative membrane polypeptides are shown as filled boxes and genes encoding putative periplasmic polypeptides are shown as striped boxes. Asterisks indicate sites of insertion mutations. Fragments used for promoter or complementation studies are shown at the bottom, indicated by the name of the corresponding plasmid.

Table 1. Nucleotide sequence analysis

ORF	Start (nt)	Finish (nt)	Overlap (nt)	Potential polypeptide (Da)
1*	1	699	0	26033*
2	887	1861	0	35451
3	1887	2156	0	9372
4	2797	3672	0	31097
5	4284	5279	0	34713
6	5319	6056	0	26716
7	6037	6810	20	27715
8	7515	9320	0	64952
9	9475	10065	0	20615
10	10065	10919	1	31032
11	11442	10966	0	15126
12	11830	11437	4	14274
13	12689	11823	8	30702
14	13153	12788	0	13201
15	13697	13134	20	20587

* Partial ORF potentially encoding C-terminal region of a larger polypeptide.

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 deduced 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 three 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 *c_L* (encoded by *mxgG*) from *M. extorquens* AM1 or other methylotrophs. This gene was designated *orf4*. Another *c*-type cytochrome, cytochrome *c₅₅₃*, 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 *c₅₅₃*. However, a comparison of the amino acid composition of the deduced mature polypeptide of *Orf4* with the amino acid content of *c₅₅₃* determined experimentally by Day *et al.* (1990) showed significant differences. For example, *Orf4* contains only one serine, while nine serines were chemically determined in *c₅₅₃*. The threonine content also differs considerably (four and nine, respectively). In addition, the predicted molecular mass for mature *Orf4* is 18 150 Da while *c₅₅₃* runs on SDS gels as a 23 kDa band (Day *et al.*, 1990). The product of the next ORF showed similarity with *XoxJ* from *P. denitrificans* (41%; Harms *et al.*, 1996) and lower similarity with *MxaJ* 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 *mxal* 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%), dihydroneopterin 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 *C₁* 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 *SphI* site in the second half of *mclA* (at nt 1525) was used for inserting a kanamycin (Km)-resistance gene, and two *AvaI* 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 succinate and then testing for their ability to grow on *C₁* and *C₂* compounds. Double-crossover mutants in *mclA* showed the expected phenotype, being unable to grow on *C₁* compounds, while double-crossover mutants in *orf2* were able to grow on *C₁* compounds. Thus, *orf2* is most probably not involved in the synthesis of active MCL or any other essential *C₁* 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 *C₁* 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 *Bgl*II–*Bam*HI 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 [$120 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$]. Activity was not detectable when the fragment was oriented divergently 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 C₁ metabolism. Insertion mutations with a Km-resistance gene were generated in all three genes, using the *Eco*RV site in the middle of *abcA* (at nt 4472), the *Sma*I site in the middle of *abcB* (at nt 5490) and the *Bam*HI 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 C₁ compounds, indicating that the putative ABC-type transporter system is not essential for growth on C₁ compounds, at least under the conditions used.

Mutations in the *orf345* region

orf345 most probably encode, respectively, a PQQ-linked dehydrogenase, an associated cytochrome and a polypeptide analogous to MxaJ (MxaJ has been postulated to be involved in assembly of MDH). Two mutations were obtained in the region. The first is an insertion into the *Bam*HI site in the beginning of *orf3* (at nt 7859). The second mutation involves a 188 nt *Bsp*MI deletion (nt 9968–10156) involving both *orf4* and *orf5*, combined with the Km-resistance gene insertion. Double-crossover mutants in both loci were able to grow normally on C₁ and C₂ compounds, indicating that these genes are not required for C₁ or C₂ 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 *mxoF* 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 methylamine.

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

DNA regions upstream of *orf3* and *orf4* were cloned into the *xylE* transcription fusion vector pHX200 (Xu *et al.*, 1993) to assess promoter activity. Two fragments upstream of *orf3* were cloned, resulting in plasmids pLCB6A (600 bp *Hind*III–*Bam*HI fragment containing

the 5' terminus of *orf3* and 180 bp upstream of it, cloned in the correct orientation with respect to the *xylE* reporter gene; Fig. 1); pLC92B-A (1.6 kb *Bam*HI 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 *xylE*; Fig. 1), and pLC92B-B (the 1.6 kb *Bam*HI fragment cloned in the opposite orientation with respect to *xylE*). One fragment upstream of *orf4* was cloned (1.6 kb *Sal*I–*Bam*HI 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 pLCB6A, pLC92B-A, pLC92B-B and pLC200G grown on succinate, methylamine, 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 C₁ enzymes, hydroxypyruvate reductase, MDH and MADH was also measured in all strains to confirm proper induction by C₁ substrates. All three enzymes were found at the expected levels in all of the strains (data not shown). In all strains, the activities of XylE were similar in cells grown on succinate, methylamine 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 pLCB6A in wild-type *M. extorquens* AM1, as well as in the regulatory mutants. XylE 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 repressible promoters upstream of both *orf3* and *orf4*. The absolute level of transcription from these putative promoters, however, was low compared to transcription from *mxoF* [up to $4 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$; C. Morris & M. E. Lidstrom, unpublished] or *mauB*, a MADH promoter (up to $6 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$; 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 isoelectrofocussed 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

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

Strain/plasmid	Putative promoter	Activity				
		AM1	MxbD	MxbM	MxcE	MxcQ
pXH200	None	4	5	3	4	4
pLCB6A	<i>orf3</i>	6	15	10	12	14
pLC92B-A	<i>orf3</i>	6	320	90	100	80
pLC92B-B	None	4	5	4	6	7
pLC200G	<i>orf4</i>	10	290	120	80	60

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 *Pst*I 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 C₁ metabolism. The *Sal*I site at nt 12870 was used to mutate *orf6*, and *orf7* was mutated by deleting the 340 nt *Bsp*MI 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 C₁ compounds, indicating that *orf6* and *orf7* are most probably not required for C₁ metabolism. *folC* must be essential for growth on C₁ 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 (*Eco*RV–*Hinc*II fragment) upstream of *folC* was cloned into pXH200 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) re-

sulted 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 C₁ 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 C₁ genes, since it seems likely that strain AM1 might contain a specific transporter to regulate the flow of C₁ compounds into the cell or to export PQQ to the periplasm for MDH assembly. However, our mutant studies showed that this putative transporter is not required for C₁ metabolism. The role of the gene cluster composed of genes similar to *mx*A,F,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 *c*₅₅₃ 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 C₁ metabolism is located in close proximity to a C₁ gene, *flhA*, 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 C₁-specific. It is expected that folate synthesis would be required during growth on multicarbon substrates. During growth on C₁ compounds, folates also play a special role in transformations of C₁ 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 C₁-gene cluster. Four new ORFs in the region remain unidentified, and they seem not to be required for C₁ metabolism.

ACKNOWLEDGEMENTS

We thank A. Chistoserdov for providing his vector, pAYC67. This work was supported by a grant from the NIH (GM36294).

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Received 4 October 1996; revised 4 December 1996; accepted 18 December 1996.