Molecular characterization of a chromosomal region involved in the oxidation of acetyl-CoA to glyoxylate in the isocitrate-lyase-negative methylotroph *Methylobacterium extorquens* AM1

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A region on the *Methylobacterium extorquens* AM1 chromosome previously shown to complement a chemically induced mutant (PCT48) unable to convert acetyl-CoA into glyoxylate was characterized in detail in order to identify the gene(s) involved in the unknown pathway for acetyl-CoA oxidation. Six complete and two partial ORFs were identified by sequencing. Sequence comparisons suggested these might code for, respectively, a dehydrogenase of unknown specificity, a polypeptide of at least 15 kDa with unknown function, a coenzyme-B₁₂-linked mutase, a catalase, an alcohol dehydrogenase (ADH) of unknown function, a polypeptide of 28 kDa, a ketol-acid reductoisomerase and a propionyl-CoA carboxylase (PCC). Insertion mutations were introduced into each ORF in order to determine their involvement in C₁ and C₂ metabolism. Mutations in three genes, encoding the mutase, ADH and PCC, resulted in a phenotype characteristic of mutants unable to oxidize acetyl-CoA, i.e. they were C₁- and C₂-negative and their growth on these compounds was restored by the addition of glycolate or glyoxylate. Mutants in the genes thought to encode catalase and PCC were found to be deficient in the corresponding enzyme activity, confirming the identity of these genes, while physiological substrates for the mutase and ADH remain unidentified. This work, in which three new genes necessary for conversion of acetyl-CoA into glyoxylate were identified, is an intermediary step on the way to the solution of the unknown pathway for acetyl-CoA oxidation in isocitrate-lyase-negative methylotrophs.

Keywords: *Methylobacterium extorquens*, isocitrate-lyase-negative methylotrophs, serine cycle, acetyl-CoA oxidation, C₁ and C₂ metabolism

INTRODUCTION

Methylobacterium extorquens AM1 is a pink-pigmented serine cycle methylotroph able to grow on methanol and methylamine, and also on a variety of multicarbon substrates including ethanol, ethylamine, lactate, pyruvate, malonate, β -hydroxybutyrate and succinate (Peel & Quayle, 1961; Tsuji *et al.*, 1990). For more than two

decades, this organism has been used as a model for studying both the biochemistry and the genetics of the serine cycle utilized for assimilation of C₁ compounds at the level of formaldehyde (Salem & Quayle, 1971; Dunstan *et al.*, 1972a, b; Dunstan & Anthony, 1973; Anthony, 1982; Chistoserdova & Lidstrom, 1992, 1994a–c). In the serine cycle, formaldehyde in the form of methylenetetrahydrofolate condenses with glycine to produce serine, which then undergoes transamination with glyoxylate, producing hydroxypyruvate, which in turn is reduced to D-glycerate. The latter is phosphorylated to phosphoglycerate, then transformed to phosphoenol pyruvate, which is carboxylated to produce malate and then malyl-CoA. This is then cleaved to glyoxylate and acetyl-CoA (Anthony, 1982). In the serine

Abbreviations: ADH, alcohol dehydrogenase; HPR, hydroxypyruvate reductase; Km, kanamycin; MCM, methylmalonyl-CoA mutase; PCC, propionyl-CoA carboxylase; Tc, tetracycline.

The GenBank accession numbers for the nucleotide sequences of the 8988 bp *HindIII*–*EcoRI* fragment and the 285 bp *EcoRI*–*HindIII* fragment reported in this paper are L48340 and L48243, respectively.

methylotrophs which lack isocitrate lyase (Icl^-), such as *M. extorquens* AM1, the second molecule of glyoxylate must be regenerated from acetyl-CoA, but the biochemical pathway leading to such a conversion remains unknown (Anthony, 1982). Studies of the fate of carbon atoms originating from ethanol, acetate or methanol have indicated that a common pathway must operate that leads from acetyl-CoA to glyoxylate during assimilation of C_1 and C_2 compounds, and also multicarbon compounds like malonate and β -hydroxybutyrate (Salem & Quayle, 1971; Dunstan *et al.*, 1972a, b; Dunstan & Anthony, 1973).

The pathway of C_2 assimilation also remains unknown in other bacterial groups (Dawes & Sutherland, 1994). Elucidation of this pathway in methylotrophic bacteria might bring about greater understanding of the fate of acetyl-CoA in other Icl^- organisms.

In *M. extorquens* AM1, three chromosomal fragments have been identified carrying genes encoding enzymes involved in assimilation of both C_1 and C_2 compounds and probably operating in the unknown pathway for glyoxylate regeneration. One of these, *orf4*, encodes a protein of unknown function and is located in a large cluster of C_1 genes that includes a number of serine cycle genes (Chistoserdova & Lidstrom, 1994c). Another gene, *glyA*, is located on a separate chromosomal fragment and encodes serine hydroxymethyltransferase. This enzyme apparently plays a dual role in *M. extorquens* AM1, firstly in the serine cycle and secondly in the oxidation of acetyl-CoA (Chistoserdova & Lidstrom, 1994b). The third chromosomal fragment not linked to the two fragments mentioned above has been shown to complement a number of chemically induced mutants such as PCT48 that are unable to grow on C_1 and C_2 compounds and are complemented by the addition of glycolate or glyoxylate (Stone & Goodwin, 1989; Smith & Goodwin, 1992; Smith *et al.*, 1995). PCT48 was shown to contain a deletion of 3.3 kb in this region (Smith & Goodwin, 1992). The goal of the present study was to characterize this last region in more detail.

MATERIALS AND 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), ethylamine (20 mM), formate (40 mM), pyruvate (40 mM) or β -hydroxybutyrate (20 mM) were used as substrates. Methanol induction of mutants was carried out as described by Dunstan *et al.* (1972b). The following antibiotic concentrations were used for *M. extorquens* AM1: tetracycline (Tc), 10 $\mu\text{g ml}^{-1}$; kanamycin (Km), 100 $\mu\text{g ml}^{-1}$; and rifamycin, 50 $\mu\text{g ml}^{-1}$. The growth responses of mutants were tested on plates containing the substrates listed above in the presence or absence of supplements of glyoxylate (5 mM) or glycolate (20 mM).

DNA-DNA hybridizations. These were carried out with dried agarose gels as described by Meinkoth & Wahl (1984) at 68 °C. SSC (0.15 M NaCl, 0.015 M sodium citrate) was used at 6 $\times$ concentration for hybridizations and 0.5 $\times$ concentration 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. The DNA sequence of both strands was determined with an Applied Biosystems automated sequencer by the Caltech Sequencing Facility.

Computer analysis. Translation and analyses of DNA and DNA-derived polypeptide sequences were carried out using PC/Gene (Genofit, Geneva) or Genetic Computer Group (Wisconsin) programs.

Enzyme assays. Enzyme activities were determined in *M. extorquens* AM1 crude extracts prepared as described previously (Chistoserdova & Lidstrom, 1992). All measurements were done at room temperature (26 °C) in a total volume of 1 ml. Hydroxypyruvate reductase (HPR) was assayed as described by Chistoserdova & Lidstrom (1991). Catalase was assayed by following A_{240} (Beers & Seizer, 1952). Propionyl-CoA carboxylase (PCC) was assayed by following the appearance of ADP. The reaction mixture contained 50 mM Tris/HCl, pH 7.5; 3 mM ATP; 10 mM MgCl_2 ; 3 mM propionyl-CoA; 0.2 mM NADH; 3 mM phosphoenolpyruvate (PEP); 20 units pyruvate kinase (PK); 7 units lactate dehydrogenase (LDH) and crude extract containing 0.1–0.3 mg protein. Methylmalonyl-CoA mutase (MCM) was measured by disappearance of ADP in a discontinuous assay coupling MCM and PCC reactions. For this assay, PCC was overexpressed by introduction of cloned *pccA* (see Results). The reaction mixture contained, in 0.5 ml 50 mM Tris/HCl, pH 7.5; 0.5 mM ADP, 5 mM MgCl_2 , 3 mM succinyl-CoA, and crude extract containing 0.1–0.3 mg protein. The reaction was allowed to proceed for 5–10 min, after which the remaining ADP was measured in LDH reaction by the addition of 0.5 ml of the following mixture: 50 mM Tris/HCl, pH 7.5; 3 mM PEP; 0.2 mM NADH; 20 units PK; and 7 units LDH. Spectrophotometric methods (Kalb & Bernlohr, 1977; Whitaker & Granum, 1980) were used for protein determination.

Isoelectrofocusing. Crude cell extracts were isoelectrofocused in a pH range of 3–9 or 4–6.5 using PhastSystem as described by the manufacturer (Pharmacia-Biotech). For alcohol dehydrogenase (ADH) assays, gels were stained using a reaction mixture consisting of a buffer (potassium phosphate, pH 7.0; Tris/HCl, pH 7.5–8.0; or pyrophosphate, pH 9.0) plus 1 mM NAD or NADP, 1 mM nitro blue tetrazolium, 0.5 mM phenazine methosulphate and 1–10 mM substrate (ethanol, 2-propanol, glycerol, acetaldehyde, propionaldehyde, glycolaldehyde, glycolate or glycerate). Catalase was visualized by incubating gels in a buffered (pH 7.0) 3% (v/v) solution of hydrogen peroxide and noting the appearance of bubbles.

Matings. Triparental or biparental matings between *E. coli* and *M. extorquens* AM1 were performed overnight on nutrient agar at 30 °C. 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

Identification of the gene impaired in mutant PCT48 and sequence of adjacent DNA regions

In *M. extorquens* AM1, a 13.5 kb *Hind*III fragment was shown to be responsible for complementation of PCT48, a mutant impaired in the conversion of acetyl-CoA to glyoxylate (Stone & Goodwin, 1989). This fragment was subcloned from a cosmid pSS48.11 (supplied by P. Goodwin) into pUC19 (Pharmacia) and sequenced. A 4.2 kb *Eco*RI fragment within this region has been sequenced elsewhere, and analysis of these data has shown that the lesion in the mutant PCT48 was in a gene (*meaA*) highly similar to genes from various sources encoding a coenzyme-B₁₂-linked enzyme, MCM (Smith *et al.*, 1995). We have independently sequenced this region, and have confirmed that finding. Here we report the sequence of regions flanking *meaA*. The physical map of the 13.5 kb chromosomal fragment employed in this study is shown in Fig. 1. Six complete and two partial ORFs found in the region are indicated. Fig. 2 shows the sequence of the 285 bp *Hind*III–*Eco*RI fragment at the left (Fig. 1a) and the 8988 bp *Eco*RI–*Hind*III fragment at the right (Fig. 1b) of the 13.5 kb fragment shown in Fig. 1. The sequence of the middle 4.2 kb *Eco*RI fragment will be published elsewhere (L. M. Smith and others, unpublished results). Analysis of the sequence revealed six complete and two incomplete ORFs. Reading from left to right on Fig. 1, the first (partial) ORF potentially encodes the C-terminal 25060 Da of a larger polypeptide, the second could encode a polypeptide of at least 15 kDa (we have not completed the double-stranded sequence of this gene) and the third encodes MeaA (78293 Da) (Smith *et al.*, 1995). The fourth ORF is potentially able to encode a protein of 55550 Da. A fifth ORF able to encode a polypeptide of 47529 Da begins 82 bp from the fourth. The next 894 bp do not seem to contain any ORFs and separate the fifth ORF from a divergently transcribed ORF, potentially able to encode a polypeptide of 28937 Da. This sixth ORF is separated by 167 bp from a seventh, transcribed in the same direction, potentially able to encode a polypeptide of 36879 Da. The eighth (partial) ORF in the region begins beyond the described fragment and could encode the C-terminal 55199 Da of a larger polypeptide. A region of 1723 bp separating the seventh ORF from the eighth one seems to be a non-coding region. A few inverted repeat sequences having the potential to form a stable stem-loop-type structure were found in the described region [hairpins with free energy exceeding -14 kcal (approx. -58.5 kJ) are underlined in Fig. 2], which could serve as terminators. The structures with the highest calculated free energies are found immediately downstream of the fourth ORF [calculated ΔG (25.5 °C) -35.8 kcal (approx. -149.6 kJ)], the eighth ORF [calculated ΔG (25.5 °C) -24.6 kcal (approx. -102.8 kJ)] and the seventh ORF [calculated ΔG (25.5 °C) -20.8 kcal (approx. -86.9 kJ)].

Amino acid sequence comparisons

The amino acid sequences deduced from complete and partial ORFs found in the region under study were

Fig. 1. Physical and genetic map of the 13.5 kb *Hind*III fragment of the *M. extorquens* AM1 chromosome able to complement mutant PCT48. Arrows show the direction of transcription, and asterisks show sites of the *Km*^r gene insertions with corresponding mutation names below.

compared against the protein databank. The first (partial) ORF showed similarity with a number of dehydrogenases, including 3-oxoacyl-[acyl-carrier-protein] reductase (about 35 % identity; Klein *et al.*, 1992; Slabas *et al.*, 1992), glucose 1-dehydrogenase (about 32 % identity; Lampel *et al.*, 1986; Heilmann *et al.*, 1988), acetoin (diacetyl) reductase (about 25 % identity; Blomqvist *et al.*, 1993) and acetoacetyl-CoA reductase (about 23 % identity; Peoples & Sinskey, 1989). This gene was designated *xdh*, for unknown dehydrogenase. No significant similarity to known protein sequences was found for the second ORF, which was designated *orf1*. The polypeptide deduced from the third ORF showed high identity with subunits of MCM from both prokaryotic and eukaryotic sources (about 35 %; Francalanci *et al.*, 1986; Jansen *et al.*, 1989; Wilkemeyer *et al.*, 1990), and has been designated *meaA* (Smith *et al.*, 1995). The next ORF, transcribed divergently from *meaA*, was shown to encode a polypeptide highly similar to catalase subunits from various sources (Murthy *et al.*, 1981; Schroeder *et al.*, 1982; Bell *et al.*, 1986; Furuta *et al.*, 1986; Orr *et al.*, 1990; Bol *et al.*, 1991) with identities ranging from 40 to 57 %, and was designated *katA*. The gene downstream of *katA* encodes a polypeptide with similarity to NAD-dependent ADHs belonging to different classes having specificity for short-chain alcohols (24–34 % identity; Russell *et al.*, 1983; Russell & Hall, 1983; Young & Pilgrim, 1985; Gwynne *et al.*, 1987; Saliola *et al.*, 1990) and was designated *adhA*. The polypeptide translated from the next ORF, transcribed divergently from *adhA* did not reveal strong identity to known proteins and was designated *orf2*. The putative product from the seventh ORF was shown to bear high similarity to *ilvC* gene products from bacteria and yeasts, with identities ranging from 32 to 54 % (Daniels *et al.*, 1992; Godon *et al.*, 1992), and was designated *ilvC*. The last (partial) ORF in the region encodes a polypeptide highly similar to the β -subunit of mammalian PCC (about 60 % identity; Kraus *et al.*, 1986; Lamhonwah *et al.*, 1986), and was designated *pccA*.

(a)	5'	AAGCTTGACCTAAGGTGCGGTGCGTCAACGGGATCGGCGCCGATCGCAAAAGCATGGCGAAGGATGGCGGCCGCTGCTCACTACGCATCGAGCAAGGCC	120
		K L D L R S R C V T G A S K G I G A A I A K A L A K D G A A V V V N Y A S S K A	
	5'	GGCGCGGATGCGGTGCGTGAAGCATACGGCGCCGGCGGCAAGGCCATCGCGGTCCAGGCCGATGCTCGCAGGCCGTCAGGCGCGGGCTTGGTTGAGGCCGCGGTGCAAGCAATTC	240
		G A D A V V E A I T A A G G K A I A V Q A D V S Q A V Q A R G L V E A A V Q Q F	
	5'	GGCGGCTCGACGTGCTGGTGAACAATTCGGCGTCTACGAATTC	285
		G R L D V L V N N S G V Y E F	
(b)	5'	GAATTCGCTCACCCTGGCTGCGGTGGCGGTCTGATGCAAGGATACCACTCATCGAGAAGATGGCCACTTCAACGGGAGCGGATCCCGAGCGGGTGGTGCACGCCAAGGCTA	120
		N S L T A G S R G P V L M Q D Y H L I E K M A H F N R E R I P E R V V H A K G	
	5'	CGCGGCTACGGCAGCTTCCGGTCAACAGAAGCTGTCCGAGTTCAACCGTGCAGAGGTGCTGACCGAGGTGGCAAGGACGTGCCGATGGTGGCGCGCTTCCACCGTCCGGCGGA	240
		Y G A Y G T F R L T R S L S E F T R A K V L T E V G K D V P M V A R F S T V G G	
	5'	GTCGGTTCGGCGCAGCGCCGCGGACCGCGGCTTCGCCCTCAAGTTCTACACCGAGGAGGCACTGGGATCTCGTCGGCAACACACCGGATCTTCTTCGTGCGCGACGCGAT	360
		E S G S A D T A R D P R G F A L K F Y T E E G N W D L V G N N T P I F F V R D A	
	5'	CAAGTTCTCGACTTCATCCGACCCAGAAGCGGATCCGCGACCCACCTCAAGCGCATTTGGCGCGCTGGGACTTCTGGAGCTGTCGCCGAGCGGATCCATCAGTCATGTTCT	480
		I K F S D F I R T Q K R D P R T H L K P H W R R W D F W S L S P E A I H Q V M E	
	5'	GTAATCGGATCGCGCAGCGCCGAATTCGGCGGTCTTATGAACGGCTACGGCTCGCACCTTCTCGCTCGGAACGACCGGGCGAGCGGCTGGTGAAGTTCGCACTTCACACCAA	600
		L Y S D R G T P K S A R F M N G Y G S H T F S L W N D R G E R H W V K F H F H T	
	5'	CGAGGCTACGAGAACTTCTCGCGGACGAAGCGGACGAGGTAGCGGGCAAGATCCGACTACTCTCCGCGGACCTCTCGACTGCCATCGAGCAGGGCGACTTCCCGAAATGGAAAGT	720
		K Q G I Q N F S A D E A D E V A G K D P D Y S S A D L S T A I E Q G D F P K W K	
	5'	CTCCGTCAGCTCATGCCGAATTTGGAGCGGACCTTACCGCATCAACCGGTTCGACCTGACCAAGGTTCGGCGCAGCGGACTATCCGTCATCGAGATTGGCGAGATGGAGCTCAA	840
		V S V Q L M P E L D A D T Y R I N P F D L T K V W P H G D Y P L I E I G E M E L	
	5'	CCGCAATCCCGAGAACTTCTCGCGGAGATCGAGCAATTCGCTTCGAGCGCTCGAAGTGGTGGCGGACGCGTTCCTCGCGGCAAGATGCTCGAGAACCGGGTCTGCTCTATGC	960
		N R N P E N Y F A E I E Q S A F E P S N V P G I G F S P H K M L Q A N C V L S Y	
	5'	CGACGCCACCGCTACCGGCTCGCGGTGAATACCAAGATCCCGGTGAACAGGCGAAACGCTGACGTTGAGAACTACCATCGCGACGGCGCATCGCGCACCGCAATCGGCAATCGG	1080
		A D A H R Y R L G V N Y Q Q I P V N Q A K N A D V Q T Y H R D G A M R T D G N H	
	5'	CGCGCAGGTGATTACGAGCGGAATCTCTCGCGGTCCGAACAGGACCGCTCTTCAGCGAGCGGCGCTCGGGATCCAGGGCGATGTCGGCGCTACGGTTCGGCGGCGGACGAGA	1200
		G A Q V D Y E P N S F G G P K Q D P S F S E P P L R I Q G D A G R Y G W P F D K	
	5'	GGATCTCTACGGTCAGCCCAAGCTGTTCTGGACCAAGGTCTGGATGAGGGCGCGCAAGCGGCTCGTCGAGAATATCGTCACCTCGATGGCGGACAGCGCGCCACATTCAGGAGCG	1320
		E D L Y G Q P K L F W T K V L D E G G R K R L V E N I V T S M G D S P R H I Q E	
	5'	GATGATCGCGCACTGTTCAAGGTGCAGGAGTTTCGGCGCGGTGTGGCGCAGGGGCTCGGCATCGATACGGCACAGGCGCGCGCAATAGTCATCTCAACATTATTTGAATACAG	1440
		R M I A H W F K V H E D F G W A Q G L G I D T A Q A A A E *	
	5'	GGGCATCGAGTGTGGTGGTGGCTTTTTGTGCGGACGAGGCAAGGCTTGCCTTTAGACCACTTGAAGGCTGGAACATCGTCTTATAGCTCGGCCAAGGTGCGCGGCTGCT	1560
		CACGCGGTGCCAAGCAACAGGAGTGCCACGATGGCTGCAAGCGCAGCACCGGCTGGACCGGGCAGACGGCGAAGCAAGGACCTTACGAGCTCGCGGAGATCCCCCGCTCGG	1680
		M A A S A A P A W T G Q T A E A K D L Y E L G E I P P L	
	5'	CCAGTGCCTGCCAAGATGTATGCTGGCGATCCCGCGGAGCGGACCGGGCGCGGAGCAGTCCGACAGCTCGAAGTGTCTCCGCTCGGGAGATCGGCGACGACGAGGTGCTCGT	1800
		G H V P A K M Y A W A I R R E R H G P P E Q S H Q L E V L P V W E I G D D E V L	
	5'	CTACGTCATGGCGCGGGGTGAATACACGCGCTTGGGCGCGGCTGGGCGAGCTATCTCGCGCTTCGACGTGCACAGGGCGAGTACACATCGCGCGCTCGGAGCGTTCGGGTAT	1920
		Y Y V M A A G V N Y N G V W A G L G E P I S P F D V H K G E Y H I A G S D A S G	
	5'	CGTCTGGAAGGTTCGGCGGCAAGGTGAAGGTTCGGAAGGTTCGGCGGACGAGGTATCGTCCATTGTAACAGGACGAGCGGACGACGAGGAGTGCACGGCGCGGATCCGATGTTCTCGCC	2040
		I V W K V G A K V K R W K V G D E V I V H C N Q D D G D D E E C N G G D P M F S	
	5'	GACCCAGCGGATCTGGGTTACGAGACCGGCGAGGTTGCTGTCGCGAGTTCTCGCGGTGCGAGTCCGCTCAGCTCATGGCCCGCCCAAGCACCTGACCTGGGAAGAGCGCGCTGCTA	2160
		P T S Q R Q L M A R P K H L T W E A A C	
	5'	CACGCTGACGCTCGCACCGCTACCGCATGCTGTTGCGCCACGCGCGCACACCGTTCGCTCGGGCGAGAACGCTGCTGATCTGGGGCGCTCCGGCGGCTCGCGGTGTTGCGGTCCA	2280
		Y T L T L A T A Y R M L F G H A P H T V R P G Q N V L I W G A S G G L G V F G V	
	5'	GCTCTGCGCGGCTCCGGCGCACGACATCGCGCTGATCTCGGACGAGTCAAGCGCGACCTACGCTGATGAGCTCGGTGCGAAGGCGCTCATCAACCGCAAGGACTTCGACTGCTGGG	2400
		Q L C A A S A N A I A V I S D E S K R D Y V M S L G A K G V I N R K D F C W	
	5'	TCAGCTCCGACCGCTCAACAGCCCGAATACACACCTGGCTCAAGGAAGCCCGAAGTTCGGCAAGCGGATCTGGGACATCACCGCAAGGCAACGACGTCGACATCTGTTCGAGCA	2520
		G Q L P T V N S P E Y N T W L K E A R K F G K A I W D I T G K G N D V D I V F E	
	5'	TCCCGGAGAGGCGAATTCGCGGTCTCGACGCTGGTGGCGCAAGCGCGCGGATGATGCTGTTCTCGCGCGCACCGGCTTCAACATCACTTCGACGCCCGGTACGCTGGATGCG	2640
		H P G E A T T F P V S T L V A K R G M I V F C A G T T G F N I T F D A R V I E A G P	
	5'	GCAGAAAGCATACAGGCTCGCACTTCGCCACCTCAAGCAGGCGCTCGCGCGCAACAGTTCGTCGATGACCGCGCGCTCGATCCCTGCATGAGCGAGTGTTCCTCGGCAAGAT	2760
		R Q K R I Q G S H F A H L K Q A S A A N Q F V M D R R V D P C M S E V F P W D K	
	5'	CCCGCGCGCCACACCAAGATGTGGAAGAACCAGCACCCCGCGGCAACATGGCGGTGCTGCTCAACTCCACCGCGCGGGTCTGCGCACGGTTCGAGGACGTGATCGAGGCGCGCGCT	2880
		I P A A H T K M W K N Q H P P G N M A V L V N S T R A G L R T V E D V I E A G P	
	5'	CAAGCGATGTGAGCGATCCAGTGCACGAAGAAGCCCGTATCCCGGTGATGCGCGGGCTTCTTTCGTTGGGGAGCGGTTACGCGCACCGGTCGATCGCCCGTCCGGTGTGC	3000
		L K A M *	
	5'	CGATGCTGTTGGAAGCGGCTTCAACGCCCGCTTCTCGAAGCGACGATGCTGTGAGAGCTGGATCGCGGATGCTCGCGCGATCTCTGCGCGCCCGGTGCTGATACCAAG	3120
		G T C G A T G C T G C A T G A C C C T C G T A T C C A G A A C G T G C C C G G C G G T A G C C C C T C G T A T C C G C C A G T A C G A G T T T C G G C A G T A G A C C G T C G	3240
		C G C A G A C G G A A C A G C T C G A A G G T G A C G G T T G T G C T C G C A C T G C C T G C T A T C C G C C A G T A C G A G T T T C G G C A G T A G A C C G T C G	3360
	5'	ATCGGCTCGAGCGATACGGACCTGTGCGCGGACCGCTGACACCGCGCAGCGGATCGACATCGACCGCTAGAGAAATGGCGCTCGGTCGGAAGAAAGTGCAG	3480
		G G A C C C C C T C T A G A C C C G A T C T G A G A A C G C A C G G T C G C C G T C C T C C C T G C G C G G A A G G C G C A A C A G C C G G T G A G A T C G C A G A A T G G T T C C A C T T G T G A T	3600
		C C T C G C G G G A G A T C T G C G C T A G A G C T C C T C G C T G C C A G C G G C A G C A G C G C C T T C C C G C G T T G T C G C C G A C A T A G C C T C C T C A C C A A G G T A G A C A A C T A	3600
	3'	G T C C A C C A C G C G C A T A T C G G A C T T T C C G A C A C G C G G G C G C A A A T G G A C C G C T A G C G C T A T C T T A C G A A C C G C C G A C A C G C T T A T T A G G C G A C T	3720
	3'	A A A C C G T G C T G A A T C A C G G C A G A A C T A G A C C C T A C A C A C G T C G C A A C G A A T T C T T T G C A G T A A G C C T A C C C G G C A C A C A C G C C C A G C C C G C T C T G T G C T	3840
		* G S I R G T T S D S T A P V F C S	
	3'	GGCGCGCGGAGCATTTGCGCGCGCCAGCTTGTACGCTCTCCGCTCGGCGCAGCAGAGTCCGGAGCGCGCGGCTGCTCGCGGACATAAGCCCTGAAGCTCCCGCGGAAGGA	3960
		G A R S T F G A P D F S H S L R R R D T E P R A A P S S R R T N R S K S A A K R	

Fig. 2. For legend see opposite.

ACCGCTGCGCGCGGAACAGGACAGGACGACTACTCGAGCGCGCGTCTAGCTCGCGGAAGGTCTCGCGCGCTCGCGCGAGAGGCGCAGCGCGCGCGCGCTGATGAACAGAGAGCTCGAAGAA
P S A R R T R T A G S A S T A P L I S G S G P R S R R R G T P R P V Y K N Q E L K K 4080

3' CATGAATACCTACATCGCGCGCGCTCACTAGGCTCTAGAAGCAAGTGTAGCTGGCGTCTCTATGAAGCGGAAGCACTAGCGCGGTAGTCGCTAGCAGCGCGCTCGCTCGGCACCTGG 4200
Y K H I Y R P C N I W I K T T V D V R V L Y K A G T Y E A M L P D D R L L S A H G

3' GACCTGCGCTACGCGACCGCGAGAGCTTCAGTATTCTGCTCGGCTAGAACCCGAGCGGAGCTACACGGGTAGCTCATCTTGTAGGCGAACCGGACCTTCGCGCCCTCTGCTGTC 4320
Q V R I R H P T E F D I F L L G I X A Q G E I N G M S Y F M A N G D F G P F V L

3' GCAGCTCGCGAGCTGGTGAAGTGTCTCGGCCAGCGCGCGGGAACATCTCTGCCAGCGCTACGGCAGCGCTAGTTGCCACCTCTCTCTTGTACAGCGCGCTCGCTACTTCGCGCG 4440
T S G D V V K V F G T P G G K Y S V T P M G D P D V T S L L F F M D R S A I F A A

3' GAGCAGCCCACTTAGCAGAGCACTTGGGTAGGACAGCTCCGCTCGAAGCCAGCAGCGGTAAAGAGGACTCCCGCGCCACCGAGGAGCTCATCTCGCTACGAGTATACGA 4560
E D P Q I T E H V D E D L R V A L K T T G F E E Q L A R H A E S Y L S A

3' GAGAGGCGCGCGCTAACGAAGCGCGGAGGACTCCCTCGCAATTCGTTTCGCGGTTCCCGGTTTTCGCGCGCGCTTTTCGCGCGAGCTGCGAGCGCGCTTGCGCGGAGTCCGTGGTG 4680
TCGCGCTCGCGCTCGCTTCGCGCGGAGGTTTCAGGCTTTTCTAGTGAAGAACGAACAGCTGGTCCCGGAAGACCTCTAGGTGGCGTAGTACGCTGCTCGAACCAGCGCTGGAAGGAGC 4800
* N K T K D V L A K E S I W P M G R L K G V E E I

3' TAGGCCACCGCGCGCTGGAACCGCGCCACCGGAACCTGCTCGACAGCGCTGCAAGAGGTGTAGTGTACACCGCACTCTACGGGCTGAOCTACGTAACTCTCGCGGAAGTAG 4920
P H A A L K A R T A K F S T Q G V K N E L M W N R T F I G S Q I D N L V R K M E

3' AGCGGGAACGAGCGCGCTGCTACGCGCGCGCTCATGAGCGGCATGACGCCCAAGCTCTAGCTCATCAAGTCAACCGCTACGCGGGAGGAGCTCTACTGCTGCTGCTAG 5040
A K T E P T V I R P G T V Y E G Y E A T N S I S Y N M N A I G G E Y I L D V I L

3' TCGAAGTGGACAGCTCCGTGAGCTTTATCCGCTAGAGCGCGCGCATCGCGCGGAGCTGGTTCGACAGCTTCGCGCGGAATAGTCAAGCTGCTCGCGCGCGCTCTCGTCCGCGAGGT 5160
K V E H L C E F Y A M E P A Y G A E V L T E F G A K I L E V L G G C L V A Q E G

3' GCGTCTGTCGACGAGCTGAGAAGGAATTCACAGAGCTACTAGCGCGCGCGCGCGGTAACCGCTACCGCATCTCTCGCGCTCCAGCCCGGACCGGCTCCGCGAGGAC 5280
F L D T E C E E K F T T E I I G A R G G G A C C Y A S L G L D H G N S C D Q Q T

3' CACTACCTCTAGTCCAGCGCTCGCGCGCGGGAACCTCATGAGCGCGCGTCCACACCGCGCCAGCGGAAGCGCGCTGGTACTCTGTCAGCTCCAGGAACCGCGCGAGCTAGTCCAAC 5400
I S I L T P V G G G K L Y E G R V T H G T G K P A V M H L V D L D K R P G I L N F

3' TTCAGTCAACTCGCGGACCGCTTGTCTCGCGCGCGGAGCAAGTCAAGCGAGGTGCTGTAGGAACCTACAGCGGAGCTGCGAGCGCGCGCTCTAGTATGGTGTGAGC 5520
H V N L G H A F L L A A G Q K M N P E L S E K Y I D G Q L E D P T L M M V V D G

3' GGGGTGAACCGCGCGCGCTGCAAGTGTGAACTTCGGGAGCAGAGTCCGAAGAACCGCCAGCGCGTGGGAGCGTCCGCTACTGCTACAGGAGCTCGGGCTTAGCGCGTCC 5640
W K A A D A V N M V K P F G E H A K K A T A S G E R L A I V I D K V G S R D L N

3' AAGTCGCGCACTGTACCGGAGCGCTCGCATCGCTGCTGCTCAAGAACGGGAACCTAGTCCAAGTCCAGCGCTAGCGCTAGCATATCTCGCGCTACCCACAGGAGAGACAC 5760
L A H A H G Q S G Y G V I V V N K G K I L N L D A D R D Y V Y R M

3' AGTCACCAAAGCAAGTCCAAAGCGGCGGCGCATCTCCCGGCTCTTGACGAGCTGCGGTGCCGCGCAGCGAGCGCTAGAGCGCGCGCATCCAGCTCGCGCAGCGCGCTCTCTTCA 5880
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CGCGGCTTCCCGCGGCTGGCACAGGCGCGACTTTCGCTTACAGCAGCAGGAAGGTCCCGGACGAGTAGAGAGAACCGCGCTGTAGGGCTCGCGCGCGCGCTAGCGGAAGCG 7560
* L P I N D H K K W P Q E M E K T R L A R A I R K R

3' CCACCTCAACCGCTACTAGTGGAGCAGTACATCGCGCGCGCGCGGCTGCTTCGCTCTCTCGCAGGAGCATAGAAGAACCGGAGCGCGCTGAGAGAGCGGTAGCGGTAGAG 7680
T S H P I V E D I Y G R E A V A S P S L F R D C C T T G C T G C T A G C A G C A G C A C T A C C A A T G C C G C C T A A G I K D A D G I E

3' CGCGCGCTTCTACTAGCTGTGCGGGAGCGCGGTAGTGGCGCTAGACGCGGACCGGCTGCGCATCAAGTCCAGCGCGCGCTGTACGAACCTCCGGTACTCGAGCATCCCGCGCGG 7800
A R F I I E V A G K A G M V A I Q A T P W A Y N L D A G V H K S A M V D Y A G

3' CTTCGGAACGCCCACTACTACAGTGTCTCGCGTGCACCGAACGACATCCGCTTCTGTCGAACCGCGGACGAATAGTCCGCGGTATAAGGAGCGCGGACGGCGCTCTCTCG 7920
F A K R T I I T V L P V T A Q S Y A F L L K A G H K I L G G Y E Q A T G Q A T G C P F L F G

3' GCGGTGAGGTGCTTCCACTGTAGCGCTAGCTTCTCGCGAACGTCTTCGCGTCTCGCGCGCGGAGCGCGCTGCGCGAGCTTGTGCTGCGCGCGGTGGTGTGCGCGGACCAACG 8040
P V D V F T V I P I S F A N C F R V F R A A C R R S A D S D L V G A K L V L P Q N A

3' CTGCTTGGCTGCACCGCGGAGCTGCGCGGCTTCGCGCACTATTATAACCGCGCATTTGGCGGAGCTAGAGCTTCTTCAGCGGAAGAGGCTGCTGGCGCGCTAGTGTAGCGGGTA 8160
V F G V T R G E V R G G A G T I I N R A Y A Q I E F F D G E D V R A I L E G M

3' CAGCATCCGGAAGGCCAACGCGCTTAGTGCAGAGCTGCTGACAGCTTCCGCACTGACAGAGCTCTGAGAGCTAGAGCGCGTGGGAGCTTACCAACAGCGCGCGCTCTCAG 8280
D Y P K N P N D P I L T D L S K D L R N V D D F S E I E P V G E I N N A P L F D

3' CTGCTCGCGCGCTAGAGCTCTAGCGGAGCTGCAGCAAGAGCTGCTTGGCAGCGCTAGTCTCTAAGCTCCACACTGGAAACCGCGCGCTCAAGGAGCGCGCATCTGGAGCA 8400
L L R R I Q L T A E V D N E F S G D A I S S K S T H V K A G G L E T A V E N

3' CCAGTCCGCAAGTGGTGCAGCGCGCGGCAAGTGTGTATACGAGCAGCTGCTGGTACTTCTACTTCAGCGAGTACCGCGCGCTCATGTGCAGCGCGCGCGCTGCGCGGGTACTA 8520
T V T K V V D P G T V F M Y S T D R V M F I F D T M A P S Y V D G G A C P G M I

3' CTGCGCTAGACGCGCTAGTGGCGGCTCCGCTGCAACCGCGCTTGTGAGCGGCACTCGCGGCTCGCGCGGCTGGGAGGAGCACTACGCGGCTGGCGCGCTAGCTTCTACGGTA 8640
V S I Q P I V G S A A V N R R F V E G Y G G L A A V G E Q I R A A G G A D F I G I

3' CTAGCGCGCGGCTAGAGTGTGCGGTACAGGACTGGAATTGTAGAACCAACACCGCGGAGCTCTGCTCGCGCGCTTGTGGCTCTCAGGAAGCTTGTCTCTCGCCACCGCGG 8760
I P A R M K L A M D Q V K V I K T T H A E S L S G G F V T D F K S F L V R

3' CAAGTGCACGGGCTCGCGACTGCTCGCGCGCGCGCTAGAAGAGGAGAGTACGGCTTAGCGCTGCGCAGAGCTGCTTGTACAGCTTGAAGAGCTTGTGGCAGAGCTTGTGGCAGAGCTC 8880
N V T G W G T V V G G G P I K Q K E M G F D A S R H Q V F M D F E E F S G H D L

3' GTCTCAAGCTACGAGGCTGCGGCACTGCAAGCGCGGAGCAGCGGAGCTCGCGGAAGAGGCGCGGCTCTGCGCGGAGCGCGCTGCGGAGGAGCTGCGAGGAGTGGAA 9000
L L O I R E R A T A C

Construction of insertion mutations

Insertion mutations were generated in all the ORFs identified in the region under study. The strategy for introduction of specific mutations was based on the exchange of DNA via homologous recombination *in vivo* between the wild-type gene and a gene that had been inactivated by an insertion mutation *in vitro* (Ruvkun & Ausubel, 1981). The 1.3 kb Km^r cassette originating from pUC4K (Vieira & Messing, 1982) was employed, cut by appropriate restriction enzymes. The Km^r cassette was always inserted in the orientation such that the direction of transcription of the *aph* gene coincided with the direction of transcription of the interrupted gene. This has been shown in the past to create non-polar insertions (Chistoserdova & Lidstrom, 1994a, c; Chistoserdov *et al.*, 1994). Plasmids in which corresponding genes had been interrupted by the insertion were then ligated with the suicide vector pAYC61 (Chistoserdov *et al.*, 1994). The resulting plasmids were transformed into *E. coli* S17-1 to produce donors, which were mated with *M. extorquens* AM1. Km^r *M. extorquens* AM1 progeny were routinely obtained on plates containing succinate as the growth substrate, except in the case of mutations in *ilvC*, when supplements of valine and isoleucine (1 mg ml^{-1} or 5 mg ml^{-1} , respectively) were also added. Colonies resistant to Km were checked for their resistance to Tc. Tc^s colonies were assumed to be double-crossover recombinants, while Tc^r colonies were assumed to be single-crossover recombinants. In all cases, the identity of the mutants was confirmed by hybridization of chromosomal DNA with corresponding specific probes and pUC4K DNA in comparison with the wild-type DNA (data not shown). Specific sites of insertions into the region are as follows (noted by asterisks in Fig. 1). Mutations 6211 and 6112KH are, approximately in the middle (*EcoRI* site) and in the end (*Tth1111* site) of *xdb*, respectively. Mutation 61EP2 is a *SphI* deletion of approximately 0.3 kb involving the 5' terminus of *orf1*. Mutation 61MCM is in the *SmaI* site approximately in the middle of *meaA*. Mutation 6128Km is in *katA* (*PstI* site at position 939, Fig. 2b). Mutation 61ADH is in *adhA* (*BsaAI* site at position 1684). Mutation 6132 is in the region separating *adhA* and *orf2* (*NruI* deletion of 389 bp, positions 3287 and 3676, Fig. 2b). Mutation 61OR2 is in *orf2* (*EcoRI* site at position 4507). Mutation 61EPKm and 61ICP are in *ilvC* (*AvaI* deletion of 190 bp, positions 5076 and 5266, and insertion into *PstI*, position 5488, respectively). Mutation 6111H is in the region separating *ilvC* and *pccB* (*BsaAI* site at position 6797). Mutation 61PCC is in *pccB* (*StuI* site at position 7805).

Phenotypic analysis of insertion mutants

Up to 300 recombinants from each mating were tested for resistance to Tc in order to separate single-crossover ($Tc^r Km^r$) and double-crossover ($Tc^s Km^r$) recombinants. Mutations in only four genes produced double-crossover recombinants (with a frequency of 10–50% in relation to the total number of recombinants): *meaA*, *katA*, *adhA* and *pccA* (Fig. 1). One of the insertions introduced into an intergenic site (mutation 6132) also resulted in double-

crossover recombinants. Mutations in the other seven sites, including one mutation in an intergenic site (6111H) produced only single-crossover recombinants. Since those types of crossover events regenerate a functional target gene, this implies that these gene products are vital for growth of *M. extorquens* AM1 on succinate. It is not clear why mutation 6111H did not result in double-crossover recombinants, since past experience with insertions of the Km^r cassette in *M. extorquens* AM1 did not cause polar effects (Chistoserdova & Lidstrom, 1994a–c; Chistoserdov *et al.*, 1994). All single-crossover mutants were able to grow on both C_1 and C_2 compounds (up to 300 colonies tested for each mutation event).

Growth responses have been determined for double-crossover recombinant insertion mutants in *meaA*, *adhA*, *katA* and *pccA*. Mutants in *katA* grew normally on C_1 (methanol and methylamine) and C_2 (ethanol and ethylamine) compounds, while mutants in *adhA*, *meaA* and *pccA* lost the ability to grow on C_1 and C_2 compounds, and also on β -hydroxybutyrate, indicating that their products are required for both C_1 and C_2 metabolism. All mutants grew normally on pyruvate. Glyoxylate and glycolate have been used previously as supplements to characterize mutants in the unknown pathway of oxidation of acetyl-CoA to glyoxylate (Dunstan *et al.*, 1972a, b; Dunstan & Anthony, 1973; Salem & Quayle, 1971; Salem *et al.*, 1973). These compounds were tested for the ability to restore growth of the mutants on methanol, formate, ethylamine and β -hydroxybutyrate. Both supplements were able to restore growth of the mutants on C_1 and C_2 compounds and on β -hydroxybutyrate. Mutants in *meaA* and *adhA* regained the ability to grow on C_1 and C_2 compounds when the 13.5 kb *HindIII* fragment was introduced in a broad-host-range vector, pRK310 (Ditta *et al.*, 1985), while mutants in *pccA* were complemented by an overlapping 5.6 kb *PstI* fragment isolated by hybridization and cloned into pRK310. This *PstI* fragment must therefore contain the complete *pccA*.

Activity of catalase in *katA* mutants

Activity of catalase was measured in a few representative *katA* mutants and in wild-type *M. extorquens* AM1 grown on methanol and succinate. In both the wild-type *M. extorquens* AM1 and the mutants, high levels of catalase activity were found, which in general seemed to be higher on succinate than on C_1 compounds (approximately 100 and 60 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$, respectively). These data suggested that *M. extorquens* AM1 may have multiple genes responsible for the synthesis of catalase. To prove the identity of the mutation in *katA*, we separated proteins on isoelectrofocusing gels using crude extracts from the wild-type cells of *M. extorquens* AM1, from two representatives of *katA* mutants, CAT38 and CAT42, and from CAT38 carrying a plasmid containing cloned *katA* (pLC310.SS) and observed catalase activity by immersing the gels into a hydrogen peroxide solution. Two activity bands with pI values of approximately 4.6 and 5.0 were present in the wild-type extract, and also in the extract of CAT38(pLC310.SS), while only one (the less acidic) band

Table 1. Activities of PCC, MCM and HPR in wild-type *M. extorquens* AM1, *pccA* and *meaA* insertion mutants, and in mutants carrying cloned *pccA* and *meaA*

Measurements were done in triplicate and values were within $\pm 25\%$ of each other.

Strain	Activity [nmol min ⁻¹ (mg protein) ⁻¹]					
	PCC		MCM		HPR	
	Succ*	MeOH†	Succ*	MeOH†	Succ*	MeOH†
Wild-type	25	20	—	—	380	1990
Wild-type (pLC310.84)	65	40	15	10	360	1880
PCC50	0	0	—	—	360	1730
PCC54	0	0	—	—	350	1600
PCC50(pLC310.84)	55	35	15	8	350	1900
MCM1(pLC310.84)	50	30	15	10	400	1600
MCM28(pLC310.84)	45	25	12	6	380	1580
MCM28(pLC310.SS84)	60	30	18	9	350	1500
MCM28(pLC310.SS84)	55	30	16	7	385	1775

—, Not measured.

* Cells grown on succinate.

† Cells grown on methanol or, in the case of methanol-negative strains, grown on succinate, washed and incubated with methanol as described by Dunstan *et al.* (1972).

was present in the *katA* mutants (data not shown). Although the calculated pI for the product of *katA* is 6.1, the corresponding activity band shows a pI of approximately 4.6. This difference could be due to the quaternary structure of catalase, which is usually composed of four subunits (Murthy *et al.*, 1981).

Activity of PCC in *pccA* mutants

PCC activity was measured in the wild-type *M. extorquens* AM1 cells and in two representatives of *pccA* mutants, PCC50 and PCC54. Cells from succinate-grown cultures or cultures grown on succinate and then induced with methanol (Dunstan *et al.*, 1972b) were employed. The activity of HPR has also been used as a positive control for induction of serine cycle enzymes by methanol. Similar levels of PCC were found in wild-type *M. extorquens* AM1 grown on methanol or succinate. PCC activity was not found in *pccA* mutants. When a plasmid carrying cloned *pccA* (pLC310.84) was introduced into the *pccA* mutants, PCC activity was found at increased levels, in accordance with the presence of multiple copies of the gene in these strains (Table 1).

Activity of MCM in *meaA* mutants

Since *meaA* appeared to be similar to genes encoding subunits of MCM, the presence of MCM was first checked in the *meaA* mutants and in the wild-type cells. Since the assay we used involved PCC as a coupling enzyme, the latter was overexpressed by the introduction of plasmids carrying *pccA* (pLC310.84 carrying the 6.5 kb *Pst*I frag-

ment containing entire *pccA* cloned into pRK310) or both *meaA* and *pccA* (pLC310.SS84 carrying the 18.5 kb *Hind*III-*Pst*I fragment). The activity was found at similar levels in mutant and wild-type cells grown on succinate or induced with methanol, and it did not seem to be overexpressed in strains carrying plasmids with cloned *meaA* (Table 1).

Search for ADH activity

The specificity of the product of *adhA* is unknown, but the enzyme reveals highest similarity with ADHs having specificity for short-chain alcohols (Russell *et al.*, 1983; Russell & Hall, 1983; Young & Pilgrim, 1985; Gwynne *et al.*, 1987; Saliola *et al.*, 1990), which are most active on ethanol. ADHs are usually readily stained in gels (Megnet, 1967). We isoelectrofocussed cell-free extracts of the wild-type *M. extorquens* AM1, two representatives of *adhA* mutants, ADH70 and ADH76, and ADH70 carrying pLC310.SS (the 13.5 kb *Hind*III fragment cloned into pRK310), in which the *adhA* product should be overexpressed. The gels were stained in various conditions, employing the following alcohols and aldehydes: ethanol, 2-propanol, glycerol, acetaldehyde, propionaldehyde, glycolaldehyde, glycolate and glycerate. As many as ten specific bands could be detected using the substrates above, and at least two of these seemed to be induced by C₁ substrates (with pI values of approximately 6.5 and 7.5). However, none of the bands disappeared in the *adhA* mutants (data not shown). Thus, the identity of *adhA* remains unknown.

DISCUSSION

In most aerobic organisms able to utilize acetate, the main assimilatory mechanism is the glyoxylate cycle, which is characterized by the presence of two enzymes: isocitrate lyase and malate synthase, in addition to enzymes of the tricarboxylic acid cycle. This pathway has been found in many bacteria, including Icl^+ serine methylotrophs, fungi, algae and protozoa (Gottschalk, 1979; Dawes & Sutherland, 1994). Icl^- serine methylotrophs, exemplified by *M. extorquens* AM1, lack isocitrate lyase, and the pathway used for assimilation of acetate, which also operates during growth on C_1 compounds and β -hydroxybutyrate, remains unknown. It has been shown previously using ^{14}C -labelled C_1 and C_2 compounds that glycolate, glyoxylate, malate and an unidentified compound (compound X) must be intermediates in this unknown pathway (Dunstan *et al.*, 1972a, b; Dunstan & Anthony, 1973). These conclusions were confirmed by the isolation of mutants unable to utilize C_1 and C_2 compounds and β -hydroxybutyrate, which were complemented by the addition of glycolate or glyoxylate (Dunstan *et al.*, 1972a, b; Dunstan & Anthony, 1973; Salem & Quayle, 1971; Salem *et al.*, 1973). However, a pathway that converts acetyl-CoA to glyoxylate in this strain has never been identified. In this study we have approached the problem using molecular genetic analysis. A fragment of the *M. extorquens* AM1 chromosome previously used to complement such a mutant, PCT48, was sequenced in order to identify the gene defective in this mutant and possibly other genes involved in the pathway. Three genes specifically involved in the unknown pathway have been identified, the one defective in PCT48 (*meaA*) encoding a coenzyme B_{12} -linked mutase and two new genes (*pccA* and *adhA*) encoding respectively PCC and an ADH with an as yet unknown function. Mutants in *meaA*, *pccA* and *adhA* have the expected phenotype, i.e. they are C_1 - and C_2 -negative, and are complemented on these substrates by glycolate or glyoxylate. These three genes, although found within a single DNA fragment, are obviously not co-transcribed and are separated from each other by non-coding DNA regions and by genes whose involvement in C_1 and C_2 metabolism we were not able to prove, since mutants in them were only obtained as single-crossover recombinants with C_1 - and C_2 -positive phenotypes. In addition to the newly identified genes apparently involved in the unknown part of the serine cycle, we have previously shown that serine hydroxymethyltransferase and the product of an unidentified gene (*orf4*) are probably involved in this pathway (Chistoserdova & Lidstrom, 1994b, c). Phenotypes of mutants in *glyA* and *orf4* are different from the phenotypes of the mutants described here, since they were only complemented on C_2 compounds by glyoxylate, and not by glycolate.

A metabolic pathway, the 3-hydroxypropionate cycle, has been described in which acetyl-CoA is converted into glyoxylate, which would involve PCC (Strauss & Fuchs, 1993). In this pathway, acetyl-CoA is carboxylated and reductively converted via 3-hydroxypropionate to propionyl-CoA, which is carboxylated and converted via

succinyl-CoA to malyl-CoA. The latter is cleaved to acetyl-CoA and glyoxylate. A different pathway was proposed to operate in the serine methylotroph *Protonobacter ruber* (Shimizu *et al.*, 1984), in which glyoxylate could be regenerated from acetyl-CoA by using part of the tricarboxylic acid cycle to convert acetyl-CoA to α -ketoglutarate. The latter is transformed to glutamate, followed by conversion of glutamate to mesaconate via β -methylaspartate, activation of mesaconate with CoA to form mesaconyl-CoA and cleavage of mesaconyl-CoA to glyoxylate and propionyl-CoA. The latter is then carboxylated to methylmalonyl-CoA and transformed to succinyl-CoA, which can be regenerated in another part of the tricarboxylic acid cycle to produce oxaloacetate, the acceptor for the next molecule of acetyl-CoA. None of the above pathways, however, would require any NAD-linked ADH, or a mutase other than MCM or serine hydroxymethyltransferase.

Two sequential reactions involving MCM and PCC are parts of both pathways described above. These are also shown to participate in β -oxidation of fatty acids and in degradation of branch-chained amino acids (Gottschalk, 1979). Both activities are present in *M. extorquens* AM1, but only PCC is proven to be specifically involved in the unknown pathway for regeneration of glyoxylate. *meaA* apparently encodes a mutase different from MCM, and MCM is not able to substitute for its activity. The physiological substrate for the alternative mutase and consequently its role remain unknown.

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