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A protein having similarity with methylmalonyl-CoA mutase is required for the assimilation of methanol and ethanol by *Methylobacterium extorquens* AM1

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A 4·0 kb region of *Methylobacterium extorquens* AM1 DNA which complements three mutants unable to convert acetyl-CoA to glyoxylate (and therefore defective in the assimilation of methanol and ethanol) has been isolated and sequenced. It contains two ORFs and the 3'-end of a third one. The mutations in all three mutants mapped within the first ORF, which was designated *meaA*; it encodes a protein having similarity with methylmalonyl-CoA mutase. However, methylmalonyl-CoA mutase was measured in extracts of one of the mutants and the specific activity was found to be similar to that in extracts of wild-type cells. Furthermore, although the predicted *meaA* gene product has the proposed cobalamin-binding site, it does not contain a highly conserved sequence (RIARNT) which is present in all known methylmalonyl-CoA mutases; *meaA* may therefore encode a novel vitamin-B$_{12}$-dependent enzyme. The predicted polypeptide encoded by the second ORF did not have similarity with any known proteins. The partial ORF encoded a protein with similarity with the 3-oxoacyl-[acyl-carrier-protein] reductases; it was not essential for growth on methanol or ethanol.

Keywords: *Methylobacterium*, methanol, mutase, serine pathway, C$_1$ assimilation

INTRODUCTION

Methylobacterium extorquens AM1 is a pink-pigmented facultative methylotroph which assimilates C$_1$ compounds by the serine pathway (Fig. 1) (Anthony, 1982). The first step in this pathway involves the condensation of a C$_1$-tetrahydrofolate derivative with glycine to form serine, catalysed by serine hydroxymethyltransferase. The serine is then converted to C$_3$ and C$_4$ carboxylic acids which can be assimilated into cell material. This involves four key enzymes – serine glyoxylate aminotransferase, hydroxypyruvate reductase, glycerate kinase and an acetyl-CoA-independent phosphoenolpyruvate carboxylase. There must also be a means of regenerating the C$_1$ acceptor, glycine. Its immediate precursor is glyoxylate and acetate is an intermediate in the recycling pathway. In the so-

called Icl$^+$ serine pathway organisms, malate thiokinase, malyl-CoA lyase, isocitrate lyase and some of the tricarboxylic acid cycle enzymes are required for the conversion of acetate to glyoxylate (Bellion & Hersh, 1972).

In *M. extorquens* AM1 there is evidence that malyl-CoA lyase and malate thiokinase are essential for the assimilation of C$_1$ compounds (Salem *et al.*, 1973a, 1974; Chistoserdova & Lidstrom, 1994b). However, isocitrate lyase has never been detected in this organism, nor in a number of other serine pathway methylotrophs (Dunstan *et al.*, 1972a; Kortstee, 1981); such organisms are known as Icl$^-$ serine pathway methylotrophs. Although several different routes for the conversion of acetate to glyoxylate in these organisms have been proposed (Dunstan *et al.*, 1972b; Kortstee, 1981; Shimizu *et al.*, 1984), none of them have been confirmed. Several mutants have been described which are unable to grow on C$_1$ compounds unless the medium is supplemented with glyoxylate, but the biochemical basis of this phenotype has not been elucidated (Dunstan *et al.*, 1972b; Salem *et al.*, 1973b;

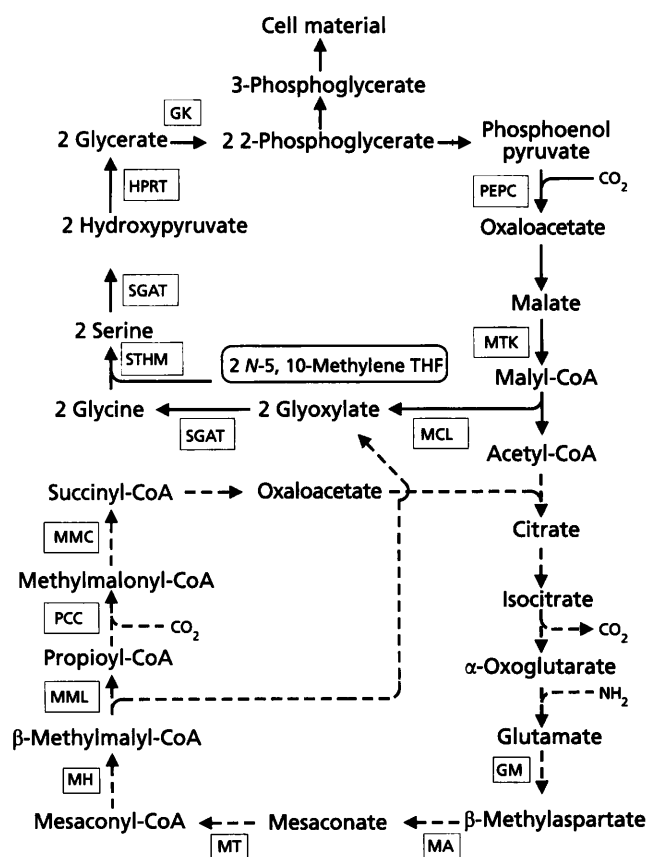† **Present address:** The Wellcome Trust, 183 Euston Road, London NW1 2BE, UK.

Fig. 1. Icl⁻ variant of the serine pathway showing the key enzymes and the route (broken line) for the regeneration of glyoxylate proposed by Shimizu et al. (1984). GK, glycerate kinase; GM, glutamate mutase; HPRT, hydroxypyruvate reductase; MA, β-methylaspartase; MCL, malyl-CoA lyase; MH, mesaconyl-CoA hydratase; MMC, methylmalonyl-CoA mutase; MML, methylmalyl-CoA lyase; MT, mesaconate thiokinase; MTK, malate thiokinase; PCC, propionyl-CoA carboxylase; PEPC, phosphoenolpyruvate carboxylase; SGAT, serine:glyoxylate aminotransferase; STHM, serine transhydroxymethylase; THF, tetrahydrofolate.

Bolbot & Anthony, 1980a, b; Stone & Goodwin, 1989). Mutants with this phenotype are also unable to grow on ethanol and on other substrates assimilated after conversion to acetate, including 1,2-propanediol, β-hydroxybutyrate and malonate. This suggests that the enzyme(s) defective in these mutants is essential for utilization of these compounds as sole carbon source (Dunstan et al., 1972b; Dunstan & Anthony, 1973; Bolbot & Anthony, 1980a, b).

Recently, Chistoserdova & Lidstrom (1994a) have isolated a mutant defective in serine hydroxymethyltransferase. Surprisingly, it was able to grow on succinate, but not on methanol or ethanol. Furthermore, when the medium was supplemented with glyoxylate it grew on ethanol, but not on methanol. This indicates that serine hydroxymethyltransferase is required for the conversion of acetyl-CoA to glyoxylate, as well as for the fixation of formaldehyde during growth on C₁ compounds. The protein encoded by an ORF (orf4) which is found within

a cluster of seven other serine pathway genes also has two functions during growth on C₁ compounds, one being that it is necessary for the conversion of acetyl-CoA to glyoxylate. The deduced amino acid sequence of this protein does not exhibit similarity with any known proteins (Chistoserdova & Lidstrom, 1994b).

Three other regions of the M. extorquens AM1 chromosome encode serine pathway genes; one complements mutants defective in glycerate kinase and the other two complement mutants which are specifically blocked in the conversion of acetyl-CoA to glyoxylate (Stone & Goodwin, 1989). In this paper, we describe the isolation and characterization of another mutant of the latter type and demonstrate that it, and two of the previously described mutants, are defective in a protein with similarity with methylmalonyl-CoA mutase.

METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1.

Growth of organisms. M. extorquens AM1 was grown at 30 °C on MacLennan's minimal salts medium (MacLennan et al., 1971). Carbon substrates were added to give the following final concentrations: 0·4% (v/v) for methanol; 0·5% (v/v) for 1,2-propanediol; 5 mM for glyoxylate; 0·2% (v/v) for ethanol; 0·2% (w/v) for other substrates. Escherichia coli strains were grown in Luria–Bertani broth at 37 °C (Sambrook et al., 1989). Where appropriate, supplements were added to the medium at the following concentrations: oxytetracycline hydrochloride, 20 µg ml⁻¹ for E. coli and 12·5 µg ml⁻¹ for M. extorquens AM1; kanamycin sulphate, 50 µg ml⁻¹ for E. coli; ampicillin, 50 µg ml⁻¹; X-Gal, 20 µg ml⁻¹; isopropyl β-D-thiogalactoside, 0·1 mM.

Isolation of mutants. A culture of wild-type M. extorquens AM1 growing on methanol medium was harvested at the mid-exponential phase of growth and resuspended at a density of approximately 10⁹ cells ml⁻¹ in 0·2 M sodium acetate buffer (pH 6·4) containing 1 mg sodium nitrite ml⁻¹ and 2 µg chloramphenicol ml⁻¹, which has been reported to enhance mutagenesis (Mishra & Tiwari, 1985). After incubation with shaking for 1 h at 30 °C, the cells were harvested and washed in succinate medium. Expression and penicillin enrichment were done as described by Tatra & Goodwin (1985). Mutants able to grow on succinate but not on methanol or ethanol were isolated by replica plating.

Preparation of cell extracts and enzyme assays. Cell extracts were prepared as described by Tatra & Goodwin (1985). Methanol dehydrogenase (EC 1.1.99.8), methylamine dehydrogenase (EC 1.4.99.3) and formaldehyde dehydrogenase (EC 1.2.99.3) were assayed polarographically (Tatra & Goodwin, 1985; Dawson et al., 1990; Ford et al., 1985). The following enzymes were assayed spectrophotometrically (Shimadzu UV 260 dual beam spectrophotometer) using published methods: formate dehydrogenase (EC 1.2.1.2; Johnson & Quayle, 1964); hydroxypyruvate reductase (EC 1.1.1.81; Goodwin, 1990); glycerate kinase (EC 2.7.1.31; Goodwin, 1990); serine:glyoxylate aminotransferase (EC 2.6.1.45; Goodwin, 1990); acetyl-CoA independent phosphoenolpyruvate carboxylase (Goodwin, 1990). Malyl-CoA lyase (EC 4.1.3.24) could not be assayed directly because the substrate, malyl-CoA, is not commercially available. The presence of this enzyme was deduced by measuring the apparent malate synthase activity, which is due to the concerted action of malyl-CoA lyase and

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant properties	Source/reference
Bacteria		
M. extorquens AM1		
Wild-type		Peel & Quayle (1961)
PCT48 ⎫		Dunstan *et al.* (1972b)
PT1005 ⎬	Mutants defective in the conversion of acetyl-CoA to glyoxylate	Stone & Goodwin (1989)
LS1 ⎭		This study
E. coli		
DSM1607	F⁻ *proA2 recA13 rpsL20* (Str^r) *hsdS20* (*hsdR hsdM*)	NCIMB 11865
S17-1	*thi pro hsdR recA*; contains the *tra* region of RP4 integrated in the chromosome	Simon *et al.* (1983)
CSR603	F⁻ *proA2 recA1*	NCIMB 11867
XL1-Blue	*endA1 hsdR17* (r$_k^-$m$_k^+$) *supE44 thi-1 recA1* (*lac*) F′ *proAB lacI^{q}Z*ΔM15 Tn*10* (Tc^r)	Bullock *et al.* (1987)
P678-54	F *thr1 ara13 leu-6 azi8 tonA2 lacY1 minA1 ginU44 gal6 minB2 rpsL135 malA1 xyl7 mtl2 thi1*	Adler *et al.* (1967)
DH5α	*supE44 lacU169* (Φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi1 relA1*	Bethesda Research Laboratories
Plasmid/cosmid		
pVK100	IncP1, Tc^r, Km^r, *cos*; broad-host-range cloning vector	Knauf & Nester (1982)
pLA2917	IncP1, Tc^r, Km^r, *cos*; broad-host-range cloning vector	Allen & Hanson (1985)
pRK415	IncP1, Tc^r, *lacZ′*; broad-host-range cloning vector	Keen *et al.* (1988)
pRK2013	Km^r mobilizing plasmid; ColE1 replicon	Figurski & Helinski (1979)
pBluescriptII KS(+)	Amp^r phagemid with ColE1 replicon and f1 phage origins of replication	Stratagene Cloning Systems
pTZ18U	Amp^r *lacZ′* cloning vector	Bio-Rad
pSS48-1, pSS48-2	Cosmids isolated from a *Hind*III clone bank of *M. extorquens* AM1 DNA in pVK100	Stone & Goodwin (1989)
pLS27C	4·0 kb *Eco*RI fragment of pSS48-1 in pRK415 ⎫	
pLS271C	1·0 kb *Pst*I fragment of pLS27C in pLA2917	
pLS273C	2·2 kb *Pst*I–*Eco*RI fragment of pLS27C in pRK415	
pLS274C	1·0 kb *Eco*RI–*Pst*I fragment of pLS27C in pRK415 ⎬ This study	
pLS27	4·0 kb *Eco*RI fragment of pSS48-1 in pBluescript KS(+)	
pLS271	1·0 kb *Pst*I fragment of pLS27 in pBluescript KS(+)	
pLS272	1·2 kb *Eco*RI–*Bgl*II fragment of pLS27 in pBluescript KS(+)	
pLS273	2·2 kb *Pst*I–*Eco*RI fragment of pLS27 in pTZ18U ⎭	

malyl-CoA hydrolase (Stone & Goodwin, 1989). Methylmalonyl-CoA mutase (EC 5.4.99.2) was measured by the method of Zagalak *et al.* (1974). Enzyme assays were repeated using at least two independent cultures. Protein concentrations were determined by the method of Lowry using BSA fraction V as the standard.

Complementation analysis. Recombinant cosmids were introduced into *M. extorquens* AM1 mutants using either biparental matings (where *E. coli* S17-1 carrying a recombinant cosmid was the donor) or triparental matings (where other *E. coli* hosts carrying a recombinant cosmid were the donors and the mobilizing plasmid pRK2013 was supplied by *E. coli* DSM1607). Matings were done using a modification of the method of Fulton *et al.* (1984). Culture of the recipient (0·1 ml; 10^8 cells) was spread onto a nutrient agar plate. The plate was dried and then 20 µl culture containing the donor (2×10^7 cells), mixed with an equal number of mobilizer cells if appropriate, was dropped onto the lawn of recipient. After incubation overnight at 30 °C, the plates were replicated onto medium containing (i) succinate plus tetracycline plus carbenicillin (to select for cosmid transfer) and (ii) methanol plus tetracycline (to select for complementation). Carbenicillin (100 µg ml⁻¹) was

added to the succinate medium to inhibit the growth of *E. coli* which occurred due to carry-over of nutrients from the mating plates. To check that complementation, rather than recombinational rescue, was occurring, single colonies which had been selected for transfer of the cosmid were removed and tested for growth on methanol plus tetracycline. Demonstration that all of these colonies grew on methanol indicated that there was complementation *in trans*.

Plasmid isolation. A modification of the alkaline lysis method of Birnboim & Doly (1979) was used for small-scale preparation of plasmid and cosmid DNA (Sambrook *et al.*, 1989). Large-scale isolation of plasmid DNA for sequencing was done as described in the Promega Protocols and Applications Guide.

Subcloning. Subcloning and agarose gel electrophoresis were done as described by Sambrook *et al.* (1989). Enzymes for restriction digestion and DNA manipulations were obtained from Sigma or Northumbria Biologicals and used according to the manufacturers' instructions. A Prep-A-Gene DNA purification kit (Bio-Rad) was used to purify DNA fragments isolated from agarose gels. Recombinant plasmids were introduced into competent *E. coli* cells by transformation as described by Sambrook *et al.* (1989).

DNA–DNA hybridization. Chromosomal DNA was isolated from *M. extorquens* AM1 using the method of Fulton *et al.* (1984). The DNA was digested with an appropriate restriction enzyme and the resulting fragments were separated by agarose gel electrophoresis and then blotted onto a Hybond-N nylon membrane (Amersham), using the Hybaid Vacu-aid according to the manufacturer's instructions.

DNA probes were prepared by random incorporation of digoxigenin-labelled deoxyuridine triphosphate (Boehringer), according to the manufacturer's instructions. Hybridization was done at 68 °C in a Hybaid incubator as described by Sambrook *et al.* (1989), using stringent washing conditions, and the hybridized probe was detected using the DIG nucleic acid detection kit (Boehringer).

Nucleotide sequencing. Plasmid pLS27 was digested with *Pst*I and the resulting 1·0 kb fragment was cloned into pBluescript KS(+), generating plasmid pLS271. The 1·1 kb *Eco*RI–*Bgl*II fragment of pLS27 was ligated into pBluescript KS(+) which had been digested with *Eco*RI and *Bam*HI, giving plasmid pLS272. The 2·2 kb *Pst*I–*Eco*RI fragment of pLS27 was subcloned into pTZ18U which had been digested with the same enzymes and a nested set of unidirectional deletions of the resulting plasmid, pLS273, was made. This was done as described by Henikoff (1984), except that mung bean nuclease was used instead of S1 nuclease. DNA fragments were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977). Primers were either obtained commercially or were custom synthesized.

Sequencing was either done with T7 DNA polymerase and [^{35}S]dATPαS using the Sequenase kit (US Biochemical) or by using a Pharmacia Automated Laser Fluorescence DNA sequencer.

Sequence data were compiled and analysed using the GCG programs (Devereux *et al.*, 1984) and the programs supplied in the PC/GENE software packages (Intelligenetics).

Preparation and labelling of *E. coli* minicells and analysis of labelled peptides. The *E. coli* minicell-producing strain P687-54 was transformed with pLS27 and with pBluescript KS(+). Minicells were isolated using a modified version of the method of Clark-Curtiss & Curtiss (1983) as described by Eggink *et al.* (1988). The protein products were labelled with [^{35}S]methionine, analysed by SDS-PAGE and visualized by fluorography (Eggink *et al.*, 1988).

RESULTS AND DISCUSSION

Isolation and characterization of mutants

Following nitrous acid mutagenesis, four mutants were isolated which had growth properties typical of mutants unable to convert acetyl-CoA to glyoxylate, i.e. were unable to grow on methanol or ethanol unless the medium was supplemented with glyoxylate. We have designated this phenotype Mea (*m*ethanol and *e*thanol *a*ssimilation deficient). Revertants were obtained at a frequency of approximately 10^{-9}. As expected, the mutants were also unable to utilize methylamine, formate, malonate or β-hydroxybutyrate as sole carbon source, but could grow on oxalate, which is assimilated by metabolism to glyoxylate and formate and then converted to phosphoglycerate by the appropriate enzymes of the serine pathway (Blackmore & Quayle, 1970). Surprisingly, pyruvate, lactate and 1,2-propanediol, which are also thought to be assimilated by

a route involving metabolism to acetyl-CoA and its subsequent conversion to glyoxylate (Salem *et al.*, 1973b; Bolbot & Anthony, 1980a, b), supported growth of the new isolates and also of two previously described mutants, PCT48 and PT1005. However, all of the mutants grew much more slowly than the wild-type on 1,2-propanediol and this may account for the failure of Bolbot & Anthony (1980b) to observe growth of PCT48 on this substrate. These results suggest that, if acetate is an intermediate in the assimilation of pyruvate, lactate and 1,2-propanediol, it must be metabolized by a route which does not involve conversion to glyoxylate.

Biochemical and complementation analysis indicated that the four new mutants were identical so the results for only one – LS1 – are given. It was grown on medium containing succinate plus methanol plus methylamine, harvested, and incubated overnight in medium containing methanol plus methylamine to induce the C_1-metabolizing enzymes. Crude extracts were then assayed for the known key enzymes of the serine pathway. Serine:glyoxylate aminotransferase, hydroxypyruvate reductase, glycerate kinase and the acetyl-CoA-independent phosphoenolpyruvate carboxylase were all detected, as were the C_1-oxidizing enzymes methanol dehydrogenase, methylamine dehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase. The presence of malyl-CoA lyase was deduced by demonstrating an apparent malate synthase activity, which is due to the concerted action of malyl-CoA lyase and malyl-CoA hydrolase. We did not assay for serine hydroxymethyltransferase, but this enzyme must be present since LS1 grew on oxalate and on methanol in the presence of glyoxylate.

Complementation analysis

Stone & Goodwin (1989) isolated two overlapping cosmids (pSS48-1 and pSS48-2) from a *Hin*dIII genomic library of *M. extorquens* AM1 DNA constructed in the broad-host-range mobilizable cosmid pVK100, and demonstrated that they complemented two mutants, PCT48 and PT1005, which were unable to convert acetyl-CoA to glyoxylate; a third mutant with the same phenotype (Cou-4) was not complemented. pSS48-1 and pSS48-2 also complemented mutant LS1.

pLS27C, a subclone of pSS48-1 which contained a 4·0 kb *Eco*RI fragment of pSS48-1, also complemented all of the mutants but subclones containing smaller DNA fragments derived from pLS27C did not (Fig. 2). However, when one of these (pLS274C) was introduced into PT1005, approximately 1 % of the transconjugants which had received the cosmid grew on methanol, indicating that recombinational rescue had occurred. Recombinational rescue also occurred when pLS273C was introduced into LS1.

Mutant PCT48 contains a chromosomal deletion

In common with Dunstan *et al.* (1972b), we were unable to isolate revertants of this mutant and we therefore investigated their suggestion that PCT48 is a deletion
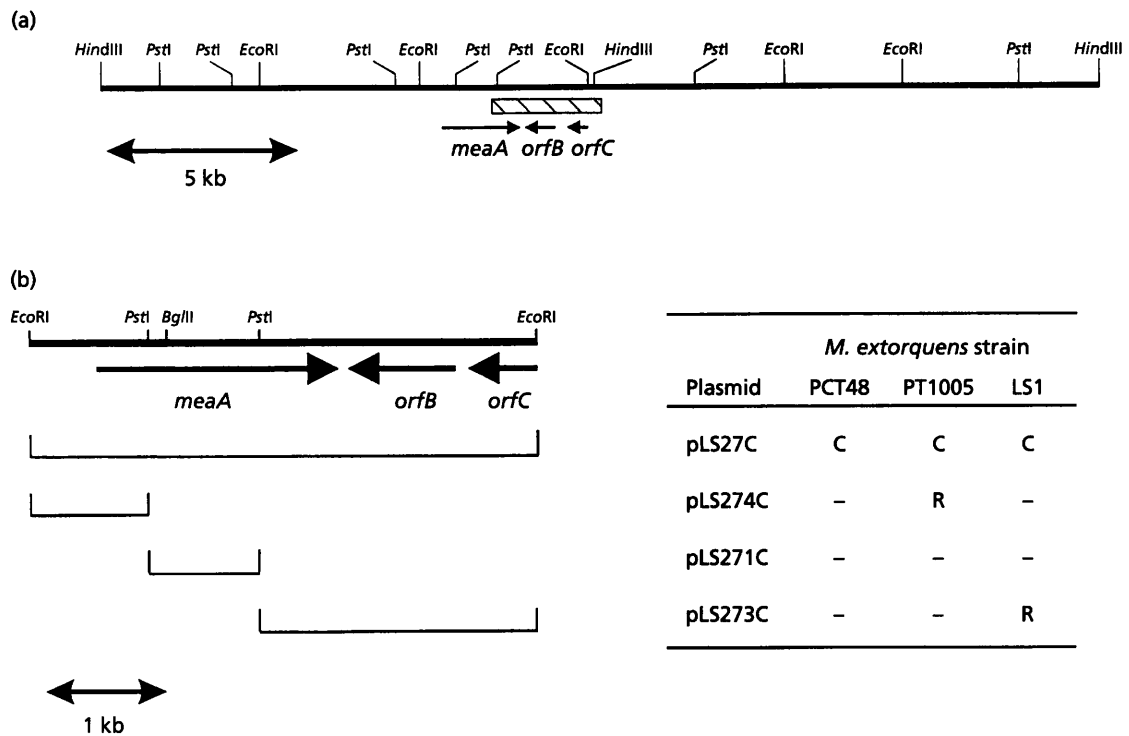
Fig. 2. (a) Restriction map of the 26 kb HindIII insert of cosmid pSS48-1. The hatched box indicates the position of the deletion in PCT48. (b) Complementation analysis of the Mea mutants using subclones of pSS48-1. C, Complementation; R, recombination; −, no complementation or recombination. The positions and directions of transcription of *meaA*, *orfB* and *orfC* are indicated by arrows.
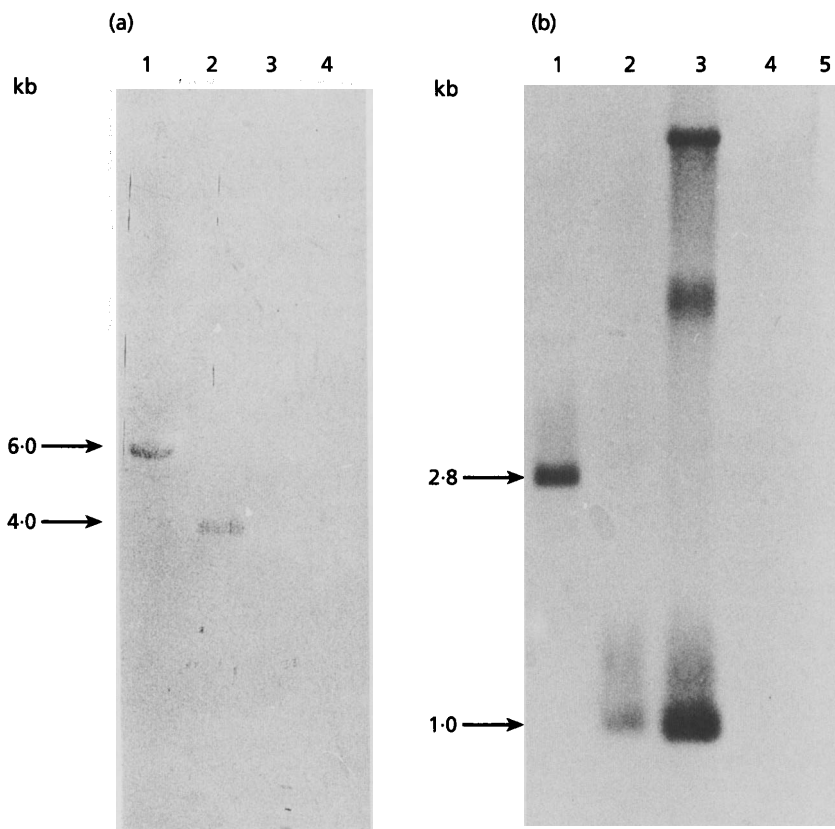
Plasmid	M. extorquens strain		
	PCT48	PT1005	LS1
pLS27C	C	C	C
pLS274C	−	R	−
pLS271C	−	−	−
pLS273C	−	−	R

Fig. 3. DNA–DNA hybridization of genomic DNA from wild-type *M. extorquens* AM1 and mutant PCT48. (a) Probed with the 4·0 kb EcoRI fragment from pLS27. Lanes: 1, EcoRI digest of genomic DNA from PCT48; 2, EcoRI digest of genomic DNA from wild-type; 3, PstI digest of lambda DNA; 4, EcoRI–HindIII digest of lambda DNA. (b) Probed with the 1·0 kb PstI fragment from pLS271C. Lanes: 1, PstI digest of genomic DNA from PCT48; 2, PstI digest of genomic DNA from wild-type; 3, PstI digest of pLS271C; 4, PstI digest of lambda DNA; 5, EcoRI–HindIII digest of lambda DNA. The PstI fragment bound to pLA2917 (data not shown) and this accounts for the bands of 21 kb (vector DNA) and 6·0 kb (degraded vector DNA).

Fig. 4. Nucleotide sequence of the *M. extorquens* AM1 chromosomal region containing *meaA*, *orfB* and the 3′-terminus of *orfC*. Amino acids are represented by the single letter code. The conceptual translation of *orfB* and *orfC* is from the reverse complement (lower-case letters). Putative ribosome-binding sites are underlined, putative transcriptional terminator sequences are double underlined and asterisks indicate stop codons. The underlined amino acid sequence represents the short-chain alcohol dehydrogenase family signature.

and probed with the 4·0 kb *Eco*RI fragment which complemented all of the mutants. It hybridized to a 4·0 kb fragment of wild-type DNA, as expected; in contrast, it hybridized to a DNA fragment of about 6 kb from PCT48 (Fig. 3a). Thus, there must be a deletion in the chromosome of PCT48 involving one of the *Eco*RI sites of the DNA fragment cloned in pLS27C.

The genomic DNA from the two strains was also digested with *Pst*I and probed with the 1·0 kb *Pst*I fragment derived from pLS27C. As expected, it hybridized to a 1·0 kb DNA fragment from the wild-type; however, it hybridized to a 2·8 kb fragment from the mutant DNA (Fig. 3b). In view of the positions of the *Pst*I and *Eco*RI restriction sites on pSS48-1, the chromosome of PCT48 must contain a deletion of 2·7 kb, which covers the 2·2 kb *Eco*RI–*Pst*I fragment (Fig. 2). The complementation analysis indicated that the 2·2 kb *Eco*RI–*Pst*I fragment of pLS27C was essential for growth on methanol and ethanol.

The *mea* locus contains a gene encoding a protein which has similarity with methylmalonyl-CoA mutase

The nucleotide sequence of the 4·0 kb *Eco*RI fragment cloned in pLS27 is shown in Fig. 4. It contained one large ORF with two possible initiation codons and translation from these putative start codons would give rise to proteins of 78 and 75 kDa. Only the second ORF has a typical ribosome-binding site upstream. Complementation analysis showed that this gene was defective in the Mea mutants PT1005 and LS1, indicating that it is essential for the conversion of acetyl-CoA to glyoxylate; we have therefore called it *meaA*. A smaller ORF of 687 bp (*orfB*) was present 118 bp downstream from *meaA* and was transcribed in the opposite direction. The 3′-end of a third ORF (*orfC*) was identified 297 bp from *orfB*. Both the intergenic regions contained inverted repeats which resembled rho-independent termination sequences (Platt, 1986). The deletion in PCT48 extends into *orfC* and, since this mutant was complemented by the 4·0 kb *Eco*RI fragment which does not contain the complete *orfC* gene, this gene cannot be considered essential for growth on methanol.

The predicted amino acid sequences of the ORFs were compared with entries in the protein database at the National Center for Biotechnology Information (NCBI) using the program BLAST (Altschul *et al.*, 1990). The *meaA* gene product had a high degree of similarity with the methylmalonyl-CoA mutases, which are adenosylcobalamin-dependent enzymes catalysing the interconversion of methylmalonyl-CoA and succinyl-CoA (Fig. 5). The enzymes from *Propionibacterium shermanii*, *Streptomyces cinnamonensis* and *Porphyromonas gingivalis* are heterodimers consisting of a large subunit (approximately 79 kDa) and a small subunit (approximately 65 kDa) (Marsh *et al.*, 1989; Birch *et al.*, 1993). In contrast, the mouse and human enzymes comprise two identical subunits of 82 kDa (Ledley *et al.*, 1988; Jansen *et al.*, 1989; Wilkemeyer *et al.*, 1990). The *sbm* gene of *E. coli* encodes

mutant. Chromosomal DNA isolated from the wild-type strain and from PCT48 was digested with *Eco*RI. The resulting fragments were separated by gel electrophoresis

```
MeaA                                                            MSAQASVAEVKRD--------KPWIIRTYAGHSTA       27
Mouse  MLRAKNQLFLLSPHYLKQLNIPSAS--RWKRLLHQQQPLHPEWAVLAKKQLKG--KNPEDLIWHTPEGISIKPLYSRAD--TLDLPEELPGVKPFTRGPYPTMYTYRPWTIRQYAGFSTV      114
P. sh         MSTLPRF-DSVDLGNAP---------VPADAARRFEELAAKAG-------TGEAWETAEQIPVGTLFNEDVYKDMDWLDTYAGIPPFVHGPYATMYAFRPWTIRQYAGFSTA       95
S. ci         MRIPEF-DDIELGAGG---------GPSGSAEQWRAAVKESVG---KSESDLLWETPEGIAVKPLYTGADVEGLDFLETYPGVAPYLRGPYPTMYVNQPWTIRQYAGFSTA       98
Human  MLRAKNQLFLLSPHYLRQVKESSGSRLIQQRLLHQQQPLHPEWAALAKKQLKG---KNPEDLIWHTPEGISIKPLYSKRD--TMDLPEELPGVKPFTRGPYPTMYTFRPWTIRQYAGFSTV      116
P. gi         MKPNY-KDIDIKSAG---------FVAKDATRWABEKGIVAD----------WRTPEQIMVKPLYTKDDLEGMEHLDYVSGLPPFLRGPYSGMYPMRPWTIRQYAGFSTA       90
E. co                                     MSNVQEWQQLANKELSRREKTVDSLVHQTAEGIAIKPLYTEADLDNLEVTGTLPGLPPYVRGPRATMYTAQPWTIRQYAGFSTA       84
                                                             ** *.** ***  **

MeaA   AESNKLYRGNLAKGQTGLSVAFDLPTQTGYDPDHELARGEVGKVGVSIAHLGDMRALFDQIPLAQMNTSMTINATAPWLLSLYLAVAEEQGAPLAALQGTTQNDIIKEYLSRGTYVFPPA      147
Mouse  EESNKFYKDNIKAGQQGLSVAFDLATHRGYDSDNPRVRGDVGMAGVAIDTVEDTKILFDGIPLEKMSVSMTMNGAVIPVLATFIVTGEEQGVPKEKLTGTIQNDILKEFMVRNTYIFPPE      234
P. sh  KESNAFYRRNLAAGQKGLSVAFDLPTHRGYDSDNPRVAGDVGMAGVAIDSIYDMRELFAGIPLDQMSVSMTMNGAVLPILALYVVTAEEQGVKPEQLAGTIQNDILKEFMVRNTYIYPPQ      215
S. ci  EESNAFYRRNLAAGQKGLSVAFDLPTHRGYDSDHPRVTGDVGMAGVAIDSIYDMRQLFDGIPLDKMTVSMTMNGAVLPVLALYIVAAEEQGVPPEKLAGTIQNDILKEFMVRNTYIYPPK      218
Human  EESNKFYKDNIKAGQQGLSVAFDLATHRGYDSDNPRVRGDVGMAGVAIDTVEDTKILFDGIPLEKMSVSMTMNGAVIPVLANFIVTGEEQGVPKEKLTGTIQNDILKEFMVRNTYIFPPE      236
P. gi  EESNAFYRRNLASGQKGLSVAFDLATHRGYDADHSRVVGDVGKAGVSICSLEDMKVLFDGIPLSKMSVSMTMNGAVLPILAFYINAGLEQGAKLEEMAGTIQNDILKEFMVRNTYIYPPE      210
E. co  KESNAFYRRNLAAGQKGLSVAFDLATHRGYDSDNPRVAGDVGKAGVAIDTVEDMKVLFDGIPLDKMSVSMTMNGAVLPVLAFYIVAAEEQGVTPDKLTGTIQNDILKEYLCRNTYIYPPK      204
        ***  *.  *.   ** ********.*  ***.*   *.** ** **.*   * ** *** * ***.*.  .*.    ***    ** ****.**.**.**.**

MeaA   PSLRLTKDVILFTTKNVPKWNPMNVCSYHLQEAGATPVQELSYALAIAIAVLDTVRDDPDFDEASFSDVFSRISFFVNAGMRFVTEICKMRAFAELWDEIAQERYGITDAKKRIFRYGVQ      267
Mouse  PSMKIIADIFQYTAQHMPKFNPISISGYHMQEAGTDAILELAYTIADGLEYCRTGLQAG-----LTIDEFAPRLSFFWGIGMNFFYMEIAKMRAGRRLWAHLIEKMFQPKNSKSLLLRAHCQ      350
P. sh  PSMRIISEIFAYTSANMPKWNSISISGYHMQEAGATADIEMAYTLADGVDYIRAGESVG-----LNVDQFAPRLSFFWGIGMNFFMEVAKLRAARMLWAKLVHQFG-PKNPKSMSLRTHSQ      330
S. ci  PSMRIISDIFAYTSQKMPRYNSISISGYHIQEAGATADLELAYTLADGVEYLRAGQEAG-----LDVDAFAPRLSFFWAIGMNFFMEVAKLRAARLLWAKLVKQFD-PKNAKSLSLRTHSQ      333
Human  PSMKIIADIFEYTAKHMPKFNSISISGYHMQEAGADAILELAYTLADGLEYSRTGLQAG-----LTIDEFAPRLSFFWGIGMNFFYMEIAKMRAGRRLWAHLIEKMFQPKNSKSLLLRAHCQ      352
P. gi  PSMRIIADIFEYTSQNMPKFNSISISGYHMQEAGATADIEMAYTLADGMQYLKAGIDAG-----IDVDAFAPRLSFFWAIGVNHFMEIAKMRAARLLWAKIVKSFG-AKNPKSLALRTHSQ      325
E. co  PSMRIIADIIAWCSGNMPRFNTISISGYHMGEAGANCVQQVAFTLADGIEYIKAAISAG-----LKIDDFAPRLSFFFGIGMDLPMNVAMLRAARYLWSEAVSGFG-AQDPKSLALRTHCQ      319
        * ..    ..   .*..*..*    .   ** .***.  .*  .  ** **        ***                .*.*** .*.  .*    ** **       .*   .*

MeaA   VNSLGLTEQQPENNVHRILIEMLAVTLSKRARARAVQLPAWNEALGLPRPWDQQWSMRMQQILAFETDLLEYDDIFDGSTVIEARVEALKEQTRAELTRIAEIGGAVTAVEAGELKRALV      387
Mouse  TSGWSLTEQDPYNNIVRTAIEAMAAVFG---GTQSLHSNSFDEALGLPTVKSARIARNTRIIIQEESGIPKVADPWGGSYMMESLTNDVYEAALKLIYEVEEMGGMAKAVAEGIPKLRIE      467
P. sh  TSGWSLTAQDVYNNVVRTCIEAMAATQG---HTQSLHTNALDEALGLPTVKSARIARNTQLFLQQESGTTRVIDPWSGSAYVEKLTYDLARRAWQHIEEVEAAGGMAQAIDAGIPKLRVE      447
S. ci  TSGWSLTAQDVFNNVTRTCVEAMAATQG---HTQSLHTNALDEALGLPTVKSARIARNTQLLIQQESGTTRTIDPWGGSAYVEKLTYDLARRAWQHIEEVEAAGGMAQAIDAGIPKLRVE      450
Human  TSGWSLTEQDPYNNIVRTAIEAMAAVFG---GTQSLHTNSFDEALGLPTVKSARIARNTQIIIQEESGIPKVADPWGGSYMMECLTNDVYDAALKLINEIEEMGGMAKAVAEGIPKLRIE      469
P. gi  TSGWSLTEQDPFNNVGRTCIEAMAAALG---HTQSLHTNALDEALALPTDFSARIARNTQIYIQEETLVCKEIDPWGGSYYVESLTNELVHKAWTLIKEVQEMGGMAKAIETGLPKLRIE      442
E. co  TSGWSLTEQDPYNNVIRTTIEALAATLG---GTQSLHTNVFDEALGLPTDFSARIARNTQIIIQEESELCRTVDPLAGSYYIESLTDQIVKQARAIIQQIDEAGGMAKAIEAGLPKRMIE      436
        .** *.  **. *.   **.*.  **    .**   *                 *****  .  ** .  **       *   ** .       .  ** **  .**    *     *

MeaA   ESNARRISAIEKGEQIVVGVNKWQQG--EPSPLTAGDGAIFTVSETVEMEAETRIREWRSKRDERAVGQALADLEQAARSG------ANIMPPSIAAAKAGVTTGEWGQRLREVPGEYRA      499
Mouse  ECAARRQARIDSGGSEVIVGVNKYHLEKEDSVHLLAID------IISLRKKQIEKLKKIKSSRDQALAEQCLSALTQCAASG------DGNILALAVDAARARCTVPEITDAFKKVFGEHKA      576
P. sh  EAAARTQARIDSGRQPLIGVNKYRLEHEPPLDVLKVD------NSTVLASQKAKLVKLRAERDPEKVKAALDKITWAAGNPDDKDPDRNLLKLCIDAGRAMATVGEMSDALEKVFGRYTA      561
S. ci  EAAARTQARIDSGRQPVIGVNKYRVDTDEQIDVLKVD------NSSVRAQQIEKLRRLREERDDAACQDALRALTAAAERGPGQGLEGNLLALAVDAARAKATVGEISDALESVVGRHAG      564
Human  ECAARRQARIDSGSEVIVGVNKYLDENDSVHLLAID------NTSVRNKQIEKLKKIKSSRDQALAEHCLAALTECAASG------DGNILALAVDSARSRARCTVGEITDALKKVFGEHKA      578
P. gi  EAAARTQARIDSHQQVIVGVNKYRLPKEDPIDILEID------NTAVRKQQIERLNDLRSHRDEKAVQEALEAITKCVETK-----EGNLLDLAVKAAGLRASLGEISDACEKVVGRYKA      551
E. co  EASAREQSLIDQGKRVIVGVNKYKLDHEDETDVLEID------NVMVRNEQIASLERIRATRDDAAVTAALNALTHAAQHN------ENLLAAAVNAARVRATLGEISDALEVAFDRYLV      544
        * **  *.    .  ..****   **      .  *       ** *   *   ** **** *   .  * ** * .* * *  *... *   .* *   .*    *   * .   *

MeaA   P----TGVTLQTVTSGA----AEDARLLIAD-LGERLGETPRLVVGKPGLDGHSNGAEQIALRARDVGFDVTYDGIRQTPTEIVAKAKERGAHVIGLSVLSGSHVPLVREVKAKLREAGL      610
Mouse  NDRMVSGAYRQEFGESK----EITSAIKRVNKFMEREGRRLGLLVAKMGKDGHDRGAKVIATGFADLGFDVDIGPLFQTPREVAHDAVDADVHAVGVSTLAAGHKTLVPELIKELTALGR      692
P. sh  QIRTISGVYSKEVKNTP----EVEEARELVEEFEQAEGRRPRILLAKMGQDGHDRGAKVIATAYADLGFDVDVGPLFQTPEETARQAVEADVHVVGVSSLAGGHLTLVPALRKELDKLGR      677
S. ci  QIRTISGVYRTEAGQSP-----SVERTRALVDAFDEAEGRRPRILVAKMGQDGHDRGAKVIASAFADLGFDVDVGPLFQTPAEVARQAVEADVHIVGVSSLAAGHLTLVPALREELAABGR      680
Human  NDRMVSGAYRQEFGESK----EITSAIKRVHKFMEREGRRPRLLVAKMGQDGHDRGAKVIATGFADLGFDVDIGPLFQTPRBVAQQAVDADVHAVGVSTLAAGHKTLVPELIKELNSLGR      694
P. gi  VIRTISGVYSSESGEDK-----DFAHAKELAEKFAKKEGRQPRIMIAKMGQDGHDRGAKVVATGVADCGFDVDMGPLFQTPEEAARQAVENDVHVMGVSSLAAGHKTLIPQVIAELEKLGR      667
E. co  PSQCVTGVIAQSYHQSEKSASEFDAIVAQTEQFLADNGRRPRILIAKMGQDGHDRGAKVIASAYSDLGFDVDLSPMFSTPEEIARLAVENDVHVVGASSLAAGHKTLIPELVEALKKWGR      664
          .*             .       *      ....**   .*     .* *  * *  **** .  *  **        .  * * . *  *...* *.    *     *  *

MeaA   DHVPVVVGGIISTEDELVLKNMGVTAVYTPKDYELDKIMVGLAKVVERALDKRAADRADTEAGVPGAPKRNESGAQVF      688
Mouse  PDILVMCGGVIPPQDYEFLYEVGVSNVFGPG-TRIPRAAVQVLDDIEKCLAEKQQSV      748
P. sh  PDILITVGGVIPEQDFDELRKDGAVEIYTPG-TVIPESAISLVKKLRASLDA      728
S. ci  DDIMIVVGGVIPPQDVEALHEAGATAVFPPG-TVIPDAAHDLVKRLAADLGHEL      733
Human  PDILVMCGGVIPPQDYEFLFEVGVSNVFGPG-TRIPKAAVQVLDDIEKCLEKKQQSV      750
P. gi  PDILVTAGGVIPAQDYDFLYQAGVAAIFGPG-TPVAYSAAKVLEILLEE      715
E. co  EDICVVAGGVIPPQDYAFLQERGVAAIYGPG-TPMLDSVRDVLNLISQHHD      714
        . . **.*. .*    *    .*      *        .  *
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Fig. 5. Alignment of the deduced amino acid sequence of MeaA with sequences of members of the methylmalonyl-CoA mutase family from other sources. P. sh, *Prop. shermanii* large subunit; S. ci, *S. cinnamonensis* large subunit; P. gi, *Porp. gingivalis* large subunit; E. co; *E. coli sbm* gene product. Identical residues are indicated by an asterisk, and conserved substitutions, according to the scheme PAGST, QNED, ILVM, HKR, YFW, C, are indicated by a dot. Numbers refer to nucleotide residues. The amino acid residues shown in bold and underlined represent the proposed vitamin-B$_{12}$-binding site and those in italics and underlined represent the conserved sequence in all methylmalonyl-CoA mutases.

a polypeptide of 78 kDa which also belongs to this family of proteins (Roy & Leadlay, 1992). Individual sequence alignments indicated that MeaA has 56–57 % similarity with the large subunits of the bacterial enzymes, the mouse and human enzymes and the *sbm* gene product. The identities were 37–37·5 % in the case of the *S. cinnamonensis* and *Porp. gingivalis* large subunits and the *sbm* gene product, and 34 % with the mouse, human and *Prop. shermanii* large subunits. In contrast, there was only about 46 % similarity and 24 % identity with the small subunits of the bacterial enzymes.

Three short, highly conserved regions are present in a number of cobalamin-dependent enzymes and it has been proposed that they are involved in cobalamin binding (Marsh & Holloway, 1992; Crane *et al.*, 1992; Drennan *et al.*, 1994). These sequences (DXHXXG, SXL and GX$_8$GGX$_{14}$G) also occur in MeaA (Fig. 5). There is another highly conserved sequence (RIARNT) in all of the methylmalonyl-CoA mutases sequenced thus far, but this is not present in *meaA* (Fig. 5).

The deduced amino acid sequence of the polypeptide encoded by *orfB* did not have significant sequence similarity with any known protein. The predicted amino acid sequence encoded by the partial ORF of *orfC* had homology with the 3-oxoacyl-[acyl-carrier-protein] reductases of *Brassica napus* (63 % similarity, 41·5 % identity), *Arabidopsis thaliana* (64 % similarity, 39 % identity) (Slabas *et al.*, 1992) and *E. coli* (62 % similarity, 37 % identity) (Cronan & Rawlings, 1992). This enzyme, which is a member of the short-chain alcohol dehydrogenase family, catalyses the first reduction step in fatty acid biosynthesis.

Translation products of *meaA*

The 4·0 kb fragment cloned in pLS27 was expressed in *E. coli* minicells and the resulting products were analysed using denaturing 8 % and 12·5 % (w/v) SDS-polyacrylamide gels, which together would have resolved both the polypeptides predicted to be expressed from this fragment. Three polypeptides, with apparent molecular
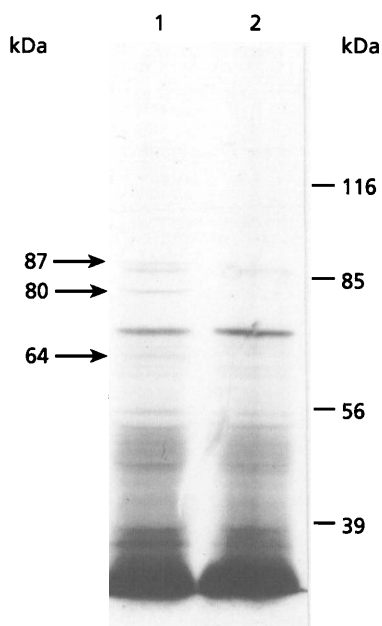
Fig. 6. SDS-polyacrylamide gel (8%) showing polypeptides synthesized by *E. coli* minicells containing a recombinant plasmid with the 4·0 kb *Eco*RI insert of pLS27 (lane 1) cloned in pBluescript. The molecular masses of marker polypeptides are shown on the right-hand side. The arrows on the left-hand side indicate polypeptides which are synthesized by the recombinant plasmid, but not the control, pBlue (lane 2).

masses of 87, 80 and 64 kDa, were present in minicells expressing this fragment, but not in controls (Fig. 6). The first is somewhat larger than the size of the predicted gene product of *meaA*, but estimates of polypeptide size from SDS-PAGE are often inaccurate. Degradation of large proteins often occurs in minicell expression systems (Eggink *et al.*, 1988) and this presumably accounts for the presence of the 80 and 64 kDa polypeptides. We did not observe any small polypeptides of the size expected of the *orfB* gene product, which is predicted to be 25·5 kDa.

Mutant PCT48 has methylmalonyl-CoA mutase activity

Methylmalonyl-CoA mutase was assayed in cell extracts of the wild-type and mutant PCT48 grown and induced as described in Methods. The specific activity of this enzyme was similar in the wild-type and in mutant PCT48 which contains a deletion in *meaA*, the value being 280 nmol min^{-1} (mg protein)$^{-1}$ ± 15%. This does not preclude the possibility that *meaA* encodes an isoenzyme of methyl-malonyl-CoA mutase which is required specifically for growth on methanol, although if this is the case it is surprising that the specific activities in the mutant and wild-type are similar. The role of the highly conserved sequence present in all of the methylmalonyl-CoA mutases but not in MeaA is unknown, but it presumably has functional significance. Thus the possibility that *meaA* codes for a novel cobalamin-binding protein needs to be explored.

Our results support the suggestion made by Shimizu *et al.* (1984) that vitamin-B_{12}-dependent enzymes are involved in the assimilation of methanol and ethanol. These authors proposed that two adenosylcobalamin-dependent enzymes, methylmalonyl-CoA mutase and glutamate mutase, were required for the conversion of acetyl-CoA to glyoxylate and that β-methylaspartate, mesaconyl-CoA, β-methylmalyl-CoA, propionyl-CoA and methylmalonyl-CoA were intermediates (Fig. 1). It is unlikely that this pathway is correct since serine hydroxymethyltransferase and an ORF encoding a polypeptide of unknown function are essential for the conversion of acetyl-CoA to glyoxylate (Chistoserdova & Lidstrom, 1994a, b), although the possibility that these proteins are required to generate an inducer of the genes involved in this part of the serine pathway cannot be excluded. We have now shown that the MeaA protein is also needed, and further work is underway to determine whether it is a mutase with an unusual substrate specificity or whether it has some other function.

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