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-B12-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, C1 assimilation

**INTRODUCTION**

*Methylobacterium extorquens* AM1 is a pink-pigmented facultative methylotroph which assimilates C1 compounds by the serine pathway (Fig. 1) (Anthony, 1982). The first step in this pathway involves the condensation of a C1-tetrahydrofolate derivative with glycine to form serine, catalysed by serine hydroxymethyltransferase. The serine is then converted to C2 and C4 carboxylic acids which can be assimilated into cell material. This involves four key enzymes—serine glyoxylate aminotransferase, hydroxy-pyruvate reductase, glycerate kinase and an acetyl-CoA-independent phosphoenolpyruvate carboxylase. There must also be a means of regenerating the C1 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 C1 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 C1 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;...
a cluster of seven other serine pathway genes also has two functions during growth on C₂ 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 glyceraldehyde 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: oxetetracycline 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 α-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 10⁸ 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 1.4.99.9) 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); glyceraldehyde 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). Maly-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

![Diagram](https://example.com/diagram.png)
### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant properties</th>
<th>Source/reference</th>
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<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td>M. extorquens AM1</td>
<td>Mutants defective in the conversion of acetyl-CoA to glyoxylate</td>
<td>Peel &amp; Quayle (1961)</td>
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<td>Wild-type</td>
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<td>PCT48</td>
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<td>PT1005</td>
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<td>Stone &amp; Goodwin (1989)</td>
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<td>E. coli</td>
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<td>This study</td>
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<td>DSM1607</td>
<td>F− proA2 recA1213 rpsL20 (Str′) hsdS20 (BkdR, hsdM); contains the tra region of RP4 integrated in the chromosome</td>
<td>NCIMB 11865</td>
</tr>
<tr>
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<td>Simon et al. (1983)</td>
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<tr>
<td>CSR603</td>
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<td>XL1-Blue</td>
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</tr>
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<td>P678-54</td>
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<td>DH5s</td>
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<td>IncP1, Te′, Km′, car; broad-host-range cloning vector</td>
<td>Knauf &amp; Nester (1982)</td>
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<td>Allen &amp; Hanson (1985)</td>
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<td>Km′ mobilizing plasmid; ColE1 replicon</td>
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<td>Amp′ phagemid with ColE1 replicon and fl phage origins of replication</td>
<td>Stratagene Cloning Systems</td>
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<td>pTZ18U</td>
<td>Amp′ lacZ′ cloning vector</td>
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<td>pSS48-1, pSS48-2</td>
<td>Cosmids isolated from a HindIII clone bank of <em>M. extorquens</em> AM1 DNA in pVK100</td>
<td>Stone &amp; Goodwin (1989)</td>
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<td>This study</td>
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<td>pLS273</td>
<td>2.2 kb PstI-EcoRI fragment of pLS27 in pTZ18U</td>
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malonyl-CoA hydrolase (Stone & Goodwin, 1989). Methylmalonyl-CoA mutase (EC 5.4.99.2) was measured by the method of Zagalsak 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 mating (where *E. coli* S17-1 carrying a recombinant cosmid was the donor) or triparental mating (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; 10⁸ cells) was spread onto a nutrient agar plate. The plate was dried and then 20 µl culture containing the donor (2 × 10⁷ 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 Hybond 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 *PstI* and the resulting 1·0 kb fragment was cloned into pBluescript KS(+) [1984], generating plasmid pLS271. The 1·1 kb *EcoRI–BglII* fragment of pLS27 was ligated into pBluescript KS(+) which had been digested with *EcoRI* and *BamHI*, giving plasmid plS272. The 2·2 kb *PstI–EcoRI* 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 [35S]dATP as using the Sequenase kit (US Biochemical) or by using a Pharmacia Automated Laser Fluorescence DNA sequencing kit.

Sequence data were compiled and analysed using the GCG 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(+) [1984]. 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 [35S]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 (methanol and ethanol assimilation deficient). Revertants were obtained at a frequency of approximately 10^{-8}. As expected, the mutants were also unable to utilize methanolamine, 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, glyceraldehyde 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 HindIII 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 *EcoRI* 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...
Methanol assimilation in *Methylobacterium*

**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.

**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).
and probed with the 40 kb EcoRI fragment which complemented all of the mutants. It hybridized to a 40 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 EcoRI sites of the DNA fragment cloned in pLS27C.

The genomic DNA from the two strains was also digested with PstI and probed with the 10 kb PstI fragment derived from pLS27C. As expected, it hybridized to a 10 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 PstI and EcoRI restriction sites on pSS48-1, the chromosome of PCT48 must contain a deletion of 2.7 kb, which covers the 2.2 kb EcoRI–PstI fragment (Fig. 2). The complementation analysis indicated that the 2.2 kb EcoRI–PstI 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 40 kb EcoRI 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 40 kb EcoRI 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 EcoRI. The resulting fragments were separated by gel electrophoresis...
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 Prop. gingivalis large subunits. The deduced amino acid sequences 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.

### Figure 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, Prop. 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-B12-binding site and those in italics and underlined represent the conserved sequence in all methylmalonyl-CoA mutases.

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 GXGXXG) 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 40 kb fragment cloned in pLS27 was expressed in E. coli minicells and the resulting products were analysed by 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 weights of 133 kDa, 116 kDa and 95 kDa, were observed.
Our results support the suggestion made by Shimizu et al. (1984) that vitamin-B<sub>12</sub>-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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