A protein having similarity with methylmalonyl-CoA mutase is required for the assimilation of methanol and ethanol by Methylobacterium extorquens AM1
Smith, Loraine M.; Meijer, Wim G.; Dijkhuizen, Lubbert; Goodwin, Pat M.

Published in:
Microbiology

DOI:
10.1099/13500872-142-3-675

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1996

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
A protein having similarity with methylmalonyl-CoA mutase is required for the assimilation of methanol and ethanol by *Methylobacterium extorquens* AM1

Loraine M. Smith,1,2 Wim G. Meijer,2 Lubbert Dijkhuizen2 and Pat M. Goodwin1†

Author for correspondence: Pat M. Goodwin. Tel: +44 171 611 8690. Fax: +44 171 611 8363. e-mail: p.goodwin@wellcome.ac.uk

1 School of Cell and Molecular Biology, NESCOT, Reigate Road, Epsom KT17 3DS, UK
2 Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

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 C₅-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, hydroxypyruvate 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;
Fig. 1. ic variant of the serine pathway showing the key enzymes and the route (broken line) for the regeneration of glyoxylate proposed by Shimizu

Cell material
3-Phosphoglycerate

2 Glycerol → 2-Phosphoglycerate → Phosphoenolpyruvate → Oxaloacetate → Malate → Malyl-CoA → Acetyl-CoA

2 Hydroxybutyrate → 2 Glycerate → 2 Phosphoenolpyruvate → Oxaloacetate → Malate → Malyl-CoA → Acetyl-CoA

Methylmalonyl-CoA

α-Oxoglutarate → Glutamate

Phosphoenolpyruvate carboxylase; SGAT, serine:glyoxylate aminotransferase; STHM, serine transhydroxymethylase; THF, kinase; GM, glutamate mutase; HPRT, hydroxypyruvate reductase; MA, β-methylaspartase; MCL, malyl-CoA lyase; MT, mesaconate thiokinase; MTK, malate thiokinase; PCC, propionyl-CoA carboxylase; PEPC, phosphoenolpyruvate carboxylase; SGAT, serine:glyoxylate aminotransferase; STHM, serine transhydroxymethylase; THF, tetrahydrofolate.

a cluster of seven other serine pathway genes also has two functions during growth on C₃ 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% (v/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 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 1.4.99.3) 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); hydroxybutyrate reductase (EC 1.1.1.81; Goodwin, 1990); glyceraldehyde 3-phosphate dehydrogenase (EC 1.1.1.31; Goodwin, 1990); β-methylaspartase (EC 2.6.1.45; Goodwin, 1990); acetyl-CoA independent phosphoenolpyruvate carboxylase (Goodwin, 1990). Methyl-CoA lyase (EC 4.1.3.24) could not be assayed directly because the substrate, malm-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...
was dropped onto the lawn of recipient. After incubation containing (i) succinate plus tetracycline plus carbenicillin (to select for cosmid transfer) and (ii) methanol plus tetracycline (to overnight at 30°C, the plates were replicated onto medium selected 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.

### Methanol assimilation in *Methyllobacterium*

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant properties</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. extorquens</em> AM1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCT48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT1005</td>
<td>Mutants defective in the conversion of acetyl-CoA to glyoxylate</td>
<td>Stone &amp; Goodwin (1989)</td>
</tr>
<tr>
<td>LS1</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSM1607</td>
<td>F⁻ pro. A2 rec. A13 rpsL 20 (Str⁺) hsdR S 20 (hdaR, hdaM)</td>
<td>NCIMB 11865</td>
</tr>
<tr>
<td>S17-1</td>
<td>thi pro hsdR rec A1; contains the tra region of RP4 integrated in the chromosome</td>
<td>NCIMB 11867</td>
</tr>
<tr>
<td>CSRI03</td>
<td>F⁻ pro A2 rec A11</td>
<td>Bullock et al. (1987)</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>end A1 hsd R 17 (r_{ex}^{+}) sup E 44 thi-1 rec A1 (lac) F' pro AB lacP Z Δ M 15 Tn 10 (Tc⁺)</td>
<td>Adler et al. (1967)</td>
</tr>
<tr>
<td>P678-54</td>
<td>F thr 1 ara 13 leu-6 azi 8 ton A2 lac Y 1 min A 1 gin U 44 gal 6 min B 2 rps L 135 mal A 1 xyl 7 met 12 thi 1</td>
<td>Bethesda Research Laboratories</td>
</tr>
<tr>
<td>DH5α</td>
<td>sup E 44 lac U 169 (Φ 80 lac Z Δ M 15) hsd R 17 rec A1 end A1 gmr A 96 thi 1 rel A 1</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmid/cosmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pVK100</td>
<td>Inc P 1, Te⁺, Km', Sm'; broad-host-range cloning vector</td>
<td>Knauf &amp; Nester (1982)</td>
</tr>
<tr>
<td>pLA2917</td>
<td>Inc P 1, Te⁺, Km', Sm'; broad-host-range cloning vector</td>
<td>Allen &amp; Hanson (1985)</td>
</tr>
<tr>
<td>pRK415</td>
<td>Inc P 1, Te⁺, lac Z⁺; broad-host-range cloning vector</td>
<td>Keen et al. (1988)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Km' mobilizing plasmid; Col E 1 replicon</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pBluescript II KS(+)</td>
<td>Amp' phagemid with Col E 1 replicon and φl phage origins of replication</td>
<td>Stratagene Cloning Systems</td>
</tr>
<tr>
<td>pTZ18U</td>
<td>Amp' lac Z⁺ cloning vector</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>pSS48-1, pSS48-2</td>
<td>Cosmids isolated from a HindIII clone bank of <em>M. extorquens</em> AM1</td>
<td>Stone &amp; Goodwin (1989)</td>
</tr>
<tr>
<td>pLS27C</td>
<td>4.0 kb Eor I fragment of pSS48-1 in pRK415</td>
<td>This study</td>
</tr>
<tr>
<td>pLS27C1</td>
<td>1.0 kb Pst I fragment of pLS27C in pLA2917</td>
<td></td>
</tr>
<tr>
<td>pLS27C3</td>
<td>2.2 kb Pst I - Eor I fragment of pLS27C in pRK415</td>
<td></td>
</tr>
<tr>
<td>pLS27C4</td>
<td>1.0 kb Pst I - Pst I fragment of pLS27C in pRK415</td>
<td></td>
</tr>
<tr>
<td>pLS27</td>
<td>4.0 kb Eor I fragment of pSS48-1 in pBluescript KS(+)</td>
<td></td>
</tr>
<tr>
<td>pLS271</td>
<td>1.0 kb Pst I fragment of pLS27 in pBluescript KS(+)</td>
<td></td>
</tr>
<tr>
<td>pLS272</td>
<td>1.2 kb Eor I - Bgl II fragment of pLS27 in pBluescript KS(+)</td>
<td></td>
</tr>
<tr>
<td>pLS273</td>
<td>2.2 kb Pst I - Eor I fragment of pLS27 in pTZ18U</td>
<td></td>
</tr>
</tbody>
</table>

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; 10⁸ cells) was spread onto a nutrient agar plate. The plate was dried and then 20 μl culture containing the donor (2 x 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 Hybaid Vacu-aid according to the manufacturer's instructions.

DNA probes were prepared by random incorporation of from L. M. Smith and Others to the manufacturer's instructions. The hybridized probe was detected using the DIG nucleic acid membrane (Amersham), using the Hybaid Vacu-aid 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(+), 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 2.5 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]dATPαS using the Sequenase kit (US Biochemical) or by using a Pharmacia Automated Laser Fluorescence DNA sequencing machine. Sequences 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 [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 methylene, 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 C₄-metabolizing enzymes. Crude extracts were then assayed for the known key enzymes of the serine pathway. Serine:glyoxylate aminotransferase, hydroxypropyruvate reductase, glyceraldehyde kinase and the acetyl-CoA-independent phosphoenolpyruvate carboxylase were all detected, as were the C₄-oxidizing enzymes methanol dehydrogenase, methylamine dehydrogenase, formamidine dehydrogenase and formate dehydrogenase. The presence of maly-CoA lyase was deduced by demonstrating an apparent malate synthase activity, which is due to the concerted action of maly-CoA lyase and maly-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 both 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).
The \textit{mea} locus contains a gene encoding a protein which has similarity with methylmalonyl-CoA mutase

The nucleotide sequence of the 4.0 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 \textit{Mea} mutants PT1005 and LS1, indicating that it is essential for the conversion of acetyl-CoA to glyoxylate; we have therefore called it \textit{meaA}. A smaller ORF of 678 bp (\textit{orfB}) was present 118 bp downstream from \textit{meaA} and was transcribed in the opposite direction. The 3'-end of a third ORF (\textit{orfC}) was identified 297 bp from \textit{orfB}. Both the intergenic regions contained inverted repeats which resembled rho-independent termination sequences (Platt, 1986). The deletion in PCT48 extends into \textit{orfC} and, since this mutant was complemented by the 4-0 kb EcoRI fragment which does not contain the complete \textit{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 \textit{BLAST} (Altschul et al., 1990). The \textit{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 \textit{Propionibacterium shermanii}, \textit{Streptomyces cinnamonensis} and \textit{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 \textit{sbm} gene of \textit{E. coli} encodes...
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 \textit{sbm} gene product. The identities were 37–37.5% in the case of the \textit{S. cinnamonensis} and \textit{Pop. gingivalis} large subunit; \textit{E. coli} \textit{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.

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 \textit{et al.}, 1992; Drennan \textit{et al.}, 1994). These sequences (DXHXXG, SXL and GX\textsubscript{3}GGX\textsubscript{4}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 \textit{meaA} (Fig. 5).

The deduced amino acid sequence of the polypeptide encoded by \textit{orfB} did not have significant sequence similarity with any known protein. The predicted amino acid sequence encoded by the partial ORF of \textit{orfC} had homology with the 3-oxoacyl-[acyl-carrier-protein] reductases of \textit{Brassica napus} (63% similarity, 41.5% identity), \textit{Arabidopsis thaliana} (64% similarity, 39% identity) (Slabas \textit{et al.}, 1992) and \textit{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 \textit{meaA}

The 40 kb fragment cloned in pLS27 was expressed in \textit{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 weights of 49, 46, 45 and 44 kDa, were detected (Fig. 6). The 49 kDa polypeptide was determined to be MeaA, on the basis of its sequence homology with the other methylmalonyl-CoA mutases.

### Fig. 5. Alignment of the deduced amino acid sequence of MeaA with sequences of members of the methylmalonyl-CoA mutase family from other sources. \textit{P. sh}, \textit{Prop. shermanii} large subunit; \textit{S. ci}, \textit{S. cinnamonensis} large subunit; \textit{P. gi}, \textit{Pop. gingivalis} large subunit; \textit{E. co}; \textit{E. coli} \textit{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.
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 80 kDa fragment, 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 methylmalonyl-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 B-methylaspartate, mesaconyl-CoA, B-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.

ACKNOWLEDGEMENTS

We thank the SERC for a PhD studentship to L.S. and NESCOT for providing facilities and assistance for this work. We are also very grateful to Dr Peter Leadlay, Cambridge, for advice on the methylmalonyl-CoA mutase assay and for the gift of coupling enzymes for the assay; and to Dr Chris Thurston, King's College London, for providing advice and facilities for some of the sequencing work.

REFERENCES


Received 15 August 1995; revised 24 October 1995; accepted 7 November 1995.