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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

Lorraine M. Smith, Wim G. Meijer, Lubbert Dijkhuizen and Pat M. Goodwin

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 glyoxylic acid and acetate is an intermediate in the recycling pathway. In the so-called Icl⁺ serine pathway organisms, malate thio kinase, 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 thio kinase 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;
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 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 & Twari, 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 1.1.1.99.8), 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). 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 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 complement 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

Cell extracts from the wild-type and the mutant were prepared by cell disruption followed by centrifugation. The crude cell extracts were dialysed against 0.1 M potassium phosphate buffer (pH 7.0) at 4 °C and then lyophilized. The extracts were stored in liquid nitrogen for long-term storage. The cell extracts were then resuspended in 50 mM potassium phosphate buffer (pH 7.0) before the enzyme assay.
was dropped onto the lawn of recipient. After incubation 
select for cosmid transfer) and (ii) methanol plus tetracycline (to 
overnight at 30 OC, the plates were replicated onto medium 
were determined by the method of Lowry using BSA fraction V 
dried and then 20 p1 culture containing the donor (2 
mobilizing plasmid pRK2013 was supplied by E. 
carrying a recombinant cosmid were the donors and the 
malonyl-CoA mutase (EC 5.4.99.2) was measured by the 
method of Fulton et al. (1984). Culture of the recipient 
duced into 
methyl- 
Enzyme assays were repeated 
substrate 
removed and tested for 
which occurred due to carry-over of nutrients from the mating 
plates. To check that complementation, rather than recombi-
national 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.
DNA probes were prepared by random incorporation of 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).

The 2.2 kb PstI-EcoRI fragment of pLS27 was had been digested with EcoRI and BamHI, giving plasmid pLS271. The 1.1 kb EcoRI-BgllI fragment of pLS27 was ligated into pBluescript KS(+) generating 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.

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 10⁻⁹. 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 C1-metabolizing enzymes. Crude extracts were then assayed for the known key enzymes of the serine pathway. Serine:glyoxylate aminotransferase, hydroxypropyruvate reductase, glycerate kinase and the acetyl-CoA-independent phosphoenolpyruvate carboxylase were all detected, as were the C1-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 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...
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 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 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 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 cinnamonosus 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; Willemeyer et al., 1990). The sbm gene of E. coli encodes 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.

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.

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

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al., proposed that they are involved in cobalamin binding another highly conserved sequence (RIARNT) in all of the methylmalonyl-CoA mutases sequenced thus far, but this is not present in GenBank entries (Marsh et al., 1994). These sequences (DXHXXG, SXL and GXGXXG) also occur in MeaA (Fig. 5). There is 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 P. shermanii gene product. Identical residues are indicated by an asterisk, and conserved similarity 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.

The identities were 37-37*5% in the case of the S. cinnamonensis and P. gingivalis gene products. Identical residues are indicated by an asterisk, and conserved similarity 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 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 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 40 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 weights...
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 methyymalonoyl-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-B₄₅-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-methylmalonyl-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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