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Cloning, Sequencing, and Expression of the Gene Encoding Methylmalonyl-Coenzyme A Mutase from *Streptomyces cinnamonensis*

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In streptomycetes, the conversion of succinyl-coenzyme A (CoA) into methylmalonyl-CoA, catalyzed by methylmalonyl-CoA mutase, most likely represents an important source of building blocks for polyketide antibiotic biosynthesis. In this work, the structural gene for methylmalonyl-CoA mutase from *Streptomyces cinnamonensis* was cloned by using a heterologous gene probe encoding the mutase from *Propionibacterium shermanii*. A 5,732-bp fragment was sequenced, within which four open reading frames were identified on one DNA strand. The two largest (*mutA* and *mutB*) overlap by 1 nucleotide and encode proteins of 616 and 733 residues showing high amino acid sequence similarities to each other and to methylmalonyl-CoA mutases from *P. shermanii* and mammalian sources. The transcriptional start of the *mutA-mutB* message, determined by S1 mapping, coincides with the first nucleotide of the translational start codon. Evidence that these two open reading frames encode a functional mutase in *S. cinnamonensis* was obtained by subcloning and expression in *Streptomyces lividans* TK64. The *mutA* and *mutB* gene products were detected in Western blots (immunoblots) with mutase-specific antibodies and by direct detection of mutase activity with a newly developed assay method. The methylmalonyl-CoA mutase was unable to catalyze the conversion of isobutyryl-CoA into *n*-butyryl-CoA, another closely related adenosylcobalamin-dependent rearrangement known to occur in *S. cinnamonensis*.

Streptomycetes produce a large number of structurally diverse polyketide antibiotics, whose carbon frameworks are constructed by the repetitive condensation of simple fatty acid derivatives, in a process similar to long-chain fatty acid biosynthesis (16). One of the building blocks most frequently used by polyketide synthases is methylmalonyl-coenzyme A (CoA), whose incorporation leads to a methyl branch in the polyketide backbone, as seen for example in the macrolide ring of erythromycin (10) and in the polyether monensin A (37). Because several methylmalonyl-CoAs are often needed to build a single polyketide (six for the macrolide in erythromycin, seven for monensin A), the production of methylmalonyl-CoA may represent a limiting step in the flow of primary metabolites into these antibiotics.

The conversion of succinyl-CoA into methylmalonyl-CoA, catalyzed by the adenosylcobalamin-dependent methylmalonyl-CoA mutase (MCM), is well known, and the enzyme has been isolated from both prokaryotic and mammalian sources (35). Partial purification of an unstable MCM from the erythromycin producer *Saccharopolyspora erythraea* has also been described (18). Apart from the direct carboxylation of propionyl-CoA, another pathway to methylmalonyl-CoA in streptomycetes was uncovered recently, by feeding labelled, branched fatty acids to antibiotic-producing organisms (32, 33, 36, 41). These experiments indicated that *n*-butyryl-CoA can be isomerized to isobutyryl-CoA before oxidation to methylmalonyl-CoA and incorporation into a polyketide antibiotic (Fig. 1). This oxidation of isobutyryl-CoA, an intermediate in valine catabolism, occurs by a route different from that seen during valine catabolism in mammals and in other bacteria (20, 25, 49), because C-1 of isobutyryl-CoA becomes C-1 of methylmalonyl-CoA, rather than being lost as carbon dioxide.

The isomerization of *n*-butyryl-CoA to isobutyryl-CoA is also catalyzed by an adenosylcobalamin-dependent enzyme in extracts of *Streptomyces cinnamonensis* (8), and its presence in many other streptomycetes has been inferred through labelling studies (32, 33, 36, 41). Although this interconversion is very similar to the MCM reaction (Fig. 1), it was not clear from earlier work (8) whether both are catalyzed by a single enzyme possessing a slack substrate specificity or whether two distinct but closely related cobalamin-dependent enzymes are present in streptomycetes. This interconversion of *n*-butyrate and isobutyrate has been detected so far only in streptomycetes (32, 33, 36, 41) and certain strictly anaerobic bacteria (26, 45).

Recently, the genes for MCM from *Propionibacterium shermanii* (23), from humans (19), and from mice (47) were cloned and sequenced. The *P. shermanii* MCM is heterodimeric, and the genes for the α-subunit (large subunit [≈79 kDa]) and β-subunit (small subunit [≈65 kDa]) are adjacent on the chromosome (23). In contrast, the human (19) and mouse (47) enzymes are homodimers. The α-subunit from *P. shermanii* shows a very high amino acid sequence similarity both to the two mammalian enzymes and, to a lesser extent, to its own β-subunit.

We describe below the first characterization of an MCM gene from a polyketide antibiotic-producing streptomycete, namely, the monensin-producer *S. cinnamonensis*, and its expression in the heterologous host *Streptomyces lividans* TK64. A new assay is described for MCM, which, along with Western blots (immunoblots), allows detection of mutase in crude extracts of *S. lividans* containing the expressed gene. The same extracts, however, do not catalyze the interconversion of *n*-butyryl-CoA and isobutyryl-CoA, indicating that this

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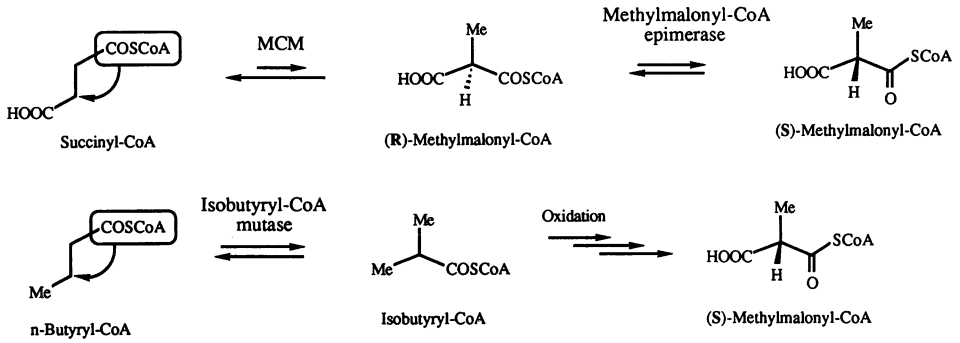

FIG. 1. The isobutyryl-CoA mutase, MCM, and methylmalonyl-CoA epimerase reactions. Oxidation of isobutyryl-CoA in streptomycetes also yields methylmalonyl-CoA.

isomerization is catalyzed by a separate mutase in *S. cinnamonensis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *S. cinnamonensis* A3823.5 (Eli Lilly) was used as the source strain and was cultured as previously described (2). *S. lividans* TK64, employed for the propagation of *Streptomyces* plasmids and as the expression host, was cultured and transformed according to the method of Hopwood et al. (15). *Streptomyces* plasmids used were pIJ702 (21), pIJ486, and pIJ487 (46). Thiostrepton (a gift of S. J. Lucania, Bristol-Myers-Squibb Research Institute, Princeton, N.J.) was used at concentrations of 50 μg/ml for solid media and 10 μg/ml for liquid media. *Escherichia coli* ED 8767 (29) and JM 103 (28) were employed as recipients in cloning experiments and for the isolation of single-stranded DNA. General culture conditions were as described by Sambrook et al. (39). The *E. coli* plasmids used were pUC18/19 (31) and pBR322 (7); transformation was performed as described by Tabak et al. (44).

DNA isolation and manipulation. DNA was isolated from streptomycetes and was manipulated as described by Birch et al. (6). DNA fragments for shotgun cloning were recovered from low-melting-point agarose (15), and those for ^{32}P-labelling were purified with Geneclean according to the manufacturer's recommendations. Blotting and Southern hybridization experiments were done as described previously (6, 39). *E. coli* colony replicas were prepared according to standard protocols (39). DNA probes were labelled to high specific activity by using the method of Feinberg and Vogelstein (11) employing random polydeoxyhexanucleotides [pd(N)$_6$] (Pharmacia).

DNA sequencing and analysis. DNA sequencing was carried out by the dideoxy chain termination method (40) with DNA fragments previously cloned in either M13mp18 or M13mp19 (50). Nested deletion sets were generated with plasmids treated with *Pst*I and *Xba*I followed by digestion with ExoIII (39) so that each insert was represented by clones differing on average by 150 to 200 bp. Sequenase was employed for chain elongation, and the entire sequence was determined from overlapping clones on both strands by using parallel dGTP and deaza-GTP reaction mixes. Compressions which remained intractable were resolved either by substituting dITP for dGTP or by employing specifically designed oligonucleotides to prime DNA synthesis closer to the problem area. Sequence confirmation of the frameshift mutation in clone pOCI444 was undertaken by using the following oligonucleotides: (5')AGTTCGCCGACATCGAC GAGTA and (5')TGTACAGCGGCTTGACCGCGAT. The University of Wisconsin Genetics Computer Group sequence analysis software package, version 7.1 (9), was used to assemble the sequence. The DNA sequence was analyzed for open reading frames (ORFs) by using CODONPREFER ENCE (9). Protein data base searches were performed with FASTA, comparisons were performed with COMPARE and DOTPLOT, and sequence alignments were performed with LINEUP and PILEUP, all from the University of Wisconsin Genetics Computer Group package.

Promoter probe experiments, RNA isolation, and S1 mapping. Promoter activity within DNA fragments was determined by subcloning fragments of interest into pIJ486 or pIJ487 (46) and transforming them into *S. lividans* TK64. The relative strengths of the promoters were assayed by plating out equivalent numbers of spores onto minimal medium plates (15) containing increasing concentrations of kanamycin (up to 900 μg/ml). RNA was isolated from 2- to 6-day-old cultures by the method of Hopwood et al. (15). DNA fragments were end-labelled by the method of Sambrook et al. (39); hybridization, S1 digestion, and analysis were performed as detailed by Geistlich et al. (12).

Protein extracts and Western blotting. Crude protein extracts were prepared from 96-h cultures grown in yeast extract-malt extract medium (15) by sonication, as detailed previously (34). For Western blots, the protein was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (22) and then the protein was transferred to polyvinyldifluoride Immobilon-P membranes with a Milliblot-Graphite electroblotter (Millipore) according to the manufacturer's instructions. After blocking with 1% bovine serum albumin in Tris-buffered saline, the membranes were incubated with rabbit anti-*P. shermanii* MCM (α-subunit) polyclonal antibodies (kindly donated by J. Rétey, University of Karlsruhe, Karslruhe, Germany) and the bound antibodies were detected with a *pico*Blue immunoscreening kit (Stratagene, La Jolla, Calif.).

Enzyme assays. MCM activity was assayed by using 25 μl (~5 mg of protein per ml) of crude protein extract diluted to 200 μl with the following buffer: 5 mM EDTA–10% (wt/vol) glycerol–50 mM potassium phosphate, pH 7.4. The solution was preincubated (10 min) in the dark with 2 μl of coenzyme-B_{12} (1 mM in H_2O) prior to addition of 10 μl of methylmalonyl-CoA (11.5 mM in H_2O). After 30 min at 30°C, the reaction was stopped by the addition of 100 μl of NaOH (2 N) containing 1 mM glutaric acid as a gas chromatography

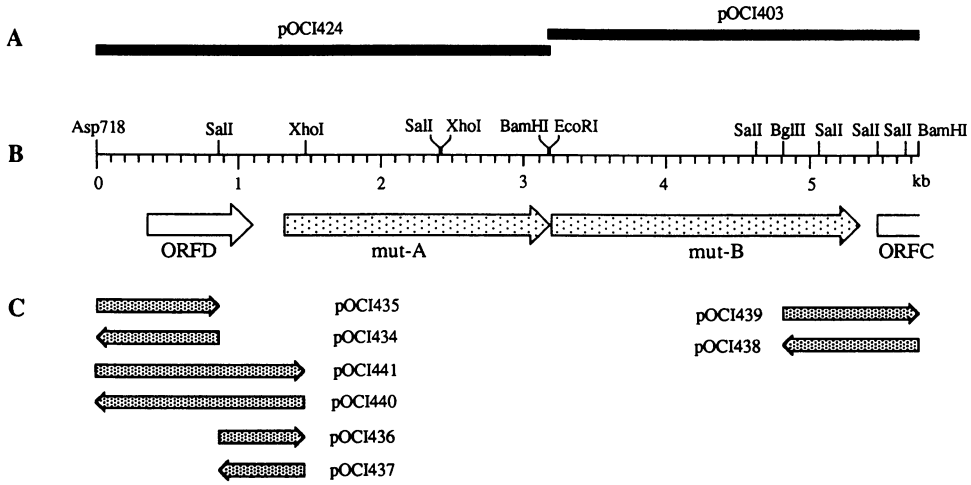

FIG. 2. Overlapping DNA fragments isolated in pOCI424 and pOCI403 (A), restriction map of the region encoding MCM from *S. cinnamonensis* and the positions and orientations of the four ORFs identified from the nucleotide sequence (B), and DNA fragments and their relative orientations used in assays of promoter activity when cloned into pIJ486 or pIJ487 (C).

standard. The mixture was acidified with 100 μl of H_2SO_4 (15%, vol/vol), saturated with NaCl, extracted with ethyl acetate (250 μl), and treated with 50 μl of CH_2N_2 (ca. 250 mM in diethylether), and the surplus was quenched by the addition of acetic acid (2 μl). The resulting solution was directly analyzed on a Hewlett-Packard HP5890 series II gas chromatograph with an HP-5 column (10 m by 0.53 mm, 2.65-μm film thickness) at column temperature profiles of 75°C for 3 min, 75 to 100°C over 2.5 min, 100 to 250°C in 3 min, and finally 250°C for 3.5 min. Retention times for dimethyl methylmalonate and dimethyl succinate were typically 1.5 and 2.3 min, respectively. Isobutyryl-CoA mutase was assayed by addition of 4 μl of *n*-butyryl-CoA (5 mM in H_2O) to protein extracts (200 μl) containing 2 μl of coenzyme-B_{12} (1 mM in H_2O). After 30 min, the reaction was stopped with 100 μl of KOH (2 N) containing valeric acid (0.184 mM), acidified with 100 μl of H_2SO_4 (15%, vol/vol), saturated with NaCl, and extracted with ethyl acetate (250 μl). The *n*- and isobutyric acids were then assayed directly by gas chromatography with an HP-FFAP column (10 m by 0.53 mm, 2.65-μm film thickness; Hewlett-Packard) at 78°C for 1.25 min, 78 to 150°C over 6 min, and finally 1.5 min at 150°C. Retention times for isobutyric acid and *n*-butyric acid were typically 2.3 and 3.0 min, respectively.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the GenBank, EMBL, and DDBJ nucleotide sequence data bases under the accession number L10064.

RESULTS AND DISCUSSION

Cloning and sequencing of the MCM gene. A DNA probe encoding the *P. shermanii* MCM large subunit (*mutB*) (kindly donated by J. Rétey, University of Karlsruhe) was labelled and was used to identify cross-hybridizing bands in a Southern blotting experiment with *S. cinnamonensis* genomic DNA digested with various restriction enzymes. The high G+C content and the biased codon usage of the gram-positive *P. shermanii* and *S. cinnamonensis* chromosomes (30, 48) should facilitate the use of genes from one organism for the cloning of similar sequences from the other. Bands were easily detected even at high stringency (washing with 0.2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% SDS, 68°C), indicating the presence of closely related sequences in the *S. cinnamonensis* genome. *Eco*RI and *Bgl*II genomic DNA fragments corresponding in size to the bands of interest were excised from preparative agarose gels and were purified and ligated to appropriately digested pBR322. Two clones were identified by colony hybridization experiments, and restriction mapping demonstrated that they represented almost 22 kb of genomic DNA with 1.6 kb of overlapping sequence. Southern blotting with labelled probes derived from DNA encoding the large and small subunits of the *P. shermanii* MCM indicated that the corresponding genes in *S. cinnamonensis* lay adjacent to each other and spanned the region of overlap. Subsequently, these hybridizing sequences were subcloned as slightly overlapping 3.2-kb *Asp*718-*Eco*RI and 2.55-kb *Bam*HI fragments in pUC18, thereby generating pOCI424 and pOCI403, respectively (Fig. 2).

The regions subcloned in pOCI424 and pOCI403 were excised as *Hin*dIII-*Eco*RI fragments and subcloned into M13mp18 and M13mp19, and overlapping sequences on both the coding and noncoding strands (Fig. 3) were determined. The resulting sequence of 5,732 nucleotides (nt) has a G+C content of 71.8%. Computer analysis of the DNA sequence by using the program CODONPREFERENCE to plot the G+C content at each of the three possible codon positions (4) revealed three complete ORFs and one incomplete ORF with typical streptomycete codon bias. These are ORFD, *mutA*, *mutB*, and ORFC and are oriented from left to right in Fig. 2.

The two central ORFs, *mutA* and *mutB*, (nt 1318 to 3165 and 3168 to 5366), possess overlapping stop and start codons, a device which is thought to lead to translational coupling (13, 52) and hence to the production of stoichiometric amounts of the respective polypeptides. The two ORFs encode proteins with very high primary sequence similarities to the two subunits of MCM from *P. shermanii*, as well as to the mouse and human MCMs (see below). These *mutA* and *mutB* genes encode polypeptides of 616 amino acids (M_r, 65,040) and 733 amino acids (M_r, 79,454), respectively. *mutB* is preceded by a potential streptomycete ribosome binding site at nt 3157 to 3160, on the basis of

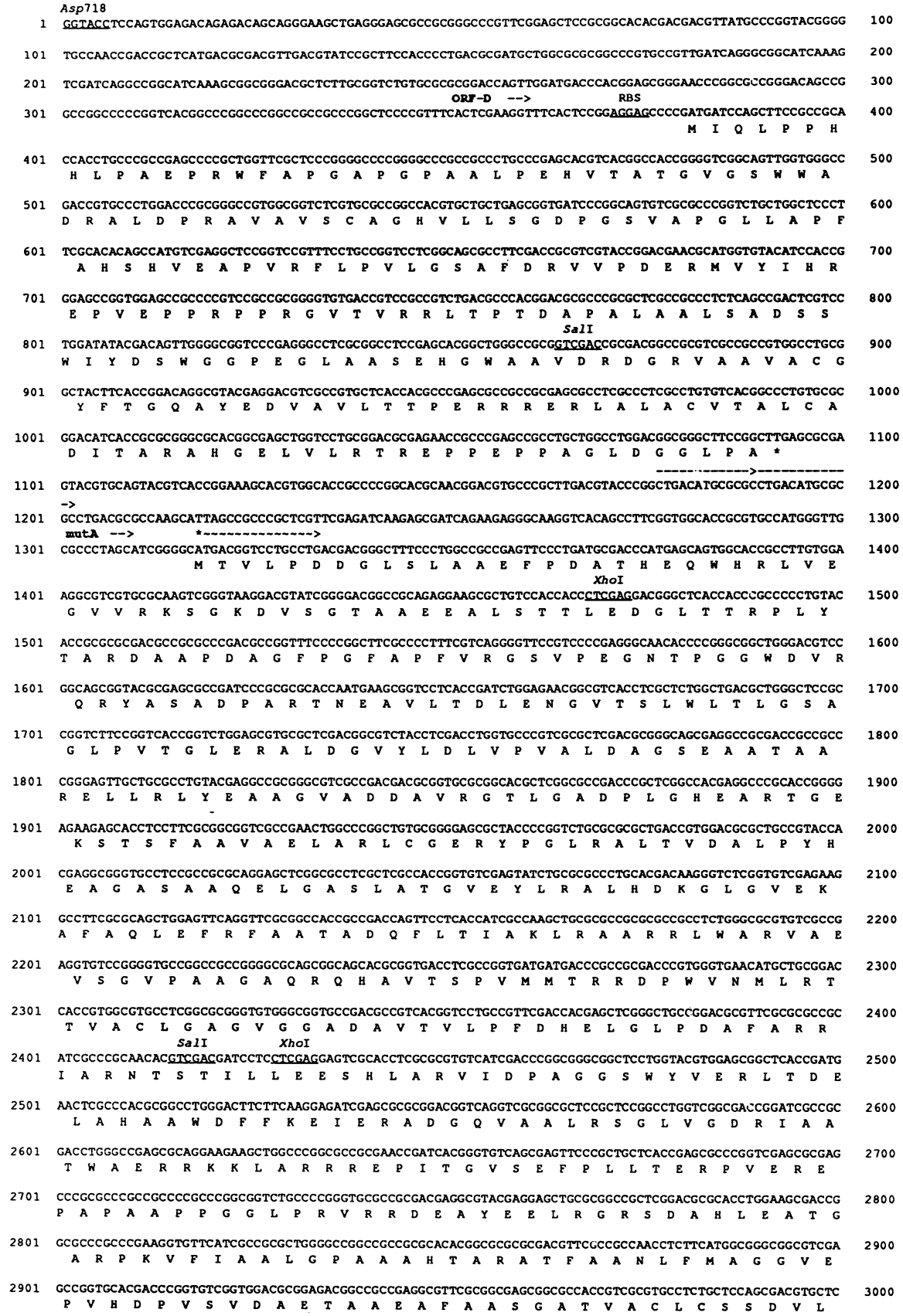

FIG. 3. The nucleotide and deduced amino acid sequences of a 5,732-bp *Asp*718-*Bam*HI fragment (see Fig. 2) from *S. cinnamonensis*, including genes for the small and large subunits (*mutA* and *mutB*, respectively) of MCM as well as two ORFs (ORF-C and ORF-D) of unknown function (see text). Putative ribosome binding sites (RBS) are underlined. The restriction endonuclease sites referred to in Fig. 2 are also underlined. The start of the *mutA-mutB* transcript, determined by S1 mapping, is indicated by the asterisked arrow. A 13-bp direct-repeat sequence upstream of the *mutA-mutB* promoter region is indicated by a broken arrow above the sequence. The two sequencing primers used to confirm the 4-bp insertional inactivation in pOCI444 are also indicated by broken arrows and are labelled primer 1 and primer 2.

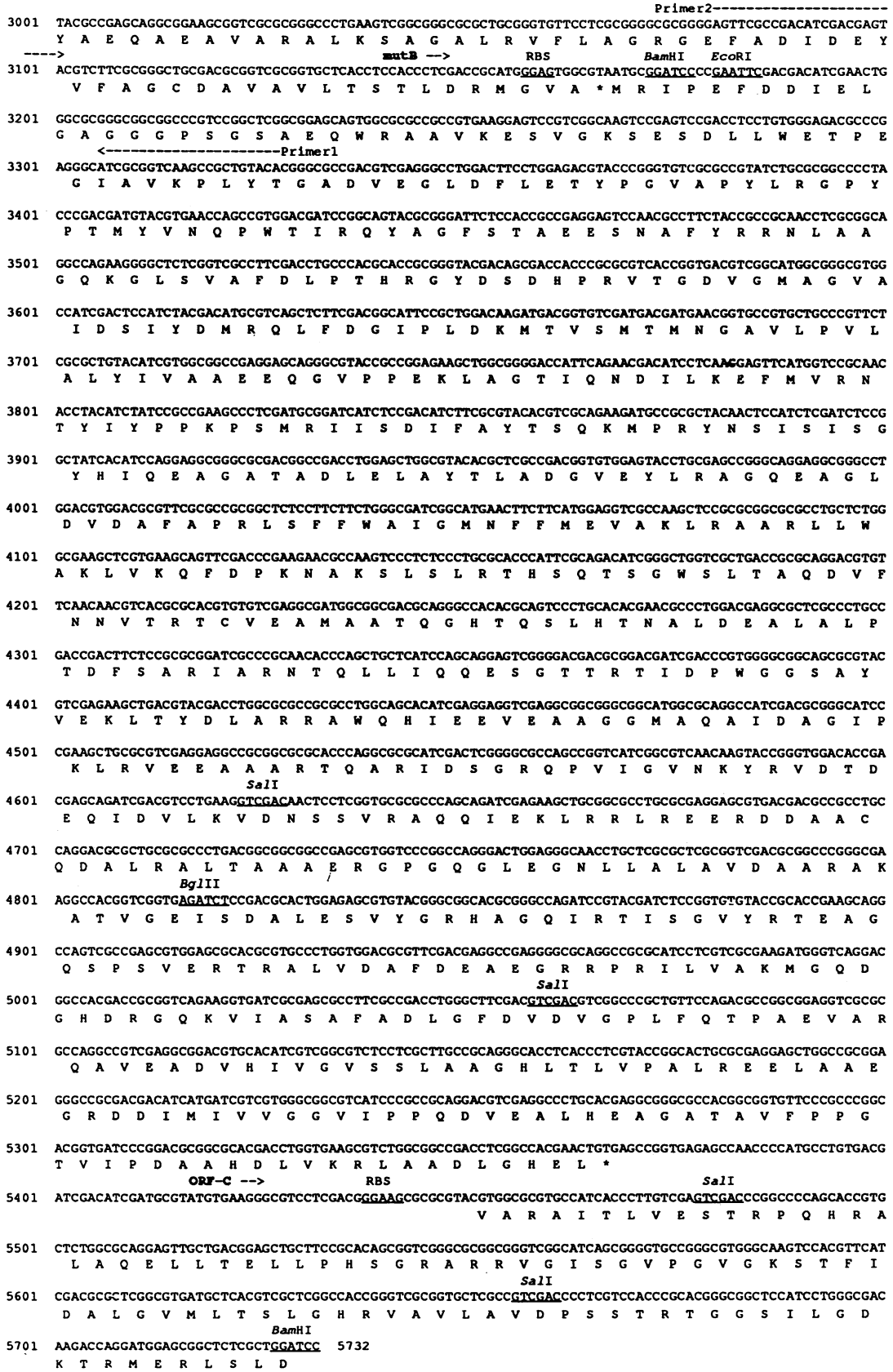
Primer2----------
mutB -->
RBS
BamHI
EcoRI
<----------Primer1
SalI
BglII
ORF-C -->
RBS
SalI
SalI
BamHI

TABLE 1. Identities and similarities among MCM α- and β-subunits[a]

Source	% Identity (% similarity) between				
	P. shermanii		*S. cinnamonensis*		Human
	α-Subunit	β-Subunit	α-Subunit	β-Subunit	
P. shermanii, β-Subunit	24 (47)				
S. cinnamonensis					
α-Subunit	72 (84)	27 (50)			
β-Subunit	27 (52)	43 (62)	29 (51)		
Human	60 (74)	26 (48)	63 (76)	27 (48)	
Mouse	57 (72)	27 (49)	61 (75)	25 (49)	92 (96)

[a] Values were derived with the GAP program from the University of Wisconsin Genetics Computer Group package, with a gap weight of 3 and a gap length of 0.1.

TABLE 2. Promoter probe analysis of the MCM coding region

Promoter probe clone	Length (bp)	Km^r (μg/ml)[a]
pOCI435	865	25
pOCI434	865	0
pOCI441	1,465	100
pOCI440	1,465	0
pOCI436	600	50
pOCI437	600	50
pOCI438	910	0
pOCI439	910	>900[b]

[a] Levels of Km^r conferred on *S. lividans* TK64 by the eight DNA fragments (Fig. 2) cloned in pIJ486 or pIJ487.
[b] Km^r was tested only to 900 μg/ml.

reasonable complementarity to the 3′ end of the 16S rRNA sequence (3, 14), whereas *mutA* shows no such complementarity.

The ORFC immediately downstream of *mutA* and *mutB* is incompletely represented in the sequence data (nt 5452 to 5732) but is preceded by a typical streptomycete ribosome binding site (nt 5438 to 5442). A search of the protein sequence data base failed to locate any protein with significant sequence similarity. However, the product of a recently sequenced gene (designated orf2 in reference 38) 0.8 kb in length lying adjacent to the *sbm* gene in *E. coli*, which encodes an MCM-like protein, shows 75% amino acid similarity to the *S. cinnamonensis* ORFC product over the available sequence. This striking cross-species conservation of gene order (*sbm* plus orf2 and *mutB* plus ORFC) and protein sequence may reflect a common biochemical function for these gene products, perhaps in the metabolism of methylmalonyl-CoA in these organisms (Fig. 1).

The codon bias of the upstream ORFD (Fig. 3, nt 381 through 1091) approximates that of a typical streptomycete ORF but is less convincing than those for the other three ORFs. The ORFD is, however, preceded by a potential ribosome binding site. A search of the protein data bases failed to reveal any similar sequences.

Comparison of MCM protein sequences. Protein sequences for the large and small subunits of MCM from *P. shermanii*, the homodimeric human and mouse MCM, and the sequence deduced for an *E. coli* MCM-like protein were extracted from the data banks and compared with the sequence deduced for the *S. cinnamonensis* MCM. These comparisons provide the first indication that a complete MCM has been cloned in this work and are also of interest for identifying residues that may play a key functional or structural role in these enzymes. Such residues are likely to be conserved across species that are, from an evolutionary standpoint, distantly related.

Computer alignment of the sequences reveals a strikingly high end-to-end sequence identity among the large subunits of the bacterial enzymes and the mammalian enzymes, as well as among the small subunits of the bacterial enzymes (Table 1). Unfortunately, the high sequence similarities thwart attempts to identify uniquely important residues that may, for example, be involved in the catalytic mechanism, and suggest that conservation of a large portion of the primary sequence is essential for maintenance of the correctly folded and functional coenzyme-B_{12}-dependent enzyme. It is notable that no cysteine residues are to be found among the many residues that occur at conserved positions in the proteins from all five organisms. Cysteine residues are of special interest because they often play an important structural role in folded proteins, and a possible direct role in coenzyme-B_{12}-dependent rearrangements has also been discussed (23, 43). Finally, the small subunit of the *S. cinnamonensis* MCM shows a high similarity to its own large subunit (51%), suggesting that the corresponding genes may have arisen via a gene duplication event, as is thought to be the case in *P. shermanii* (24).

Promoter analysis. An in vivo promoter analysis was undertaken by cloning the eight DNA fragments depicted in Fig. 2C into the promoter probe vectors pIJ486 and pIJ487, which contain a promoterless kanamycin resistance (Km^r) gene (46). Promoter activity is determined by inserting DNA fragments into a multiple cloning site immediately upstream of this Km^r gene. Km^r levels give an indication of the relative strength of any promoters present in the DNA fragment. Because these vectors do not replicate in *S. cinnamonensis*, the assays were carried out with *S. lividans* TK64. The fragment containing sequences upstream of and including the start of ORFD showed only very low activity (Table 2) in the sense direction (pOCI435), whereas the fragment containing sequences upstream of and including the start of *mutA* possessed low activity in both orientations (pOCI436 and pOCI437). However, pOCI441, which spans this entire region and reads into the *mutA* gene, conferred significant Km^r activity. To allow a more precise localization of the *mutA* promoter and to express the MCM gene in *S. lividans*, the inserts of pOCI424 and pOCI403 (Fig. 2) were subcloned into the high-copy-number streptomycete vector pIJ702 so that the native configuration of *mutA* and *mutB* was regenerated in the plasmid pOCI433. For this, the region encoding *mutB* was excised from pOCI403 as an *Eco*RI fragment, by using an *Eco*RI site present in the multiple cloning site of pUC18, and was ligated into *Eco*RI-digested pOCI424. A clone with the correct insert orientation (pOCI428) was then digested with *Asp*718 to release the insert, and this was subcloned into *Asp*718-digested pIJ702 to yield pOCI433.

Low-resolution S1 mapping was performed with an end-labelled 1.55-kb *Sal*I fragment (nt 866 to 2417, Fig. 3) and RNA isolated from 5- and 6-day-old cultures of *S. lividans* TK64 harboring pOCI433. A 1.1-kb protected fragment was observed, indicating that the mRNA start point lay in the neighborhood of the translation start. No other protected species were seen (data not presented). High-resolution S1

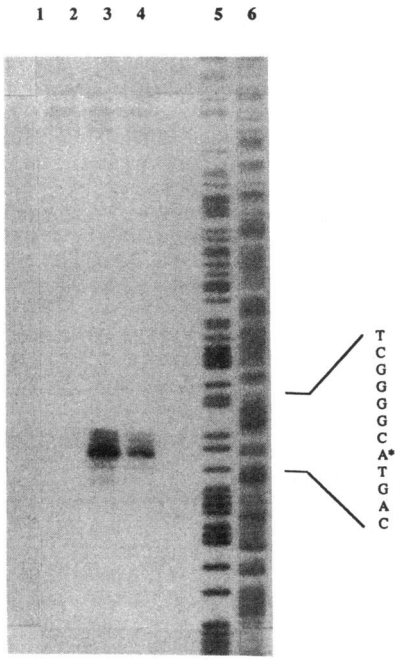

FIG. 4. High-resolution S1 mapping of the 5′ end of the MCM transcript from pOCI433 in *S. lividans* TK64. A labelled 603-bp *Xho*I (nt 1472)-*Sal*I (nt 869) fragment (Fig. 2) was hybridized to RNA (62°C, 3 h) and was subsequently digested with 200 U of S1 nuclease (37°C, 45 min). RNA from 5- and 6-day-old *S. lividans* TK64 cultures containing pOCI433 protected identical fragments (lanes 3 and 4), whereas RNA from a 5-day-old *S. lividans* TK64 culture containing pIJ702 failed to yield any protection (lane 2), as did the tRNA control (lane 1). The position of the likely transcription start point (allowing for slower migration, by 1.5 nt, of the S1-protected fragment compared with that of the Maxam and Gilbert sequence ladder [27]) is indicated by an asterisk alongside the sequence of the non-coding strand. Maxam and Gilbert sequence ladders derived from the same end-labelled fragment are present in lanes 5 and 6 (A/G and C).

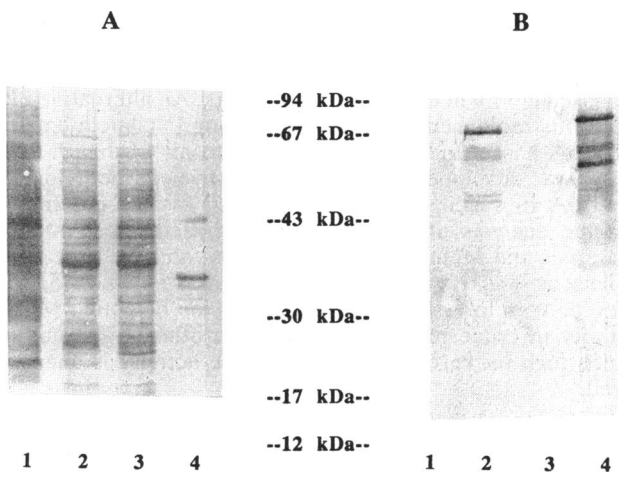

FIG. 5. Detection of MCM by Western blotting. (A) Coomassie brilliant blue-stained SDS–12% PAGE of the following crude protein extracts (by lane): 1, *S. lividans* TK64 (with pIJ702), 73 μg of protein; 2, *S. lividans* TK64 (with pOCI433), 43 μg of protein; 3, *S. lividans* TK64 (with pOCI444), 47 μg of protein; 4, partially purified *P. shermanii* MCM, 2.5 μg of protein. (B) Western blot of a parallel gel (lanes are as described above) hybridized with purified rabbit polyclonal antibodies raised against the *P. shermanii* MCM large subunit. The antibodies cross-react with a single protein (approximately 75 kDa) present in *S. lividans* extracts containing the cloned *S. cinnamonensis* MCM (pOCI433). Cross-reactivity is abolished by a 4-bp insertional inactivation in the large subunit gene (pOCI444).

mapping showed that the transcription start point most probably coincides with the first nucleotide of the *mutA* ATG start codon. The slowest migrating band shown in Fig. 4 is likely to represent the start of transcription, while the smaller fragments are presumably the result of degradation. The position of the most intense band varied between experiments (with the same RNA preparation), suggesting that this may be a function of the different digestion conditions used. Although the mapped transcript originates from a plasmid-borne gene and not from the gene in its native environment, the precise point of transcription initiation is most likely conserved between chromosome and plasmid. It is thus likely that the mapped transcript also reflects the 5′ mRNA start in the *S. cinnamonensis* genome. Difficulties in preparing RNA from *S. cinnamonensis* frustrated attempts to perform S1 mapping experiments with this strain. So far, 11 streptomycete promoters in which translation and transcription are proposed to initiate at the same nucleotide have been characterized (42). Two of these promoters (*ermE* [5] and *sta* [17]) show a resemblance to the *mutA* and *mutB* promoters around the −10 region.

Interestingly, there is no stem-loop structure present in the small intergenic region between *mutB* and ORFC (82 nt), which would be typical of a rho-independent terminator. The promoter probe experiments, however, indicated the presence of a very strong promoter reading into ORFC (from clone pOCI439; Fig. 2C and Table 2). A preliminary Northern (RNA) analysis of this region (data not presented) revealed a high-abundance transcript originating here. These data indicate that the polycistronic *mutA mutB* transcript most likely terminates in this intergenic region and does not include ORFC. No stem-loop structures, indicative of transcriptional termination, are present between ORFD and *mutA-mutB*.

Expression of the MCM gene and assay of enzyme activity. Plasmid pOCI433 contains the *mutA* and *mutB* genes on a 5.732-kb *Asp* 718 fragment cloned into the unique *Asp*718 site (isoschizomer of *Kpn*I) of pIJ702 (15). The MCM gene should, in this construct, be expressed from its own promoter in the well-characterized host strain *S. lividans* TK64. To further support the assignment of *mutA* and *mutB* as the MCM structural genes, an insertional inactivation experiment was carried out. Plasmid pOCI433 was linearized at the unique *Eco*RI site which lies at the 5′ end of *mutB* (Fig. 2 and 3), and the sticky ends were filled in with T4 DNA polymerase Klenow fragment and were religated to yield pOCI444. This resulted in a 4-bp insertion at this point (confirmed by sequencing) and produced a frameshift mutation which rendered the *mutB* polypeptide nonfunctional.

Rabbit polyclonal antibodies against the *P. shermanii* large subunit have been shown to detect cross-reacting protein in crude protein extracts of *S. cinnamonensis* (8). These antibodies also recognized protein in Western blots prepared from crude extracts of *S. lividans* TK64 containing pOCI433 but failed to detect any expression when the strain contained just the vector pIJ702 or pOCI444, in which the large subunit has been disrupted by a frameshift mutation (Fig. 5). These blotting experiments indicate the production of a stable, functional MCM in *S. lividans* TK64 containing

pOCI433. However, attempts to detect enzyme activity by using the usual enzyme-coupled assay system (51) were hampered by the presence of high background NADH-oxidase activity in crude protein extracts. An alternative but laborious radiochemical assay employing [^{14}C]methylmalonyl-CoA has been used (18, 35), but to avoid this, a new assay was developed in which the turnover of methylmalonyl-CoA to succinyl-CoA is monitored by gas chromatographic analysis of the corresponding methyl esters (see Materials and Methods). The method is rapid, reliable, and at least as sensitive as the usual optical assay method (51). It should, therefore, be generally useful for detecting MCM activity in crude protein extracts from other organisms in which high background NADH-oxidase activity is encountered.

Extracts of *S. lividans* containing pOCI433 showed MCM activity that was at least 5- to 10-fold higher than that from the same strain bearing pOCI444 or pIJ702. Low background MCM activity is present in *S. lividans* TK64, but the levels of MCM in the presence of pOCI433 were typically 0.025 μmol/min/mg of protein. Taken with the other experiments described above, these results provide strong evidence that the *mutA* and *mutB* sequences encode the two subunits of a functional *S. cinnamonensis* MCM.

The last question addressed here is whether the *S. cinnamonensis* MCM also catalyzes the rearrangement of isobutyryl-CoA to *n*-butyryl-CoA. A modified form of the gas chromatography-based assay described above (see Materials and Methods) provided a convenient and sensitive method for the detection of isobutyryl-CoA mutase from *S. cinnamonensis*. Whereas crude protein extracts of *S. lividans* TK64 with pOCI433 consistently demonstrated high MCM activity, isobutyryl-CoA mutase activity was completely absent. These results provide the first indication that two distinct coenzyme-B_{12}-dependent mutases catalyzing closely related rearrangements (Fig. 1) are indeed present in this microorganism. The presence of a distinct isobutyryl-CoA mutase in *Streptomyces* species is also of enzymological interest, and work is presently under way to isolate and characterize the isobutyryl-CoA mutase and MCM from *S. cinnamonensis*.

Knowledge of the MCM gene structure may facilitate future investigations on its regulation and role in polyketide antibiotic biosynthesis. For example, recent studies (1) have provided a base for the genetic manipulation of *S. cinnamonensis*, so the effects upon monensin production of introducing extra copies of the MCM gene into this strain or of disrupting the chromosomal copy can now be investigated.

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