0273-2289/99/82/0209/$14.25

Molecular Analysis and Heterologous Expression of the Gene Encoding Methylmalonyl–Coenzyme A Mutase from Rifamycin SV–Producing Strain *Amycolatopsis mediterranei* U32

Weiwen Zhang,*,†,¶ Ling Yang,¶ Weihong Jiang, Guoping Zhao,* Yinliu Yang, and Juishen Chiao

Department of Microbiology, Shanghai Institute of Plant Physiology, Academia Sinica, 200032 Shanghai, P.R. China, E-mail: wzhang@hsc.vcu.edu

Received September 8, 1999; Revised December 14, 1999; Accepted December 16, 1999

Abstract

The conversion of succinyl-coenzyme A (CoA) into methylmalonyl-CoA, catalyzed by adenosylcobalamin-dependent methylmalonyl-CoA mutase (MCM), represents an important source of building blocks for rifamycin SV biosynthesis. The structural gene for MCM from rifamycin SV–producing strain *Amycolatopsis mediterranei* U32 was isolated by using a heterologous gene probe encoding the MCM of *Streptomyces cinnamonensis*. A 7.8-kbp fragment was sequenced and four complete open reading frames (ORFs) and two incomplete ORFs were found. Two central ORFs, ORF3 and ORF4, overlap by four nucleotides and were found to encode MCM small (602 residues) and large (721 residues) subunits, respectively. Comparison showed that the MCM gene of *A. mediterranei* U32 was quite similar to those from other sources. The functionally unknown ORF5, immediately downstream of the *mut*AB gene, was quite similar to the ORFs downstream of *mut*AB from *S. cinnamonensis* and *Mycobacterium tuberculosis*. Such a striking cross-species conservation of gene order suggested that ORF5 could also be involved in the metabolism of methylmalonyl-CoA. MCM gene was overexpressed in

*Authors to whom all correspondence and reprint requests should be addressed.
†Present address: ISBDD, Suite 212B, Virginia Biotechnology Park, 800 East Leigh Street, Richmond, VA 23219.
¶Both authors contributed equally to this paper.

Escherichia coli under T7 promoter, and MCM activity could be detected in the recombinant *E. coli* clone harboring MCM gene after the addition of coenzyme B_{12}. A purification procedure based on the B_{12} affinity column was established to purify the MCM from *E. coli*. The molecular weight of purified MCM from *E. coli* was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, which corresponds to that calculated from the MCM protein sequence and is also the same size as that of the enzyme purified directly from *A. mediterranei* U32. MCM gene was overexpressed in polyketide monensin producing *S. cinnamonensis*, and the total monensin production was increased by 32%.

Index Entries: Methylmalonyl–Coenzyme A mutase; cloning; expression; rifamycin SV; monensin biosynthesis.

Introduction

Rifamycin belongs to the group of macrolide antibiotics whose general structure consists of a chromophore and an ansa chain. Early isotope studies with ^{13}C-enriched precursors *(1)* suggested that the ansa chain is derived from two acetate units and eight propionate units, possibly via malonyl–coenzyme A (CoA) and methylmalonyl-CoA, respectively. The subsequent condensation of activated acyl units to form the macrolide lactonic ring is catalyzed by a polyketide synthetase system, for example, by the synthesis of the macrolide ring of erythromycin *(2)* and the polyether monensin A *(3)*. The metabolic routes to methylmalonyl-CoA are of great interest because they represent a limiting step in the flow of primary metabolites into these antibiotics *(3)*. Studies with ^{13}C-labeled nuclear magnetic resonance analyses showed that methylmalonyl-CoA was probably synthesized mainly from succinyl-CoA, catalyzed by an adenosylcobalamin-dependent methylmalonyl-CoA mutase (MCM), in rifamycin, leukomycin, and tylosin-producing strains *(4)*.

In recent years, the MCM genes from *Propionibacterium shermanii (5)*, human *(6)*, mouse *(7)*, *Sinorhizobium meliloti (8)*, and *Porphyromonas gingivalis (9)* have been cloned and sequenced. The only MCM gene from antibiotic-producing streptomycete was cloned from polyether monensin A–producing *Streptomyces cinnamonensis (10)*. All the MCMs occur in two classes. The first MCM class is a large subunit homodimer (~80 kDa) encoded by a single gene in the mammalian case. By contrast, all prokaryotic MCMs are heterodimers, encoded by two genes (large subunit, ~75 to 79 kDa; small subunit, ~65 to 68 kDa). Three prokaryotic MCM-like proteins—*sbm* from *Escherichia coli (11)*, *mea*A from *Methylobacterium extorquens (12)*, and *Streptomyces collinus (13)*—appear as a single polypeptide of ~78 or ~80 kDa. The large subunits of MCM from prokaryotic sources show a very high similarity in amino acid sequence to the mammalian MCM enzymes.

MCM from a rifamycin SV producing *Amycolatopsis mediterranei* U32 has been purified and characterized. It consists of two subunits of 75 and 65 kDa *(14)*. Metabolic analysis showed that MCM is the main enzyme responsible for methylmalonyl-CoA formation for rifamycin SV produc-

tion in *A. mediterranei* U32, although methylmalonyl-CoA could also be produced by methylmalonyl-CoA transcarboxylase or propionyl-CoA carboxylase pathways *(15,16)*. In this article, we report on the cloning and sequencing of MCM gene and its heterologous overexpression in *E. coli* and *S. cinnamonensis*, as well as its effects on monensin production. We compare the deduced amino acid sequence with the known MCM-like sequences of both prokaryotes and eukaryotes and discuss the possible implications of these findings. (The nucleotide sequence data reported herein have been submitted to Genebank under the accession no. AF117980.)

Materials and Methods

Materials and General Molecular Biology Methods

(α-^{32}P)CTP was purchased from Amersham. The restriction enzymes *Taq* polymerase and T4 ligase were from Promega (Madison, WI). Small- and large-scale plasmid DNA preparation was done with Wizard Plus DNA purification systems (Promega, Madison, WI). Competent cell preparation, transformation, restriction digestion, phenol extraction, ethanol and isopropanol precipitation, treatment of DNA with Klenow fragment of DNA polymerase and alkaline phosphatase, and T4 DNA ligase were done according to the standard methods described in ref. *17*.

Cloning and Sequencing of mut*AB Gene*

A. mediterranei U32 was grown in medium consisting of the following components: 10.0 g/L of glycerol, 2.0 g/L of tryptone, 1.0 g/L of yeast extract, and 1.0 g/L of beef extract, pH 7.2. Cosmid library was constructed from *A. mediterranei* U32 DNA that was partially digested with *Sau*3A1, and then size fractionated by sucrose gradient centrifugation to give fragments of about 20–30 kbp. The DNA fragments were ligated into the *Bam*HI site of cosmid vector pLAFR3. Competent *E. coli* DH5α cells were transformed with the gene libraries and plated onto Xgal-isopropyl-β-D-thiogalactoside (IPTG) Luria agar supplemented with 25 µg/mL of tetracycline. The resulting gene libraries contained 3000–4000 individual recombinant colonies. A 4.8-kbp fragment containing *mut*AB of *S. cinnamonensis* (kindly donated by Prof. J. A. Robinson, University of Zurich-IRCHEL) was labeled with (α-^{32}P)CTP using a nick translation kit (Promega) and was used as a probe in heterologous hybridization experiments. Hybridization was carried out in 6X saline sodium citrate (SSC) at 68°C for 16 h. The nylon membranes were then washed in 2X SSC and 1X SSC for 45 min at 68°C. DNA sequencing was performed with an ABI PRISMTM 373 DNA Sequencer (Perkin Elmer, Branchburg, NJ) according to the manufacturer's recommendations. Sequence was analyzed for open reading frames (ORFs) by using CODONPREFERENCE in Genetics Computer Group or FramePlot 2.3* software (available at http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl, from the homepage of The Society for Actinomycetes, Japan). Protein database searches were performed with BLAST and FASTA, comparison

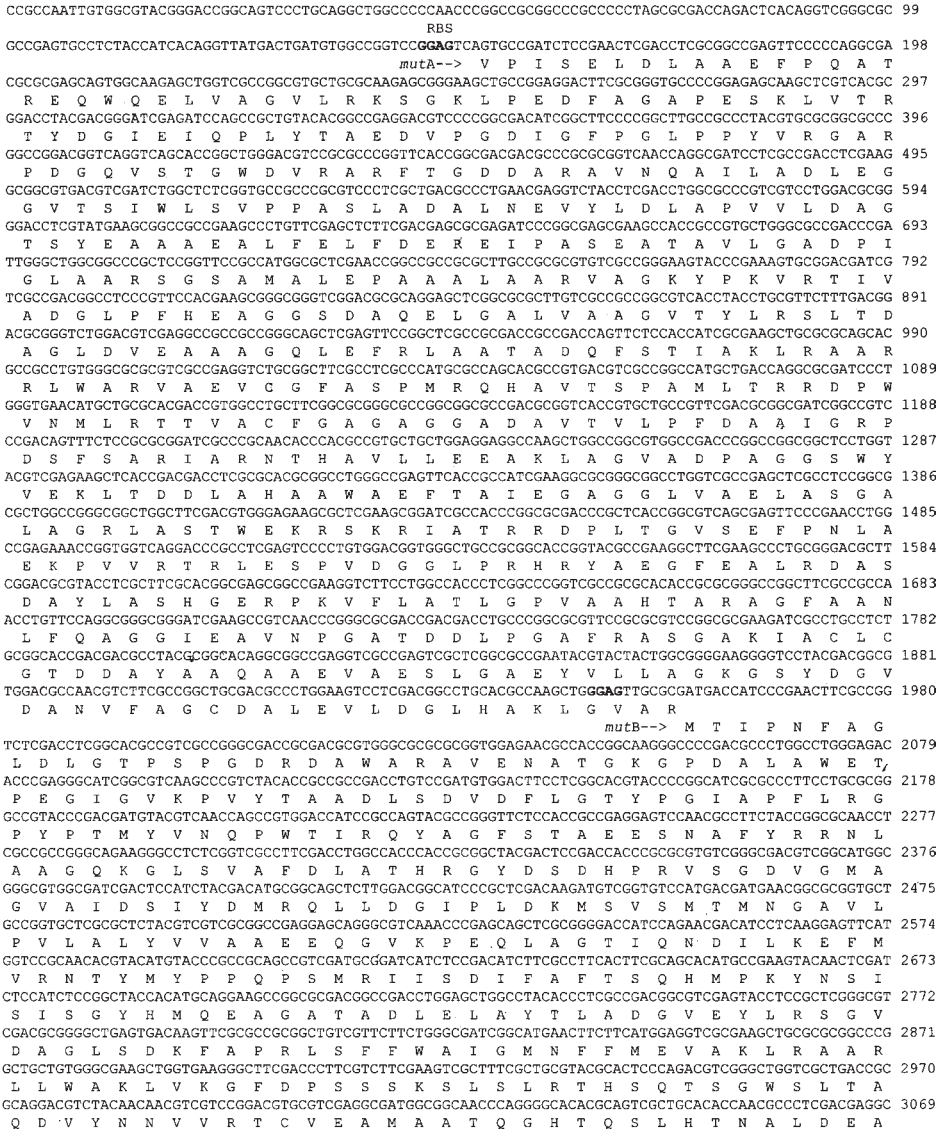

Fig. 1. The nucleotide and deduced amino acid sequences of a 5509-bp fragment from *A. mediterranei* U32, including genes for small and large subunits (*mut*A and *mut*B) of MCM and its downstream ORFs. The putative ribosome-binding sites (RBS) are in boldface. The nucleotide sequence data were submitted to Genebank under accession no. AF117980.

with COMPARE, and sequence alignments with LINEUP and PILEUP, all from the University of Wisconsin Genetics Computer Group Package (Madison, WI).

Expression in E. coli *and Gene Product Purification*

An *Sac*I/*Pst*I fragment containing *mut*AB was cloned into the *Sma*I/*Pst*I sites of pALTER-1 (Promega, Madison, WI), and an *Nde*I site was intro-

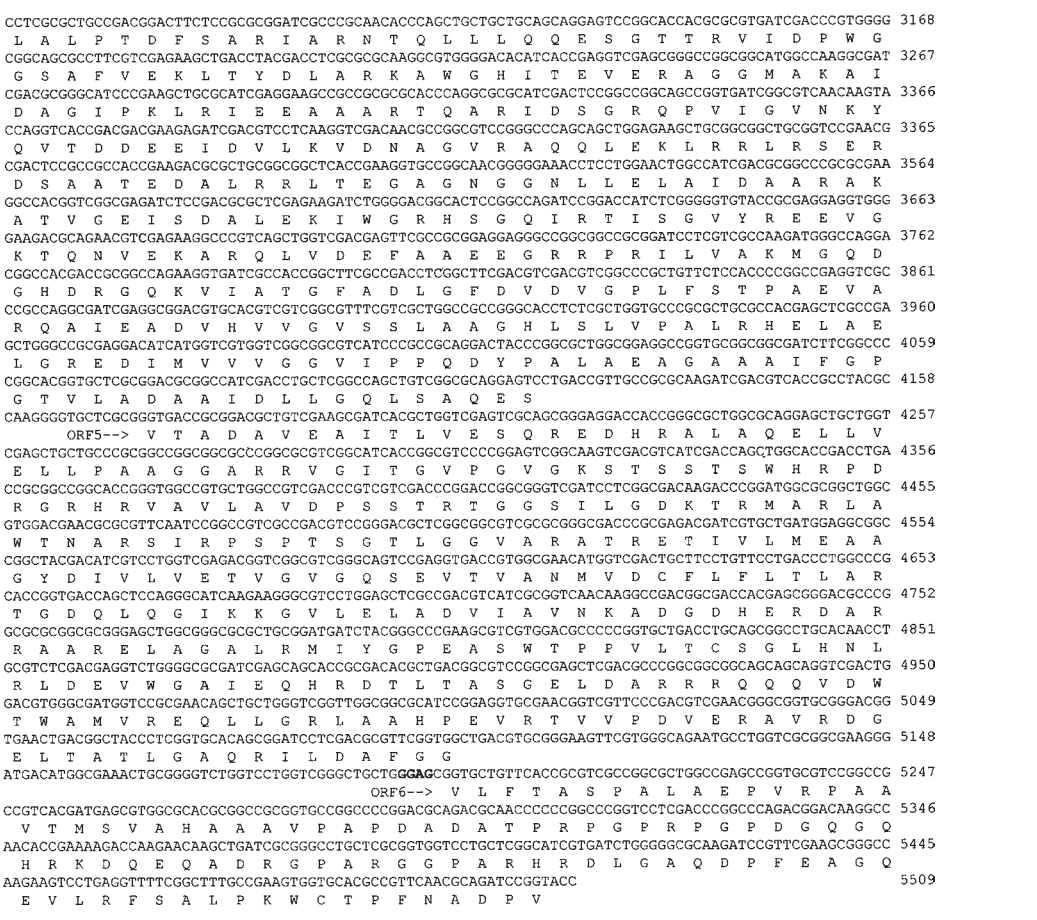

Fig. 1. *(continued)*.

duced before the *mut*AB ORF by site-directed mutagenesis, with Altered Sites® II in vitro mutagenesis systems (Promega, Madison, WI). The mutagenesis oligo 5' CTACCATCACAGCATATGACTGATGT 3' synthesized corresponds to the DNA sequence nucleotide (nt) 111–136 (*Nde*I site is underlined) in Fig. 1. The mutagenesis was done according to the conditions suggested in the manufacturer's manual (Promega). The *Nde*I/*Hind*III fragment from pALTER-1 recombinant plasmid was then cloned into *Nde*I/*Hind*III sites of pET28b(+) (Novagen, Madison, WI), resulting in pYL28-MCM for expression. A 0.1-mL overnight culture of *E. coli* BL21(DH3) (pYL28-MCM) was inoculated into 200 mL of Luria-Bertani medium (with 12 µg/mL of kanamycin), and then incubated at 37°C for 2 to 3 h. IPTG was added to a final concentration of 0.4 m*M* and the medium was further incubated for 3–5 h at 25°C. Harvested cells were washed twice in buffer A (50 m*M* NaH_2PO_4, pH 7.5) and then disrupted by sonication. The crude cellular extract of *E. coli* BL21(DE3) (pYL28) was obtained after centrifugation at 36,000*g* for 20 min. The cellular extract was precipitated with $(NH_4)_2SO_4$ at 90% saturation. The protein collected was dialyzed with 2 L

of buffer A for 2 h and then was loaded into a coenzyme B_{12} affinity column (Ø1.5 cm × 6.5 cm) (Sigma, St. Louis, MO). The column was washed completely with 50 mL of 50 m*M* phosphate buffer containing 1.0 *M* NaCl. The MCM was then eluted with 50 m*M* phosphate buffer containing 1.0 *M* NaCl and 3 m*M* coenzyme B_{12} *(14)*.

The MCM assay was based on the rate of conversion of succinyl-CoA to methylmalonyl-CoA, which can be assayed spectrophotometrically by means of a series of coupled reactions *(18)*. The following were combined in a final volume of 1.0 mL: 2.5 µmol of succinyl-CoA, 2.5 µmol of reduced glutathione, 10 µmol of sodium pyruvate, 0.1 U of methymalonyl-CoA transcarboxylase and 0.1 U of methylmalonyl-CoA racemase (both purified from *A. mediterranei* U32; *[14,16]*), 3×10^{-4} nmol of coenzyme B_{12} (pH 7.0), 50 mmol phosphate buffer, and enzyme solution. Succinyl-CoA was omitted for the blank. The reaction was stopped by adding 0.2 mL of 10% (w/v) trichloroacetic acid, the precipitate was removed by centrifugation, and the supernatant was then neutralized by adding 1 *N* NaOH. Then 2.5 µmol of NADH and 0.1 U of malic dehydrogenase (Sigma) were added to measure produced oxaloacetate at 340 nm. Succinyl-CoA was prepared chemically by the method of Simon and Shemin *(19)* or purchased from Sigma. One unit of enzyme is defined as the consumption of 1.0 µmol of substrate/min at 25°C.

Expression in S. cinnamonensis *and Monensin Production*

*Nde*I/*Hind*III fragment from pYL28-MCM was cloned into pIJ4123. *S. cinnamonensis* protoplast was prepared and transformed according to the methods described by Hopwood et al. *(20)*. Monensin production was checked by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) according to the methods described by Reynolds et al. *(21)* and Beran and Zima *(22)*, respectively. A two-stage fermentation cycle was used. A vegetative culture consisted of 0.25% glucose, 1.5% soybean, 0.3% $CaCO_3$, 0.03% $FeSO_4$, and 0.003% $MnCl_2$ was grown at 30°C for 30 h. In the second stage, medium contained the same constituents as seed medium except 5% glucose was used. Fermentation was carried out at 30°C for 6 d. Monensin was extracted with chloroform after the whole broth was homogenized with methanol. Chloroform fractions containing monensin A and B were evaporated in a spray drier followed by suspension of brownish powder in methanol. After filtration, the methanolic solution was directly injected for HPLC analysis as described by Beran et al. *(22)*. The total production of monensin A and B was calculated by comparing the monensin A and B peaks with monensin standard.

Results and Discussion

Cloning of MCM Gene

A DNA probe encoding the *S. cinnamonensis mut*AB gene (kindly donated by Prof. J. A. Robinson) was labeled and used to identify cross-

hybridizing bands in a Southern blotting experiment with *A. mediterranei* U32 genomic DNA digested with various restriction enzymes. Bands were easily detected even at high stringency (washing with 0.2X SSC-0.1% sodium dodecyl sulfate at 68°C for 45 min twice), indicating the presence of similar sequences in the *A. mediterranei* U32 genome. Two gene libraries were constructed from *A. mediterranei* U32 DNA. A 4.8-kbp fragment containing *mut*AB of *S. cinnamonensis* was labeled with α-^{32}P(dCTP) using a nick translation kit (Promega) and was used as a probe in heterologous hybridization experiments. Hybridization was conducted in 6X SSC at 68°C for 16 h. The nylon membranes were then washed in 2X SSC and 1X SSC for 45 min at 68°C. Two positive clones with inserts of ~22 kbp were obtained. Restriction mapping of the clones showed that they overlapped by 8 kbp and that they both contained complete *mut*AB gene. One clone (pCZ31) was selected for further work.

Sequencing and Analysis of MCM Gene

An ~7.8-kbp *Kpn*I fragment from pCZ31 was subcloned into pUC18, generating pCZ8. The complete nucleotide sequence of pCZ8 was determined. It contained two incomplete and four complete ORFs with typical streptomycete codon bias. The two central overlapping ORFs, ORF3 and ORF4, were found to contain the *mut*A and *mut*B genes. ORF2, upstream of *mut*AB, which is transcribed in the opposite direction from *mut*AB, has been identified as a novel serine/threonine protein kinase-like gene (data not given). Herein, we reported the sequence analysis of an ~5.5-kbp fragment containing *mut*AB and its downstream ORF5 (Fig. 1).

ORF3, which starts at nt 153 (GTG) and ends at nt 1961, appears to encode a 602 amino acid small subunit of MCM, and has a G+C% of 73%. ORF4 starts from nt 1958 (ATG) to nt 4123, encodes a 721 amino acid large subunit of MCM, and has a G+C% of 68%. These two ORFs possess overlapping stop and start codons at nt 1958–1961 (ATGA), the device that is thought to lead to translational coupling and hence the production of stoichiometric amounts of the respective polypeptides, also found in *S. cinnamonensis mut*AB gene *(10)*. The small subunit of *A. mediterranei* U32 MCM shows 53.3% similarity (29.0% identity) to its own large subunit. Very high similarity (identity) between two subunits occurs especially in the middle region of two subunits, amino acids 220–410 of the small subunit and amino acids 240–440 of the large subunit, suggesting that the two genes may have arisen via a gene duplication event *(5)*. *mut*A and *mut*B are both preceded by potential streptomycete ribosome binding sequences GGAG at nt 146–149 and nt 1947–1950, on the basis of reasonable complementarity to the 3' end of the 16*S* rRNA sequence *(23)*. No putative *E. coli* σ^{70}-like promoter element was found upstream of the *mut*AB gene *(24)*.

Protein sequences for some of the MCMs available in the database were extracted and compared with the protein sequence deduced for *A. mediterranei* U32 MCM (Fig. 2). α-Subunit of MCM from *A. mediterranei* U32 showed the highest similarity (identity) to that of *S. cinnamonensis*,

```
          1                                                                                                  80
Human     MLRAKNQLFL LSPHYLRQVK ESSGSRLIQQ RLLHQQQPLH PEWAALAKKQ LKGKNPEDLI WHTPEGISIK PLYSKRDTMD
Mouse     MLRAKNQLFL LSPHYLKQLN IPSASRW..K RLLHQQQPLH PEWAVLAKKQ LKGKNPEDLI WHTPEGISIK PLYSRADTLD
S.c       .......... ......MRIP EFDDIEL... .GAGGGPSGS AEQWRAAVKE SVGKSESDLL WETPEGIAVK PLYTGADVEG
A.m       .......... ......MTIP NFAGLDL... .GTPS..PGD RDAWARAVEN ATGKGPDALA WETPEGIGVK PVYTAADLSD
M.t       .......... .MTTKTPVIG SFAGVPLHSE RAAQSPTEAA VHTHVAAAAA AHGYTPEQLV WHTPEGIDVT PVYIAADRAA
P.s       .......... .....MSTLP RFDSVDL... ..GNAPVPAD AARRFEELAA KAGTGE...A WETAEQIPVG TLFNEDVYKD
P.g       .......... .......MKP NYKDIDIKS. ....AGFVAK DATRWAEEKG IVAD...... WRTPEQIMVK PLYTKDDLEG
E.c       .......... .......... .......... ....MSNVQE WQQLANKELS RREKTVDSLV HQTAEGIAIK PLYTEADLDN
M.e       .......... .......... .......... .......... .......... .......... .......... ..........

          81                                                                                                 160
Human     LP......EE LPGVKPFTRG PYPTMYTFRP WTIRQYAGFS TVEESNKFYK DNIKAGQQGL SVAFDLATHR GYDSDNPRVR
Mouse     LP......EE LPGVKPFTRG PYPTMYTYRP WTIRQYAGFS TVEESNKFYK DNIKAGQQGL SVAFDLATHR GYDSDNPRVR
S.c       LDF....LET YPGVAPYLRG PYPTMYVNQP WTIRQYAGFS TAEESNAFYR RNLAAGQKGL SVAFDLPTHR GYDSDHPRVT
A.m       VDF....LGT YPGIAPFLRG PYPTMYVNQP WTIRQYAGFS TAEESNAFYR RNLAAGQKGL SVAFDLATHR GYDSDHPRVS
M.t       AEAEGYPLHS FPGEPPFVRG PYPTMYVNQP WTIRQYAGFS TAADSNAFYR RNLAAGQKGL SVAFDLATHR GYDSDHPRVQ
P.s       MDW....LDT YAGIPPFVHG PYATMYAFRP WTIRQYAGFS TAKESNAFYR RNLAAGQKGL SVAFDLPTHR GYDSDNPRVA
P.g       ME....HLDY VSGLPPFLRG PYSGMYPMRP WTIRQYAGFS TAEESNAFYR RNLASGQKGL SVAFDLATHR GYDADHSRVV
E.c       LEVT....GT LPGLPPYVRG PRATMYTAQP WTIRQYAGFS TAKESNAFYR RNLAAGQKGL SVAFDLATHR GYDSDNPRVA
M.e       .......MSA QASVAEVKRD ........KP WIIRTYAGHS TAAESNKLYR GNLAKGQTGL SVAFDLPTQT GYDPDHELAR
                    *             * * ** *** * *   **  *   *   ** ** ****** *   *** *
                                              -                   -

          161                                                                                                240
Human     GDVGMAGVAI DTVEDTKILF DGIPLEKMSV SMTMNGAVIP VLANFIVTGE EQGVPKEKLT GTIQNDILKE FMVRNTYIFP
Mouse     GDVGMAGVAI DTVEDTKILF DGIPLEKMSV SMTMNGAVIP VLATFIVTGE EQGVPKEKLT GTIQNDILKE FMVRNTYIFP
S.c       GDVGMAGVAI DSIYDMRQLF DGIPLDKMTV SMTMNGAVLP VLALYIVAAE EQGVPPEKLA GTIQNDILKE FMVRNTYIYP
A.m       GDVGMAGVAI DSIYDMRQLL DGIPLDKMSV SMTMNGAVLP VLALYVVAAE EQGVKPEQLA GTIQNDILKE FMVRNTYMYP
M.t       GDVGMAGVAI DSILDMRQLF DGIDLSTVSV SMTMNGAVLP ILALYVVAAE EQGVAPEQLA GTIQNDILKE FMVRNTYIYP
P.s       GDVGMAGVAI DSIYDMRELF AGIPLDQMSV SMTMNGAVLP ILALYVVTAE EQGVKPEQLA GTIQNDILKE FMVRNTYIYP
P.g       GDVGKAGVSI CSLEDMKVLF DGIPLSKMSV SMTMNGAVLP ILAFYINAGL EQGAKLEEMA GTIQNDILKE FMVRNTYIYP
E.c       GDVGKAGVAI DTVEDMKVLF DQIPLDKMSV SMTMNGAVLP VLAFYIVAAE EQGVTPDKLT GTIQNDILKE YLCRNTYIYP
M.e       GEVGKVGVSI AHLGDMRALF DQIPLAQMNT SMTINATAPW LLSLYLAVAE EQGAPLAALQ GTTQNDIIKE YLSRGTYVFP
          * **  ** *     *   *    ***      ***         *          ***         ** **** **    *     *
                                          -  - -                     -

          241                                                                                                320
Human     PEPSMKIIAD IFEYTAKHMP KFNSISISGY HMQEAGADAI LELAYTLADG LEYSRTG.LQ AGLTIDEFA. ..PRLSFFWG
Mouse     PEPSMKIIAD IFQYTAQHMP KFNPISISGY HMQEAGTDAI LELAYTIADG LEYCRTG.LQ AGLTIDEFA. ..PRLSFFWG
S.c       PKPSMRIISD IFAYTSQKMP RYNSISISGY HIQEAGATAD LELAYTLADG VEYLRAG.QE AGLDVDAFA. ..PRLSFFWA
A.m       PQPSMRIISD IFAFTSQHMP KYNSISISGY HMQEAGATAD LELAYTLADG VEYLRSG.VD AGLS.DKFA. ..PRLSFFWA
M.t       PKPSMRIISD IFAYTSAKMP KFNSISISGY HIQEAGATAD LELAYTLADG VDYIRAG.LN AGLDIDSFA. ..PRLSFFWG
P.s       PQPSMRIISE IFAYTSANMP KWNSISISGY HMQEAGATAD IEMAYTLADG VDYIRAG.ES VGLNVDQFA. ..PRLSFFWG
P.g       PEFSMRIIAD IFEYTSQNMP KFNSISISGY HMQEAGATAD IEMAYTLADG MQYLKAG.ID AGIDVDAFA. ..PRLSFFWA
E.c       PKPSMRIIAD IIAWCSGNMP RFNTISISGY HMGEAGANCV QQVAFTLADG IEYIKAA.IS AGLKIDDFA. ..PRLSFFFG
M.e       PAPSLRLTKD VILFTTKNVP KWNPMNVCSY HLQEAGATPV QELSYALAIA IAVLDTVRDD PDFDEASFSD VFSRISFFVN
          *  *                 *   *     * *  ***          *                        *     * ***
                                          -

          321                                                                                                400
Human     IGMNFYMEIA KMRAGRRLWA HLIEKMFQPK NSKSLLLRAH CQTSGWSLTE QDPYNNIVRT AIEAMAAVF. ..GGTQSLHT
Mouse     IGMNFYMEIA KMRAGRRLWA HLIEKMFQPK NSKSLLLRAH CQTSGWSLTE QDPYNNIVRT AIEAMAAVF. ..GGTQSLHS
S.c       IGMNFFMEVA KLRAARLLWA KLV.KQFDPK NAKSLSLRTH SQTSGWSLTA QDVFNNVTRT CVEAMAATQ. ..GHTQSLHT
A.m       IGMNFFMEVA KLRAARLLWA KLV.KGFDPS SSKSLSLRTH SQTSGWSLTA QDVYNNVVRT CVEAMAATQ. ..GHTQSLHT
M.t       IGMNFFMEVA KLRAGRLLWS ELV.AQFAPK SAKSLSLRTH SQTSGWSLTA QDVFNNVART CIEAMAATQ. ..GHTQSLHT
P.s       IGMNFFMEVA KLRAARMLWA KLV.HQFGPK NPKSMSLRTH SQTSGWSLTA QDVYNNVVRT CIEAMAATQ. ..GHTQSLHT
P.g       IGVNHFMEIA KMRAARLLWA KIV.KSFGAK NPKSLALRTH SQTSGWSLTE QDPFNNVGRT CIEAMAAAL. ..GHTQSLHT
E.c       IGMDLFMNVA MLRAARYLWS EAV.SGFGAQ DPKSLALRTH CQTSGWSLTE QDPYNNVIRT TIEALAATL. ..GGTQSLHT
M.e       AGMRFVTEIC KMRAFAELWD EIAQERYGIT DAKKRIFRYG VQVNSLGLTE QQPENNVHRI LIEMLAVTLS KRARARAVQL
           *           **   **                      *    *      **  *   **  *    *  *
```

Fig. 2. Multiple alignment of *A. mediterranei* U32 MCM protein large subunit and other known MCM and MCM-like proteins. The alignment was generated using GCG Pileup. The positions conserved in all proteins are marked with an asterisk. The amino acids that have been shown to be involved in substrate binding in *P. shermanii* are underlined *(26)*. Sites that are important for B_{12} coenzyme binding in methionine synthase are marked with a plus sign *(25)*. S.c, *S. cinnamonensis* (GeneBank accession no. L10064); *P. shermanii* (X14965); M.t, *M. tuberculosis* (Z79701); P.g, *P. gingivalis* (L30136); E.c, *E. coli sbm* gene (X66836); M.e, *M. extorquens* (U28335); Mouse, (X51941); Human, (M65131); A.m, *A. mediterranei* U32 (AF117980).

```
        401                                                                 480
Human   NSFDEALGLP TVKSARIARN TQIIIQEESG IPKVADPWGG SYMMECLTND VYDAALKLIN EIEEMGGMAK AVAEGIPKLR
Mouse   NSFDEALGLP TVKSARIARN TRIIIQEESG IPKVADPWGG SYMMESLTND VYEAALKLIY EVEEMGGMAK AVAEGIPKLR
S.c     NALDEALALP TDFSARIARN TQLLIQQESG TTRTIDPWGG SAYVEKLTYD LARRAWQHIE EVEAAGGMAQ AIDAGIPKLR
A.m     NALDEALALP TDFSARIARN TQLLLQQESG TTRVIDPWGG SAFVEKLTYD LARKAWGHIT EVERAGGMAK AIDAGIPKLR
M.t      ALDEALALP TDFSARIARN TQLVLQQESG TTRPIDPWGG SYYVEWLTHR LARRARAHIA EVAEHGGMAQ AISDGIPKLR
P.s     NSLDEAIALP TDFSARIARN TQLFLQQESG TTRVIDPWSG SAYVEELTWD LARKAWGHIQ EVEKVGGMAK AIEKGIPKMR
P.g     NALDEAIALP TDFSARIARN TQIYIQEETL VCKEIDPWGG SYYVESLTNE LVHKAWTLIK EVQEMGGMAK AIETGLPKLR
E.c     NVFDEALGLP TDFSARIARN TQIIIQEESE LCRTVDPLAG SYYIESLTDQ IVKQARAIIQ QIDEAGGMAK AIEAGLPKRM
M.e     PAWNEALGLP RPWDQQWSMR MQQILAFETD LLEYDDIFDG STVIEARVEA LKEQTRAELT RIAEIGGAVT AVEAGELKRA
            *** **                          *   * *   *                           *   *   *

        481                                                                 560
Human   IEECAARRQA RIDSGSEVIV GVNKYQLEKE DAV.....EV LAIDNTSVRN RQIEKLKKIK SSRDQALAEH CLAALTECAA
Mouse   IEECAARRQA RIDSGSEVIV GVNKYHLEKE DSV.....HL LAIDIISLRK KQIEKLKKIK SSRDQALAEQ CLSALTQCAA
S.c     VEEAAARTQA RIDSGRQPVI GVNKYRVDTD EQI.....DV LKVDNSSVRA QQIEKLRRLR EERDDAACQD ALRALTAAA.
A.m     IEEAAARTQA RIDSGRQPVI GVNKYQVTDD EEI.....DV LKVDNAGVRA QQLEKLRRLR SERDSAATED ALRRLTEGA.
M.t     IEEAAARTQA RIDSGQQPVV GVNKYQVPED HEI.....EV LKVENSRVRA EQLAKLQRLR AGRDEPAVRA ALAELTRAAA
P.s     IEEAAARTQA RIDSGRQPLI GVNKYRLEHE PPL.....DV LKVDNSTVLA EQKAKLVKLR AERDPEKVKA ALDKITWAA.
P.g     IEEAAARTQA RIDSHQQVIV GVNKYRLPKE DPI.....DI LEIDNTAVRK QQIERLNDLR SHRDEKAVQE ALEAITKCV.
E.c     IEEASAREQS LIDQGKRVIV GVNKYKLDHE DET.....DV LEIDNVMVRN EQIASLERIR ATRDDAAVTA ALNALTHAA.
M.e     LVESNARRIS AIEKGEQIVV GVNKWQQGEP SPLTAGDGAI FTVSET.VEM EAETRIREWR SKRDERAVGQ ALADLEQAA.
          *   *     *         ****                                    **          *

        561                                                                 640
Human   ........SG DGNILALAVD ASRARCTVGE ITDALKKVFG EHKANDRMVS GA....YRQE FGESKEITSA IKRVHKFMER
Mouse   ........SG DGNILALAVD AARARCTVPE ITDAFKKVFG EHKANDRMVS GA....YRQE FGESKEITSA IKRVNKFMER
S.c     ..ERGPGQGL EGNLLALAVD AARAKATVGE ISDALESVYG RHAGQIRTIS GV....YRTE AGQSPSVERT RALVDAFDEA
A.m     ..GNG..... .GNLLELAID AARAKATVGE ISDALEKIWG RHSGQIRTIS GV....YREE VGKTQNVEKA RQLVDEFAAE
M.t     EQGRAGADGL GNNLLALAID AARAQATVGE ISEALEKVYG RHRAEIRTIS GV....YRDE VGKAPNIAAA TELVEKFAEA
P.s     ..GNPDDKDP DRNLLKLCID AGRAMATVGE MSDALEKVFG RYTAQIRTIS GV....YSKE VKNTPEVEEA RELVEEFEQA
P.g     .......ETK EGNLLDLAVK AAGLRASLGE ISDACEKVVG RYKAVIRTIS GV....YSSE SGEDKDFAHA KELAEKFAKK
E.c     ........QH NENLLAAAVN AARVRATLGE ISDALEVAFD RYLVPSQCVT GVIAQSYHQS EKSASEFDAI VAQTEQFLAD
M.e     ........RS GANIMPPSIA AAKAGVTTGE WGQRLREVFG EYRAPTGVT. ........LQ TVTSGAAEDA RLLIADLGER
                    *         *         *

        641                                                                 720
Human   EGRRPRLLVA KMGQDGHDRG AKVIATGFAD LGFDVDIGPL FQTPREVAQQ AVDADVHAVG VSTLAAGHKT LVPELIKELN
Mouse   EGRRLGLLVA KMGKDGHDRG AKVIATGFAD LGFDVDIGPL FQTPREVAHD AVDADVHAVG VSTHAAGHKT LVPELIKELT
S.c     EGRRPRILVA KMGQDGHDRG QKVIASAFAD LGFDVDVGPL FQTPAEVARQ AVEADVHIVG VSSLAAGHLT LVPALREELA
A.m     EGRRPRILVA KMGQDGHDRG QKVIATGFAD LGFDVDVGPL FSTPAEVARQ AIEADVHVVG VSSLAAGHLS LVPALRHELA
M.t     DGRRPRILIA KMGQDGHDRG QKVIATAFAD IGFDVDVGSL FSTPEEVARQ AADNDVHVIG VSSLAAGHLT LVPALRDALA
P.s     EGRRPRILLA KMGQDGHDRG QKVIATAYAD LGFDVDVGPL FQTPEETARQ AVEADVHVVG VSSLAGGHLT LVPALRKELD
P.g     EGRQPRIMIA KMGQDGHDRG AKVVATGYAD CGFDVDMGPL FQTPEEAARQ AVENDVHVMG VSSLAAGHKT LIPQVIAELE
E.c     NGRRPRILIA KMGQDGHDRG AKVIASAYSD LGFDVDLSPM FSTPEEIARL AVENDVHVVG ASSLAAGHKT LIPELVEALK
M.e     LGETPRLVVG KPGLDGHSNG AEQIALRARD VGFDVTYDGI RQTPTEIVAK AKERGAHVIG LSVLSGSHVP LVREVKAKLR
                  *** **** *    **    *  ****        ** *     *     *  *  *     *   *       *
                      + +  +                                                 + +

        721                                                                 800
Human   SLGRPDILVM CGGVIPPQDY EFLFEVGVSN VFGP.GTRIP KAAVQVLDDI EKCLEKKQQS V..... .....
Mouse   ALGRPDILVM CGGVIPPQDY EFLYEVGVSN VFGP.GTRIP RAAVQVLDDI EKCLAEKQQS V..... .....
S.c     AEGRDDIMIV VGGVIPPQDV EALHEAGATA VFPP.GTVIP DAAHDLVKRL AADLGHEL.. ..... .....
A.m     ELGREDIMVV VGGVIPPQDY PALREAGAAA IFGP.GTVLA DAAIDLLGQL SAQES .... ..... .....
M.t     QVGRPDIMIV VGGVIPPGDF DELYAAGATA IFPP.GTVIA DAAIDLLHRL AERLGYTLD. ..... .....
P.s     KLGRPDILIT VGGVIPEQDF DELRKDGAVE IYTP.GTVIP ESAISLVKKL RASLDA.... ..... .....
P.g     KLGRPDILVT AGGVIPAQDY DFLYQAGVAA IFGP.GTPVA YSAAKVLEIL LEE..... ..... .....
E.c     KWGREDICVV AGGVIPPQDY AFLQERGVAA IYGP.GTPML DSVRDVLNLI SQHHD..... ..... .....
M.e     EAGLDHVPVV VGGIISTEDE LVLKNMGVTA VYTPKDYELD KIMVGLAKVV ERALDKRAAD RADTEAGVPG APKRNESGAQVF
          *         ** *   *        *
                    ++                    +
```

Fig. 2. *(continued)*.

89.2 (82.0%), followed by 84.4 (75.7%) to its counterpart in *Mycobacterium tuberculosis*, 83.3 (74.6%) to that of *P. shermanii*, 79.3 (65.5%) to that of *P. gingivalis*, 77.7 (64.1%) to that of human, 76.9 (61.6%) to that of mouse, 76.5 (60.2%) to *sbm* in *E. coli*, 73 (59%) to that of *S. meliloti*, 58.1 (37.6%) to MCM-like gene *mea*A of *M. extorquens*, and 53 (37%) to *mea*A in *S. collinus*. Such a high similarity between α-subunits from both bacterial and eukaryotic sources suggests that MCM has been rather conserved during evolution.

Identification of Putative B_{12} and Substrate Binding Domains

The alignment of large subunit peptide sequences of some MCMs, *E. coli sbm*, and *M. extorquens mea*A gene product was utilized to identify the possible B_{12} coenzyme and substrate-binding sites (Fig. 2). High identity of amino acid sequences was particularly prevalent in two regions, amino acids 109–410, and amino acids 651–754. In earlier research with methionine synthase, Drennan et al. *(25)* indicated that sequences $DxH^{759}xxxG$, SxL^{806}, and $G^{833}G^{834}$ of *P. shermanii* are diagnostic for cobalamin binding, which corresponds to the second conserved region. The lack of this motif in the small subunit is consistent with only 1 mol of B_{12} coenzyme binding per heterodimer.

The recently published MCM crystal structure of *P. shermanii (26)* showed a $(\beta/\alpha)_8$ or TIM-barrel structure at the substrate–enzyme binding region. In the structure, the center of the barrel is typical filled with large, often branched, hydrophobic side chains. In the large subunit of *P. shermanii* MCM, the barrel is lined by Thr^{85}, Ser^{114}, Ser^{162}, Ser^{164}, Thr^{166}, Thr^{195}, Ser^{239}, Ser^{285}, and Ser^{362}, creating a hole through which the CoA can thread, and providing hydrogen bonds to the amide groups of CoA. His^{244} is in a position to bind to the carbonyl oxygen in the rearrangement reaction and is likely to be the major catalytic residue. Arg^{207} and Tyr^{89} in the small subunit of mutase are also involved in substrate binding. All of these amino acids are conserved in the first conserved region (Fig. 2). It seems that this region is involved in the formation of suitable catalytic architecture of MCM. Because a very high similarity was seen throughout the whole sequence, no very clear motif for substrate binding could be identified.

Roy *(27)* found that Cys^{535} in the large subunit of *P. shermanii* MCM is important for the enzymatic activity, but for MCMs of other sources, this position is replaced by alanine. Although it is conserved in all the amino acid residues thought to be important for B_{12}-dependent enzymes, *mea*A from *M. extorquens* presents a huge difference between other MCM and MCM-like proteins, suggesting that this enzyme may have a function other than as MCM.

Sequence Analysis of Downstream Region of mut*AB*

ORF5, immediately downstream of *mut*AB, starts at nt 4175 and ends at nt 4104. No typical streptomycete RBS was found before the translational start codon and no putative *E. coli* σ^{70}-like promoter elements were found upstream of ORF5 *(24)*. A search of the protein sequence database failed to correlate the gene with any specific function. The gene does have a very high similarity to the incomplete ORF3 of *S. cinnamonensis*, which is also located immediately downstream *mut*AB *(10)*. An ~0.9-kbp downstream ORF of *M. tuberculosis mut*AB, a ~0.8-kbp ORF2 lying downstream of *E. coli sbm*, and a functionally unknown ~0.9-kbp ORF from *Caulobacter crescentus (28)* (Fig. 3) also showed significant similarity to ORF5. The striking cross-species conservation of gene order (*mut*AB plus ~0.9-kbp ORF) and protein

```
     1                                                                    70
S.c  .......... .......... .....MARAI TLVESTRPQH RALAQELLTE LLPHSGRARR VGISGVPGVG
M.t  MMAASHDDDT VDGLATAVRG GDRAALPRAI TLVESTRPDH REQAQQLLLR LLPDSGNAHR VGITGVPGVG
A.m  .......... .......... .VTADAVEAI TLVESQREDH RALAQELLVE LLPAAGGARR VGITGVPGVG
C.c  ....MIPALD IDSLEHRLVA GDRAALARAI TLVESRRADH QVAARTLLSR LMPLTGRAQR IGITGVPGAG
E.c  .......... .......... .........M TLVESRHPRH QALSTQLLDA IMPYCGNTLR LGVTGTPGAG
                                      *****    *       **     *  *   *  *  * ** *

     71                                                                  140
S.c  KSTFIDALGV MLTSLGHRVA VLAVDPSSTR TGGSILGDKT RMERLSLDP. .......... ..........
M.t  KSTAIEALGM HLIERGHRVA VLAVDPSSTR TGGSILGDKT RMARLAVHPN AYIRPSPTSG TLGGVTRATR
A.m  KSTSSTSWHR PDRGR.HRVA VLAVDPSSTR TGGSILGDKT RMARLAWTNA RSIRPSPTSG TLGGVARATR
C.c  KSTTIERFGC KLVEAGHRVA VLAVDPSSGR HGGSILGDKT RMEQLSVQAN AFIRPSPSGG ALGGVARKTR
E.c  KSTFLEAFGM LLIREGLKVA VIAVDPSSPV TGGSILGDKT RMNDLARAEA AFIRPVPSSG HLGGASQRAR
     ***               ** * ******    ********* **  *        *** *  *  ***     *

     141                                                                 210
S.c  .......... .......... .......... .......... .......... .......... ..........
M.t  ETVVLLEAAG FDVILIETVG VGQSEVAVAN MVDTFVLLTL ARTGDQLQGI KKGVLELADI VVVNKADGEH
A.m  ETIVLMEAAG YDIVLVETVG VGQSEVTVAN MVDCFLFLTL ARTGDQLQGI KKGVLELADV IAVNKADGDH
C.c  EAMLLCEAAG FDVVIIETVG VGQSETVVAD MVDIFLALLI PGGGDELQGI KKGLIELADL LVINKADADP
E.c  ELMLLCEAAG YDVVIVETVG VGQSETEVAR MVDCFISLQI AGGGDDLQGI KKGLMEVADL IVINKDDGDN
     *   * ****  *    **** *****  **  *** *  *      ** **** ****** **     ** *

     211                                                                 280
S.c  .......... .......... .......... .......... .......... .......... ..........
M.t  HKEARLAARE LSAAIRLIYP REALWRPPVL TMSAVEGRGL AELWDTVERH RQVLTGAGEF DARRRDQQVD
A.m  ERDARRAARE LAGALRMIYG PEASWTPPVL TCSGLHNLRL DEVWGAIEQH RDTLTASGEL DARRRQQQVD
C.c  AKAER.SARD YRNALHILTP AHPDWTPPVL TASGLTGQGL DVLWTQILRH REVMTANGAR AARRADQDAR
E.c  HTNVAIARHM YESALHILRR KYDEWQPRVL TCSALEKRGI DEIWHAIIDF KTALTASGRL QQVRQQQSVE
                 *           * * ** *             *           *  *      *  *

     281                                                            334
S.c  .......... .......... .......... .......... .......... ....
M.t  WTWQLVRDAV LDRVWSNPTV RKVRSELERR VRAGELTPAL AAQQILEIAN LTDR
A.m  WTWAMVREQL LGRLAAHPEV RTVVPDVERA VRDGELTATL GAQRILDAFG G ..
C.c  WMWAMVRDRL EDAFKTAPAV AALVPDLETA VREGEVPASA AADRLLAAFG L...
E.c  WLRKQTRRRS TESPVRE... .......... .......... .......... ....
      *     *          * *       *   ** **          *
```

Fig. 3. Multiple alignment of *A. mediterranei* U32 ORF5 and other similar proteins. The alignment was generated using GCG Pileup. The positions conserved in all proteins are marked by with an asterisk. S.c, *S. cinnamonensis* (L10064); E.c, *E. coli sbm* (X66836); A.m, *A. mediterranei* U32 (AF117980); M.t, *M. tuberculosis* (Z79701); C.c, *C. crescentus* (M91449).

sequence may reflect a common biochemical function for gene products, perhaps in the metabolism of methylmalonyl-CoA in the aforementioned microorganisms (Fig. 3). No stem-loop structure, corresponding to a transcriptional termination, was found in the small intergenic region between *mut*AB and ORF5. Further experiments are under way in our group to determine whether *mut*AB and ORF5 are organized in one operon. A search of the protein database failed to reveal any sequences with similarity to the incomplete ORF6.

Overexpression in E. coli *and Gene Product Purification*

*mut*AB containing fragment was cloned into pBluescript and transformed into *E. coli* DH5α to detect activity *(18)*. In this case, the recombinant *E. coli* DH5α (*mut*AB) cell extract gave a very low MCM specific activity of

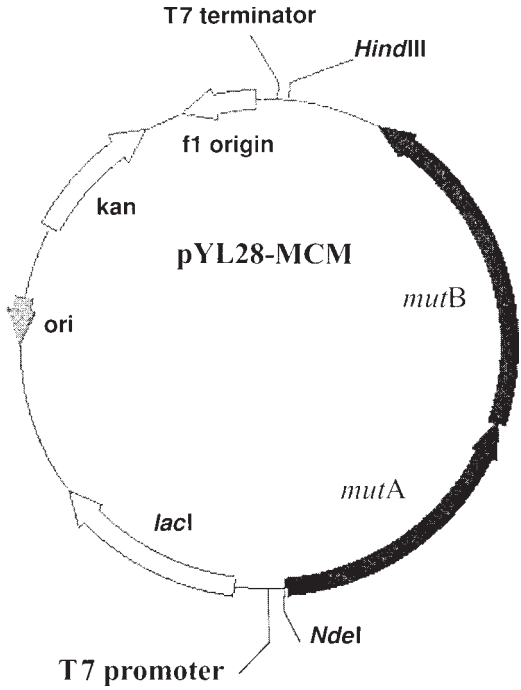

Fig. 4. Physical map of the expression plasmid pYL28-MCM. The *Sac*I/*Pst*I fragment containing *mut*AB was cloned into the *Sma*I/*Pst*I sites of pALTER-1 (Promega), an *Nde*I site was introduced before the *mut*AB ORF by site-directed mutagenesis, and the *Nde*I/*Hind*III fragment from pALTER-1 recombinant plasmid was then cloned into *Nde*I/*Hind*III sites of pET28b(+) (Novagen), resulting in pYL28-MCM for expression.

0.012–0.030 U/mg, which is similar to the activity level in cell extracts of *A. mediterranei* U32 *(15)*, and no obvious extra protein band could be detected in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Omission of B_{12} coenzyme from the assay system reduced expressed MCM activity to zero, indicating that the MCM being produced in *E. coli* was an apoenzyme.

Better overexpression was achieved by using pET28b(+) expression vector (Novagen, Madison, WI). To provide the convenience to the cloning, site-directed mutagenesis was performed to introduce an *Nde*I site before the ORF of *mut*A. Then an ~4.9-kbp *Nde*I-*Hind*III fragment was cloned into pET28b(+), and the resulting expression plasmid, pYL28-MCM, was transformed into *E. coli* BL21(DE3) for expression (Fig. 4). A 0.1-mL overnight culture of *E. coli* BL21(DH3) (pYL28-MCM) was inoculated into 200 mL of LB medium (with 12 μg/mL of kanamycin) and incubated at 37°C for 2 to 3 h (when OD_{600} value reached 0.4). The cultures were transferred to a 25°C incubator for a further 1–5 h after IPTG was added to a final concentration of 0.4 m*M*. Overexpression was confirmed by SDS-PAGE, in which two clear extra bands of ~75 and ~65 kDa were found (Fig. 5A). Analysis by scanning densitometry of the SDS-PAGE of

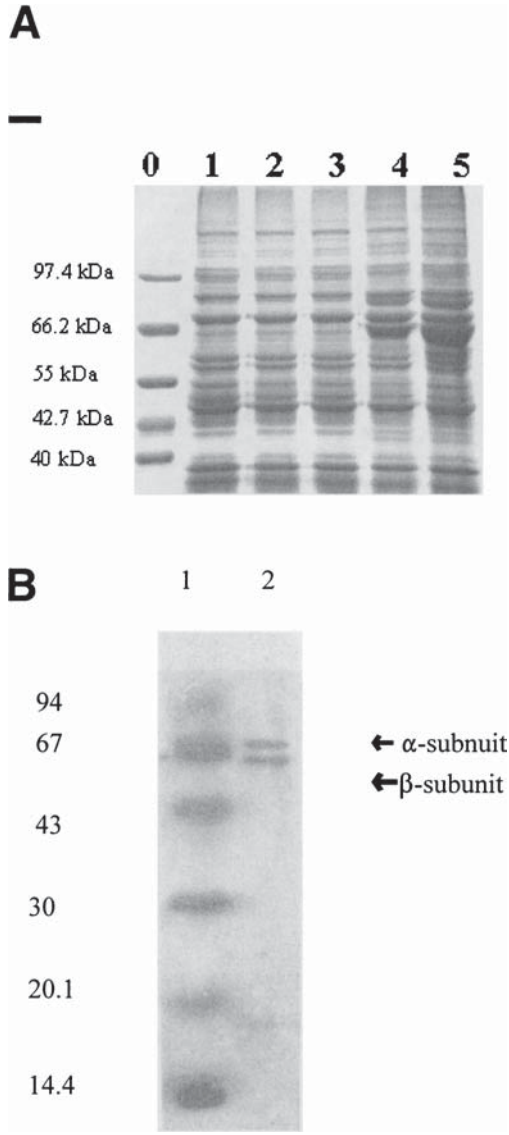

Fig. 5. **(A)** SDS-PAGE of the overexpression of MCM in *E. coli* BL21(DE3) (pYL28-MCM). Lane 0, molecular mass standard (kDa); lane 1, *E. coli* BL21(DE3) with pET28(+), not induced by IPTG; lanes 2 and 3, *E. coli* BL21(DE3) with pET28b(+), induced by IPTG for 3 h; lane 4, *E. coli* BL21(DE3) with pYL28-MCM, induced by IPTG for 3 h; lane 5, *E. coli* BL21(DE3) with pYL28-MCM, induced by IPTG for 5 h. **(B)** SDS-PAGE of purified MCM. Lane 1, molecular mass standard; lane 2, purified MCM.

the crude extract indicated that insoluble mutase constituted approx 30% of the total soluble protein (Fig. 5A).

The purification procedure developed previously (*see* ref. *[15]*) was used to purify the MCM from *E. coli* BL21(DE3) (pYL28-MCM). The purification centered on an affinity column of coenzyme B_{12} Sepharose (Fig. 5B).

SDS-PAGE of the purified MCM separated the native enzyme into two bands corresponding to a mol wt of ~75 and ~65 kDa (Fig. 5B), which is the same pattern as the MCM purified directly from *A. mediterranei* U32 *(14)*.

Kinetic Analysis of Purified MCM from E. coli

The effects of the concentration of succinyl-CoA and coenzyme B_{12} on the activity of MCM were studied with enzyme purified from *E. coli*. The reactions were time and concentration dependent. The reciprocal plots showed normal Michaelis-Menten kinetics with the apparent K_m of 14.2 μ*M* for succinyl-CoA and 0.5504 μ*M* for B_{12} coenzyme. These values were similar to those of enzyme directly purified from *A. mediterranei* U32 (9.72 μ*M* for succinyl-CoA and 0.1277 μ*M* for coenzyme B_{12}) *(14)*.

Overexpression of mut*AB* *in* S. cinnamonensis *and Monensin Production*

We have conducted many experiments to transform rifamycin SV–producing strain *A. mediterranei* U32 in our laboratory, but until now no success has been achieved with this industrially used strain. The lack of a suitable gene expression system impedes our further attempt to study the *mut*AB function in *A. mediterranei* U32. Alternatively, the *mut*AB gene was overexpressed in polyketide monensin producing *S. cinnamonensis*. The *Nde*I/*Hind*III fragment containing complete *mut*AB genes was cloned into pIJ4123 expression plasmid. The resulting construct, pZW22, was transformed into *S. cinnamonensis*. Transformant was checked for monensin production and MCM activity (Table 1). In the complex fermentation medium, the *S. cinnamonensis* (*mut*AB) strain gave a 4.2-fold greater increase in MCM activity, and a 32% greater increase in overall production of monensin than the control strains grown under the same conditions. The smaller increase in monensin production compared to the change in MCM activity might have resulted for the following reasons. First, MCM catalyzes the transformation of succinyl-CoA to *R*-methylmalonyl-CoA, another enzyme. Methylmalonyl-CoA racemase is necessary to transform *R*-methylmalonyl-CoA to *S*-methylmalonyl-CoA, which is the substrate with the correct conformation recognized by polyketide synthase. Our earlier biochemical and physiological studies showed that methylmalony-CoA racemase activity is at the same level as MCM in *A. mediterranei* U32 *(15)*. It is speculative that methylmalonyl-CoA racemase becomes the limiting step for the *S*-methylmalonyl-CoA supply in the *mut*AB overexpressed strain. Second, monensin polyketide synthase can utilize either methylmalonyl-CoA or ethylmalonyl-CoA as its starter units, which results in the production of a mixture of monensin A and B; monensin A contains a butyryl unit in its polyketide chain. In this case, the availability of ethylmalonyl-CoA could also be a limiting factor for monensin A production. To prove this prediction, we isolated and checked the individual monensin A and B production. The results in Fig. 6 show that overexpressed

Table 1
MCM Activity and Total Monensin Production
in Wild-Type and *mut*AB Overexpression *S. cinnamonensis* Strains[a]

	Wild type	Wild type (with pIJ4123)	Wild type (with pZW22)
MCM activity (U/mg protein)	0.045	0.047	0.2340
Monensin AB production (mg/500 mL medium)	83	79	109

[a]Data are the average of four individual experiments.

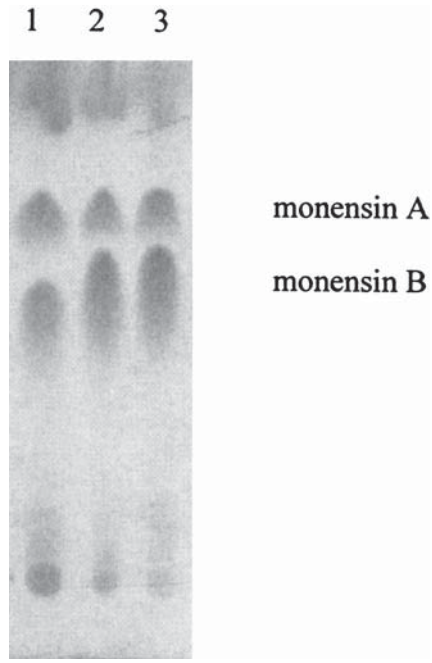

Fig. 6. TLC of monensin A and B. The mobile phase was composed of ethylacetate:methylene chloride (3:1), and the monensin was detected by staining with vanillin. Lane 1, monensin A and B production from *S. cinnamonensis* strain; lanes 2 and 3, monensin A and B production from *mut*AB overexpression of *S. cinnamonensis*.

*mut*AB resulted in an increased production of mainly monensin B, whereas little change can be seen in the production of monensin A.

Conclusion

We have reported herein the complete sequence of methylmalonyl-CoA gene from a rifamycin SV–producing strain, *A. mediterranei* U32. The gene was expressed in *E. coli* and the active recombinant protein was purified. The deduced amino acid sequence showed very high homology to other MCM from both prokaryotic and eukaryotic sources. The *mut*AB

gene was overexpressed in monensin producing *S. cinnamonensis*, which resulted in an increase in total production of monensin by 32%. This is the first report of *mut*AB gene being used to increase the production of antibiotics. The results showed that manipulation of the *mut*AB enzymatic level through a genetic approach could be a useful method to increase the production of polyketide antibiotics. In the future, the *mut*AB gene will be expressed in *A. mediterranei* U32, and its effects on rifamycin SV production will be investigated in detail.

Acknowledgment

We are grateful to Professor J. A. Robinson of the University of Zurich for providing *S. cinnamonensis* methylmalonyl-CoA mutase gene as cloning probe and to Dr. Fiona Stuart for critical reading of the manuscript. This work was supported by a grant from the National Natural Science Foundation of P.R. China grant no. 39630010.

References

1. White, R. J., Martinelli, E., Gallo, G. G., and Lancini, G. (1973), *Nature (London)* **243,** 273–277.
2. Donadio, S., Stever, M. J., McAlpine, J. B., Swanson, S. J., and Katz, L. J. (1991), *Science* **252,** 675–679.
3. Robinson, J. A. (1992), *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **332,** 107–114.
4. Omura, S., Takeshima, H., Nakagawa, A., Miyazawa, J., Piriou, F., and Lukacs, G. (1977), *Biochemistry* **16,** 2860–2866.
5. March, E. N., McKie, N., Davis, N. K., and Leadlay, P. F. (1990), *Biochem. J.* **260,** 345–352.
6. Jansen, R., Kalousek, F., Fenton, W. A., Rosenberg, L. E., and Ledley, F. (1989), *Genomics* **4,** 198–205.
7. Wilkemeyer, M. F., Crane, A. M., and Leadley, F. D. (1990), *Biochem. J.* **271,** 449–455.
8. Charles, T. C. and Aneja, P. (1999), *Gene* **226,** 121–127.
9. Jackson, C. A., Kirszbaum, L., Dashper, S., and Reynolds, E. C. (1995), *Gene* **167,** 127–132.
10. Birch, A., Leiser, A., and Robinson, J. A. (1993), *J. Bacteriol.* **175,** 3511–3519.
11. Roy, I. and Leadley, P. F. (1992), *J. Bacteriol.* **174,** 5763, 5764.
12. Smith, L. M., Meijer, W. G., Dijkhuizen, L., and Goodwin, P. M. (1996), *Microbiology* **142,** 675–684.
13. Han, L. and Reynolds, K. A. (1997), *J. Bacteriol.* **179,** 5157–5164.
14. Zhang, W. and Chiao, J. S. (1996), *Acta Microbiologica Sinica* **36,** 199–206.
15. Zhang, W. and Chiao, J. S. (1996), *Acta Microbiologica Sinica* **36,** 276–282.
16. Zhang, W. and Chiao, J. S. (1996), *Chin. J. Biochem.* **22,** 167–172.
17. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989), *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring, NY.
18. Kellermeyer, R. W. and Wood, H. G. (1969), *Methods Enzymol.* **13,** 207–214.
19. Simon, E. J. and Shemin, D. (1953), *J. Am. Chem. Soc.* **75,** 2520.
20. Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M., and Schrempf, H. (1985), *Genetic Manipulation of Streptomyces: A Laboratory Manual*, John Inns Foundation, Norwich, England.
21. Reynolds, K. A., O'Hagan, D., Gani, D., and Robinson, J. A., (1988), *Chem. Soc. Perkin. Trans.* **1,** 3194–3207.
22. Beran, M. and Zima, J. (1993), *Chromatographia* **35,** 206–208.
23. Bibb, M. J. and Cohen, S. N. (1982), *Mol. Gen. Genetics* **187,** 265–277.

24. Strohl, W. R. (1992), *Nucleic Acids Res.* **20,** 961–974.
25. Drennan, C. L., Matthews, R. G., and Ludwig, M. L. (1994), *Curr. Opin. Struct. Biol.* **4,** 919–929.
26. Mancia, F., Keep, N. H., Nakagawa, A., Leadley, P. F., McSweeney, S., Rasmussen, B., Bosecke, P., Diat, O., and Even, P. R. (1996), *Structure* **4,** 339–350.
27. Roy, I. (1996), *FEMS Lett.* **394,** 126–128.
28. Wang, S. P., Sharma, P. L., Schoenlein, P. V., and Ely, B. (1993), *Proc. Natl. Acad. Sci. USA* **90,** 630–634.