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MeaA, a Putative Coenzyme B_{12}-Dependent Mutase, Provides Methylmalonyl Coenzyme A for Monensin Biosynthesis in *Streptomyces cinnamonensis*

WEIWEN ZHANG AND KEVIN A. REYNOLDS*

Institute for Structural Biology and Drug Discovery and Department of Medicinal Chemistry, Virginia Commonwealth University, Richmond, Virginia 23219

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The ratio of the major monensin analogs produced by *Streptomyces cinnamonensis* is dependent upon the relative levels of the biosynthetic precursors methylmalonyl-coenzyme A (CoA) (monensin A and monensin B) and ethylmalonyl-CoA (monensin A). The *meaA* gene of this organism was cloned and sequenced and was shown to encode a putative 74-kDa protein with significant amino acid sequence identity to methylmalonyl-CoA mutase (MCM) (40%) and isobutyryl-CoA mutase (ICM) large subunit (36%) and small subunit (52%) from the same organism. The predicted C terminus of MeaA contains structural features highly conserved in all coenzyme B_{12}-dependent mutases. Plasmid-based expression of *meaA* from the *ermE promoter in the *S. cinnamonensis* C730.1 strain resulted in a decreased ratio of monensin A to monensin B, from 1:1 to 1:3. Conversely, this ratio increased to 4:1 in a *meaA* mutant, *S. cinnamonensis* WM2 (generated from the C730.1 strain by insertional inactivation of *meaA* by using the erythromycin resistance gene). In both of these experiments, the overall monensin titers were not significantly affected. Monensin titers, however, did decrease over 90% in an *S. cinnamonensis* WD2 strain (an *icm meaA* mutant). Monensin titers in the WD2 strain were restored to at least wild-type levels by plasmid-based expression of the *meaA* gene or the *Amycolatopsis mediterranei mutAB* genes (encoding MCM). In contrast, growth of the WD2 strain in the presence of 0.8 M valine led only to a partial restoration (<25%) of monensin titers. These results demonstrate that the *meaA* gene product is significantly involved in methylmalonyl-CoA production in *S. cinnamonensis* and that under the tested conditions the presence of both MeaA and ICM is crucial for monensin production in the WD2 strain. These results also indicate that valine degradation, implicated in providing methylmalonyl-CoA precursors for many polyketide biosynthetic processes, does not do so to a significant degree for monensin biosynthesis in the WD2 mutant.**

Streptomycetes produce a large number of structurally diverse polyketide antibiotics by a process similar to long-chain fatty acid biosynthesis (20). Polyketide biosynthesis, catalyzed by polyketide synthases, uses carboxylated acyl thioesters, such as malonyl-coenzyme A (CoA), methylmalonyl-CoA, or ethylmalonyl-CoA, as extender units. These precursors form the polyketide carbon backbone and side chains, as seen in the examples of rifamycin (2), erythromycin (11), and monensin (6). Malonyl-CoA and ethylmalonyl-CoA are likely derived from the carboxylation of acetyl-CoA and butyryl-CoA, respectively (16, 35), while methylmalonyl-CoA can be produced from a variety of different pathways (6). As several methylmalonyl-CoA molecules are required to build a single polyketide (six for erythromycin, seven for monensin A, and eight for rifamycin), the levels of methylmalonyl-CoA under certain conditions may represent a limiting factor in production titers. For monensin biosynthesis, either ethylmalonyl-CoA or methylmalonyl-CoA can be used at the same stage of elongation to generate monensin A or monensin B, respectively (14, 24). Thus, in this organism, changes in the levels of methylmalonyl-CoA affect not only the titers but also the ratio of monensin A to monensin B. This feature makes *Streptomyces cinnamonensis* an excellent organism for probing pathways that contribute in both minor and major ways to generating methylmalonyl-CoA for polyketide biosynthesis.

Three generally accepted routes to methylmalonyl-CoA are (i) the isomerization of succinyl-CoA, catalyzed by the coenzyme B_{12}-dependent methylmalonyl-CoA mutase (MCM) (28, 43); (ii) carboxylation of propionyl-CoA, catalyzed by either propionyl-CoA carboxylase (PCC) (7, 35) or methylmalonyl-CoA transcarboxylase (MMT) (22); and (iii) a multistep oxidation of isobutyryl-CoA (34) (Fig. 1). The *mutAB* genes encoding MCM involved in the first of these pathways have been cloned from monensin A-producing *S. cinnamonensis* (6), as well as from rifamycin SV-producing *Amycolatopsis mediterranei* U32 (49) and other prokaryotic and mammalian sources (27, 47). A *mutAB* disruption has no effect on the monensins A and B total production and ratio in *S. cinnamonensis* cells (44). Plasmid-based overexpression of the *mutAB* genes in *S. cinnamonensis*, on the other hand, has been reported to yield both a slight increase in total monensin production and a decreased ratio of monensin A to monensin B (49). These observations suggest that under these conditions, methylmalonyl-CoA is a limiting factor in monensin biosynthesis and that the natural levels of MCM activity generated from the *mutAB* genes do not contribute significantly to this process. Genes encoding either PCC or MMT, enzymes capable of generating methylmalonyl-CoA from propionyl-CoA, have not yet been cloned from *S. cinnamonensis*, and thus their role in providing this precursor through the second pathway has not been evaluated.

* Corresponding author. Mailing address: ISBDD, Suite 212B, 800 East Leigh St., Richmond, VA 23219. Phone: (804) 828-5679. Fax: (804) 827-3664. E-mail: kareynol@hsc.vcu.edu.

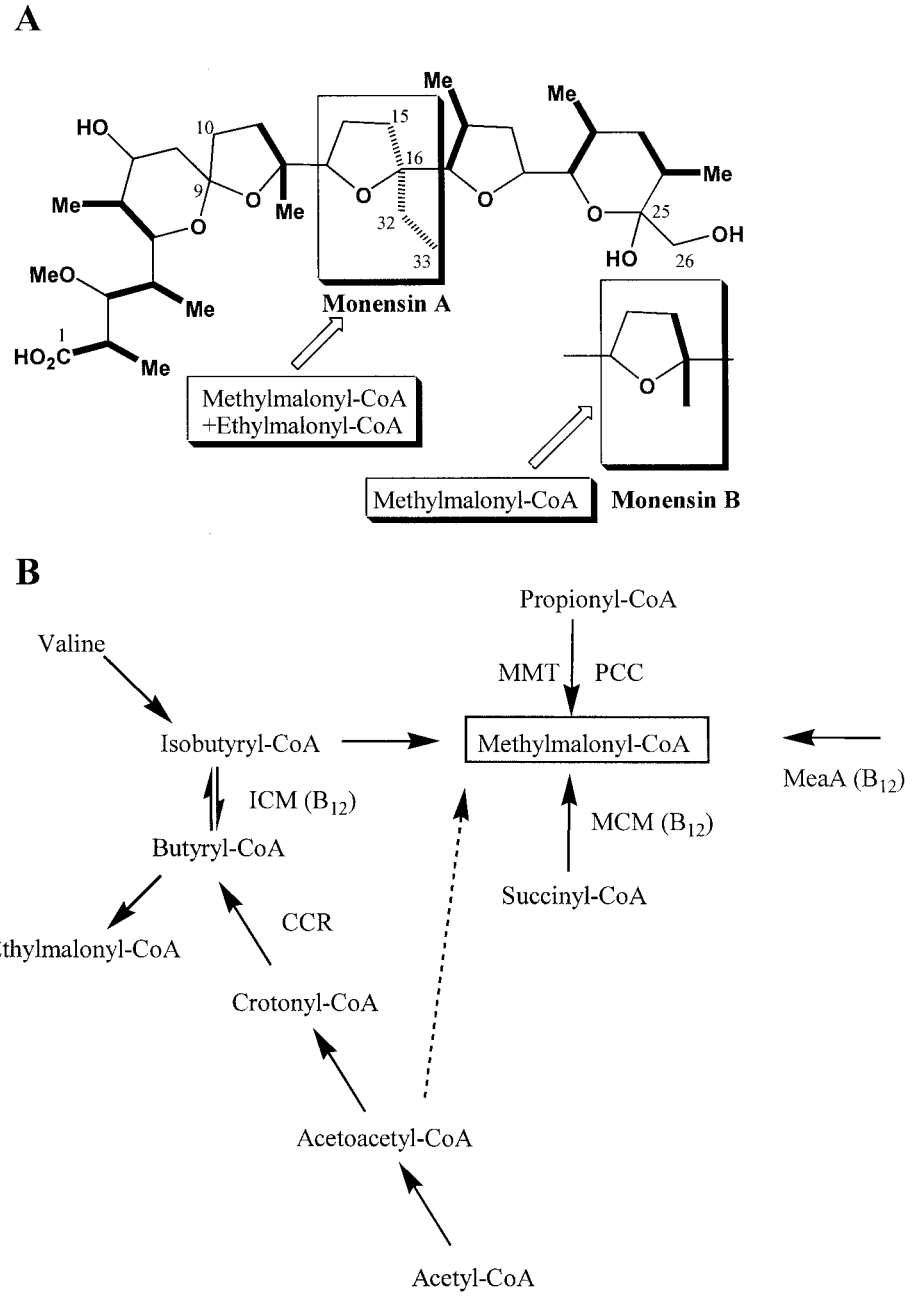

FIG. 1. (A) The structures of monensins A and B. Methylmalonyl-CoA positions in both monensins A and B are marked in bold, and the ethylmalonyl-CoA position in monensin A is hatched. (B) Proposed pathways for methylmalonyl-CoA formation in *S. cinnamonensis*. The dotted arrow indicates a pathway that does not require the *meaA, icm*, or *mutAB* genes cloned from this organism. The substrate for MeaA is unknown. Ethylmalonyl-CoA is used for monensin A biosynthesis, while methylmalonyl-CoA is used for both monensin A and B biosynthesis. B_{12} indicates known or putative coenzyme B_{12}-dependent mutase. MCM, methylmalonyl-CoA mutase; ICM, isobutyryl-CoA mutase; MMT, methylmalonyl-CoA transcarboxylase; PPC, propionyl-CoA carboxylase; CCR, crotonyl-CoA reductase.

A PCC encoded by the *pcc* gene has been cloned and characterized for *Streptomyces coelicolor* A3(2) (7, 35) and erythromycin-producing *Saccharopolyspora erythraea* (12). Disruption of the *pcc* gene in *S. erythraea* has no effect on erythromycin production (12). However, even in this organism, the role of carboxylation of propionyl-CoA in methylmalonyl-CoA formation is not clear, as there are probably an MMT and additional acyl-CoA (or propionyl-CoA) carboxylases (6, 7, 18, 35).

The third pathway from methylmalonyl-CoA, clearly established from numerous biosynthetic studies with monensin (34) and tylosin (30) and other polyketide antibiotic producing organisms, is oxidation of isobutyryl-CoA (Fig. 1). Isobutyryl-CoA can be formed either from valine catabolism or from butyryl-CoA by carbon skeleton rearrangement catalyzed by coenzyme B_{12}-dependent isobutyryl-CoA mutase (ICM) (34). The *icm* genes encoding *S. cinnamonensis* ICM were recently

TABLE 1. Strains, plasmids, and cosmids used in this study[a]

Strain or plasmid	Description	References or source
E. coli		
XL1-Blue	*supE44 recA1 hsdR17 endA1 gyrA46 thi relA1 lac* F′(*proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15* Tn*10* Tet^r)	8
DH5α	*supE44 DlacU169* (ϕ80*lacZ*Δ*M15*) *hsdR17 recAl endA1 gyrA96 thi-1 relA1*	17
ET12567	F$^-$ *dam-13*::Tn*9 dcm-6*	25
S. cinnamonensis		
C730.1	Monensin overproducer (wild-type strain for this study)	34
icm mutant	*icmA*::*hyg*	44
mcm mutant	*mutB*::*hyg*	44
WM2	*meaA*::*ermE*	This study
WD2	*icmA*::*hyg, meaA*::*ermE*	This study
L1	*ccr*::*hyg*	24
Major plasmids and cosmids		
pBluescript	Amp^r	1
SuperCos I	Amp^r	Stratagene
pKC1139	*Streptomyces-E. coli* shuttle vector, Am^r	5
pSE34	pWHM3 with *ermE** promoter, Thio^r	Pfizer Inc.
pIJ4026	pUC18 carrying *ermE*; Amp^r	Pfizer Inc.
pHL1	pUC119 with 5.7-kb *Pst*I insert containing *S. cinnamonensis ccr*, Amp^r	24
pME291	*meaA*-positive cosmid clone, Amp^r	This study
pME291K	pBluescript with 5.2-kb *Bgl*II/*Kpn*I insert containing complete *meaA*, Amp^r	This study
pZR8	pSE34 with complete *meaA*, Thio^r	This study
pZR22	pKC1139 with *Xba*I/*Hin*dIII 5.6-kb *meaA*::*ermE* fragment, Ery^r Am^r	This study
pZC32	pSE34 with MCM-encoding genes *mutAB* from *A. mediterranei*, Thio^r	49, 50

[a] Abbreviations: Amp^r, ampicillin resistant; Am^r, apramycin resistant; Ery^r, erythromycin resistant; Thio^r, thiostrepton resistant; *hyg*, hygromycin resistance gene; *ermE*, erythromycin resistance gene.

cloned and sequenced, and insertional inactivation of *icm* has been shown to have no detectable effect on monensin production (33, 44, 48). Thus, it appears that either ICM or MCM activities can be removed from *S. cinnamonensis* without significantly affecting the pools of methylmalonyl-CoA for monensin biosynthesis. Methylmalonyl-CoA in these strains might thus be obtained directly from the oxidation of valine-derived isobutyryl-CoA (44) or from the carboxylation of propionyl-CoA. A third possibility was raised by the unexpected observation that [1,3-$^{13}C_2$]acetoacetyl-CoA can be converted intact into [1,2-$^{13}C_2$]methylmalonyl-CoA in *S. cinnamonensis* in the absence of either ICM or MCM. There are no other known pathways beyond that involving ICM which would explain such an intact interconversion, and these data suggested the presence of another coenzyme B_{12}-dependent mutase and/or unidentified pathway(s) involved in methylmalonyl-CoA formation (44).

A novel *meaA* gene encoding an MCM-like protein with unknown function has been found in both *Streptomyces collinus* (15) and *Methylobacterium extorquens* AM1 (9, 37). The predicted MeaA amino acid sequence contains the distinctive coenzyme B_{12}-binding domain and exhibits high end-to-end homology to the large subunit of MCM and both subunits of ICM. The *meaA* gene has been shown to be involved in acetate assimilation in both of these organisms (9, 15, 37). In both *S. collinus* and *S. coelicolor*, the *meaA* gene was found to be 20 to 40 bp downstream of the crotonyl-CoA reductase (CCR)-encoding gene *ccr*, with the same transcriptional orientation. CCR plays a key role in the catalysis of the last reductive step in the biosynthesis of butyryl-CoA from acetyl-CoA in *S. collinus* (15) and a significant role in providing butyryl-CoA from monensin A biosynthesis in *S. cinnamonensis* (24). The genetic organization of *meaA* and *ccr* implies a possible role of *meaA* in the butyryl-CoA or methylmalonyl-CoA pathways. Our interest in the clarification of the role of various methylmalonyl-CoA formation pathways in polyketide-producing streptomycetes prompted us to further examine this novel mutase gene.

In this study, we report the cloning and sequencing of *meaA* from monensin-producing *S. cinnamonensis* cells. Gene disruption, gene overexpression, and labeling studies clearly demonstrate that MeaA is involved in methylmalonyl-CoA formation, but that this process does not involve an acetoacetyl-CoA–butyryl-CoA intermediate. Furthermore, the generation and analysis of an *meaA icm* mutant of *S. cinnamonensis* has clearly demonstrated that under the tested conditions, the expression of these two genes is crucial for providing the majority of this methylmalonyl-CoA used for monensins A and B production. Surprisingly, valine catabolism does not contribute significantly to providing methylmalonyl-CoA in this mutant.

MATERIALS AND METHODS

Chemicals. Chemicals were purchased from Fisher Scientific (Pittsburgh, Pa.). Ethyl [3,4-$^{13}C_2$]acetoacetate and perdeuterated valine were from Cambridge Isotope Laboratories Inc. (Andover, Md.). Monensin A standard was from Sigma Co. (St. Louis, Mo.). The *S. cinnamonensis* C730.1 strain was provided by Eli Lilly & Company.

Bacterial strains, plasmids, and cultural conditions. Table 1 contains a list of the strains and plasmids used in this work. *Escherichia coli* XL1-Blue, DH5α, and ET12567 were grown at 37°C in Luria-Bertani medium supplemented with either ampicillin (Amp; 100 μg/ml) or apramycin (50 μg/ml) when necessary (18). Cultures of *S. cinnamonensis* were grown in YEME medium (19) at 30°C for the isolation of genomic and plasmid DNAs, preparation of protoplasts, and fatty acid analysis. R_2YE medium (19) was used for the preparation of *Streptomyces* spore suspensions and for the regeneration of protoplasts after transformation. For the DL-perdeuterated valine feeding experiments, the following liquid medium was used: yeast extract (0.3%)–malt extract (0.5%)–peptone (0.3%)–glucose (1.0%), pH 7.2.

Molecular cloning. Isolation of *S. cinnamonensis* genomic DNA was performed according to standard methods (19). Genomic DNA was partially digested by *Sau*3A1 to yield a majority of fragments of around 30 to 40 kb. Sucrose gradient centrifugation was run at 36,000 rpm for 16 h at 4°C in a Sorvall TH-641

Rotor (Sorvall Ultracentrifuge; Du Pont, Wilmington, Del.). Fractions containing 30-to 40-kb fragments were collected and precipitated. Cosmid SuperCos I DNA was prepared according to the instruction manual (Stratagene, La Jolla, Calif.). Twenty microliters of a ligation mixture of genomic DNA (1 μg) and SuperCos I (0.2 μg) was packaged with the Gigapack III XL lambda extract kit (Stratagene) and then transfected into the *E. coli* XL1- Blue strain. The *E. coli* XL1-Blue cells competent for transfection were prepared in Luria-Bertani medium with 0.2% (wt/vol) maltose–10 mM $MgSO_4$. About 3,600 recombinant colonies were chosen as a working cosmid library of *S. cinnamonensis* and subsequently screened using a radiolabeled pHL1 probe that included the *ccr* gene and 0.5 kb of *meaA* gene (24). Radioactive DNA labeling was performed with a RTG DNA labeling beads (-dCTP) kit from Pharmacia (Piscataway, N.I.), with [α-^{32}P]dCTP (3,000 Ci/mmol) from Amersham Life Science (Arlington Heights, Ill.). Nonradioactive probing of cosmid subclones with pHL1 was accomplished using digoxigenin DNA labeling and detection kits (Boehringer, Mannheim, Germany). Southern and colony hybridizations were performed according to established methods (36) with a Porablot NY amp nylon membrane (Macherey-Nagel, Duren, Germany). Conditions for prehybridization and hybridization were slightly modified from standard protocols, with final washes for Southern hybridization and colony hybridization being carried out at 68°C in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Small-scale isolation of high-copy-number plasmid DNA from *E. coli* was accomplished by the TENS method (51). Large-scale *E. coli* plasmid preparation was carried out with the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, Wis.). Low-copy-number cosmid DNA was isolated with Nucleobond AX100 cartridges (Macherey-Nagel). Isolation of DNA fragments from agarose gels was carried out with QIAquick Gel Extraction Kit (Qiagen, Santa Clarita, Calif.). Restriction digestion, phenol extraction, ethanol and isopropanol precipitation, treatment of DNA with the Klenow fragment of DNA polymerase I and alkaline phosphatase, and T4 DNA ligation were accomplished by following standard protocols (36). Preparation and transformation of competent *E. coli* cells were performed by standard methods (36).

Nucleotide sequence analysis. Positive cosmids identified with pHL1 were selected, and subclones were prepared with the high-copy-number vector pBluescript SK. DNA sequencing was performed with an ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Foster City, Calif.) according to the conditions recommended by manufacturer. The sequence was analyzed for open reading frames (ORFs) by using CODONPREFERENCE in Genetics Computer Group or FramePlot 2.3* software available on the Internet (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl). Protein database searches were performed with BLAST and FASTA, protein comparisons were made with COMPARE, and protein sequence alignments were made with LINEUP and PILEUP (all from the University of Wisconsin Genetics Computer Group Package).

Protein overexpression. *Streptomyces* protoplasts were transformed in the presence of 25% polyethylene glycol 1000 (19). For overexpression of the *meaA* gene in *S. cinnamonensis*, plasmid pZR8 was constructed by PCR with two synthetic oligonucleotides as primers, using cosmid DNA pME291 as a template. Primer 1 (5′-ATACTCTAGAGGAACATGACAGAGC-3′) and primer 2 (5′-ATCTAAGCTTACGAAGGCGGTTGATGG-3′) contained *Xba*I and *Hin*dIII sites (underlined), respectively. The PCR product was purified directly from the gels (Qiagen), digested, and subcloned into pSE34 to produce pZR8. *S. cinnamonensis* protoplasts were then transformed with pZR8. The *mutAB* genes from *A. mediterranei* were released from pYL28 (49) and cloned into the *Xba*I/*Hin*dIII sites of pSE34, resulting in pZC32.

Insertional inactivation of *S. cinnamonensis meaA*. A shuttle vector pKC1139 containing the temperature-sensitive *Streptomyces* origin of replication from pSG5 was used to construct a *meaA* gene disruption plasmid (5, 25). A *Bam*HI site suitable for the insertion of the erythromycin resistance gene (*ermE*) was created within the *meaA* ORF by PCR. Two fragments corresponding to the first and second halves of the *meaA* gene were PCR amplified from the positive cosmid clone pME291 by using the following primers: primer 1 (described above), primer 2 (described above), primer 3, 5′-ATCTGGATCCAAGATGTCCTCGTAC-3′, and primer 4, 5′-ATCTGAATTCCGACGGCTCGCACGTCGT-3′. Primer 3 contained a *Bam*HI site, and primer 4 contained an *Eco*RI site (underlined). Primers 1 and 3 produced a 1.0-kb PCR fragment with *Xba*I and *Bam*HI cohesive ends, and primers 2 and 4 produced a 1.0-kb PCR fragment with *Eco*RI and *Hin*dIII cohesive ends. The two fragments were cloned into the corresponding sites of pBluescript, resulting in the formation of pZR6. A 1.6-kb *Bgl*II *ermE* gene fragment was excised from pIJ4026 and cloned into the *Bam*HI site of pZR6. The resulting pZR7 plasmid, with *ermE* transcribed in the same direction as *meaA*, was then isolated and confirmed by restriction analysis. A 3.6-kb *Xba*I/*Hin*dIII fragment containing the disrupted *meaA* gene and *ermE* insert was released from pZR7 and cloned into pKC1139 to yield pZR22, a *meaA* gene disruption plasmid. In order to increase transformation and homologous recombination efficiency, this plasmid was denatured by 1.0 N NaOH according to the method described by Oh et al. (29) before it was used to transform *S. cinnamonensis* protoplasts. The *meaA* mutant of *S. cinnamonensis* was then obtained following standard protocols (24) and confirmed by PCR.

Fatty acid analysis. *S. cinnamonensis* cultures were grown in YEME medium at 30°C for 72 h, and the fatty acids were extracted and analyzed as described previously (45). When required, perdeuterated valine (200 mM) was added to the media at the time of inoculation.

Production and quantitation of monensins A and B. Fermentations of *S. cinnamonensis* C730.1 cells and mutant derivatives were carried out in a two-stage fermentation process as described previously. The second-stage production medium was either a glucose-soybean meal (24) or an oil-based medium composed of soybean meal (1.5%), glucose (2.5%), soybean oil (1.5%), methyl oleate (2.0%), lard oil (1.0%), $CaCO_3$ (0.3%), $FeSO_4 \cdot 7H_2O$ (0.03%), KCl (0.01%), and $MnCl_2$ $4H_2O$ (0.003%). When required, valine (0.1 or 0.8 M) was added in three equal portions at 24, 60 and 72 h during fermentation. Monensins were isolated and quantitated by high-performance liquid chromatography (HPLC) analysis as described previously (34).

Isotope labeling experiments with ethyl [3,4-$^{13}C_2$] acetoacetate. The conditions for the production of monensins A and B used in the labeling experiments were identical to those described above. A 30 mM 1:3 mixture of ethyl [3,4-$^{13}C_2$]acetoacetate and unlabeled ethyl acetoacetate was added batchwise to the liquid cultures in three equal portions after 24, 28, and 72 h of growth. Monensin A was purified from organic extracts of fermentations as described previously (34) and analyzed by ^{13}C spectroscopy with proton decoupling.

Nucleotide sequence accession number. The complete sequence of *S. cinnamonensis meaA* reported here has been deposited in the GenBank database under the accession no. AF303662.

RESULTS

Cloning and sequence analysis of *S. cinnamonensis meaA*. The plasmid pHL1 (24), containing the *ccr* gene and 0.5 kb of *meaA* of *S. cinnamonensis*, was used as the DNA probe to screen a cosmid library. Eight positive cosmid clones with inserts of ~35 kb were obtained. One of the positive cosmid clones, designated pME291, was chosen for further study. Restriction mapping of pME291 showed that *meaA* was contained within a 5.2-kb *Bgl*II/*Kpn*I fragment. This fragment was cloned into pBluescript to generate pME291K. The complete nucleotide sequence of the *meaA* gene was determined. This ORF has a typical streptomycete codon bias (G+C content of 69.5%), extends from nucleotide 122 (ATG) to 2149 in the deposited sequence, and encodes a protein of 675 residues. The previously identified *ccr* gene is located 36 bp upstream of *meaA* (24), while the downstream sequence contained an incomplete downstream ORF, ORF1, encoding a protein with 48% sequence identity to malyl-CoA lyase of *Methylobacterium extorquens* (37) and 33% identity to citrate lyase of *E. coli*. The same gene order has previously been identified for *S. collinus* (16) and *S. coelicolor* (www.sanger.ac.uk). *meaA* possesses a stop codon overlapping the start codon of ORF1 at nucleotides 2147 to 2149 (ATGA), which is suggestive of translational coupling (17). The *meaA* and ORF1 ORFs are both preceded by a potential streptomycete ribosome binding sequence, GGAG, at nucleotides 107 to 110 and 2134 to 2137, respectively (4). No putative *E.coli* σ^{70}-like promoter element was found upstream of either the *mea*A gene or ORF1 (40).

A phylogenetic tree of the coenzyme B_{12}-dependent mutases, MCM, ICM, and MeaA, was made (data not shown). MeaA from *S. cinnamonensis* showed the highest amino acid sequence identity to corresponding proteins in *S. coelicolor* (89%), *S. collinus* (90%), and *M. extorquens* (60%). *S. cinnamonensis* MeaA is the third MCM-like coenzyme B_{12}-depen-

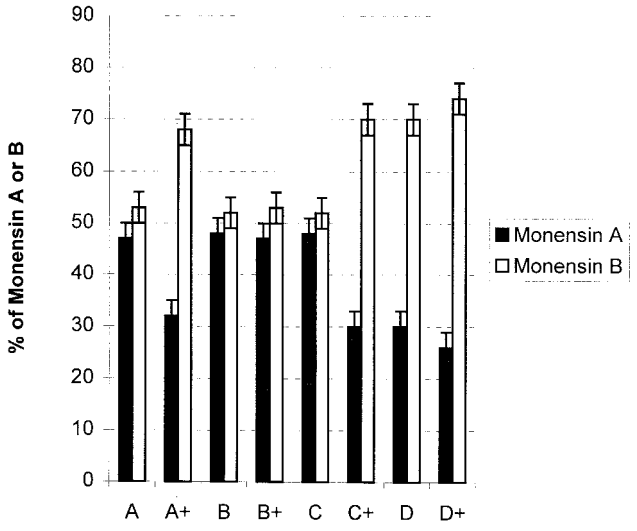

FIG. 2. Effects of plasmid-based *meaA* expression (pZR8) on the monensin A-to-monensin B ratio. (A) C730.1, wild-type strain; A+, C730.1/pZR8; B, *icm* mutant; B+, *icm* mutant/pZR8; C, *mut* mutant; C+, *mut* mutant/pZR8; D, *ccr* mutant (L1); D+, L1/pZR8.

dent mutase from this organism to be studied and has amino acid sequence homology to the MCM large subunit (52% similarity, 41% identity), ICM large subunit (48% similarity, 33% identity), and ICM small subunit (62% similarity, 52% identity). Similar homologies were observed with the corresponding subunits of the *S. coelicolor* ICM enzyme. The motifs DXHXXG, SXL, and GG, which have been indicated as diagnostic characteristics for cobalamin binding from the *E. coli* methionine synthase (13) and the *Propionibacterium freudenreichii* subsp. *shermanii* MCM (26) crystal structures, are found perfectly conserved at the C-terminal end of MeaA. A crystal structure of MCM from *P. freundenreichii* subsp. *shermanii* with bound substrate has recently been revealed, in which amino acids in the active site form an interaction with methylmalonyl-CoA (26). An alignment of *P. freundenreichii* subsp. *shermanii* MCM with *S. cinnamonensis* MeaA revealed that many of these residues are conserved (data not shown).

Overexpression of *S. cinnamonensis meaA*. Earlier work demonstrated that the C730.1 strain produces monensins A and B in a ratio of about 1:1 under standard laboratory growth conditions (24,34). Neither *icm* inactivation nor *mutAB* inactivation has any effect on the monensin A-to-monensin B ratio or the total monensin titer (24, 44). A *ccr* mutant, however, causes a substantial decrease in this monensins A-to-B ratio from 1:1 to approximately to 1:4 (24). This change was attributed to a decrease in the pools of ethylmalonyl-CoA (formed by the action of CCR) relative to those of methylmalonyl-CoA (24). The *S. cinnamonensis meaA* gene was PCR amplified and used to generate pZR8, in which the *meaA* gene was expressed from the strong constitutive *ermE** promoter (46). This plasmid was transformed into *S. cinnamonensis* C730.1 (wild type), as well as into *icm* (44), *ccr* (L1) (24), and *mut* (44) mutants. The monensin titers and monensin A-to-monensin B ratio for each of the resulting transformants was determined (Fig. 2). In all experiments, the overall monensin titers were not significantly altered. Plasmid-based overexpression of *meaA*, however, did significantly decrease the monensin A-to-monensin B ratio in the C730.1 strain and the *mutAB* mutant. These results are consistent with the expression of *meaA* leading to an increase in the pool of methylmalonyl-CoA relative to that of ethylmalonyl-CoA and suggestive of a role of MeaA in methylmalonyl-CoA formation. In the *ccr* mutant (L1), this ratio is already significantly reduced from that of the wild-type strain and only marginally increased by plasmid-based *meaA* overexpression. Surprisingly, *meaA* overexpression in the *icm* mutant did not change the monensin A-to-monensin B ratio.

Targeted disruption of *S. cinnamonensis meaA* and phenotype analysis. An insertional inactivation strategy was used to disrupt the *meaA* gene in both *S. cinnamonensis* C730.1 and the *icm* mutant (44). The plasmid pZR22 used to create the *meaA* mutants was generated in *E. coli* ET12567 and denatured by alkali treatment (29) before it was used for the transformation of *S. cinnamonensis* cells. Colonies resistant to both apramycin and erythromycin (Erm^r, Am^r) were obtained. Mutants in which a single crossover between pZR22 and genomic DNA had occurred were selected by cultivating these transformants at 40°C in the presence of both antibiotics. Following several rounds of propagation of these single-crossover mutants in the absence of any antibiotics at 30°C, colonies resistant to erythromycin but sensitive to apramycin (Erm^r, Am^s) were obtained. The *meaA*::*ermE* disruption in C730.1 and the *icm* mutation of *S. cinnamonensis* were designated WM2 and WD2 strains, respectively. The double-crossover event in both these clones ware confirmed by PCR using primers 1 and 2 (see Materials and Methods).

When grown in either a carbohydrate-based medium or an oil-based medium, the mutants WM2 (*meaA* mutation) and WD2 (*icm* and *meaA* mutations) and wild-type C730.1 strains grew equally well and displayed no morphological differences. The monensin titers and monensin A-to-monensin B ratio produced by each of these strains was analyzed (Fig. 3). The *meaA* mutant produced a significantly higher monensin A-to-monensin B ratio (4:1) compared to the C730.1 strain (1:1) in either production media. This ratio in the WM2 was more than

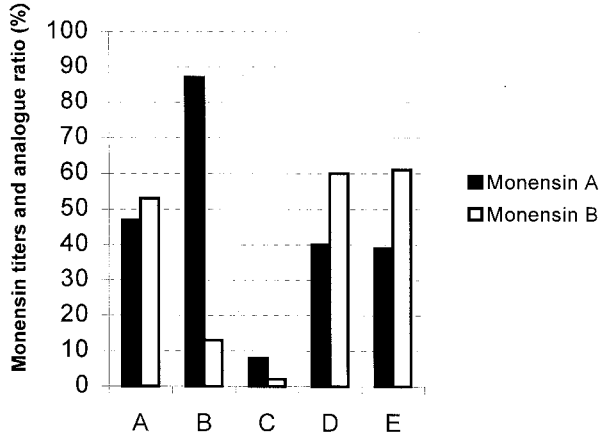

FIG. 3. Monensin titers and analog ratios for various *S. cinnamonensis* mutants in carbohydrate-based fermentation medium. (A) C730.1, wild-type strain; B, *meaA* mutant (WM2); C, *icm meaA* mutant (WD2); D, WM2/pZR8 (plasmid-based expression of *meaA*); E, WD2/pZR8. Total monensin titers (monensins A and B) are expressed as a percentage of that obtained using the C730.1 strain. The same pattern of changes in ratios and titers was observed using the oil-based media.

restored with plasmid-based expression of the *meaA* gene. In fact, the *S. cinnamonensis* WM2/pZR8 strain produced slightly more monensin B than monensin A, presumably due to higher levels of *meaA* expression. These results are also consistent with a role of MeaA in production of methylmalonyl-CoA in *S. cinnamonensis*.

The *meaA* mutant produced monensin titers comparable to those of the C730.1 strain, indicating that while the ratio of methylmalonyl-CoA to ethylmalonyl-CoA may have decreased, there was still sufficient methylmalonyl-CoA to support monensin production. The levels of methylmalonyl-CoA were limiting, however, in the *icm meaA* mutant (WD2), which produced only 10% of the monensin titers (predominantly as monensin A) seen with either the C730.1 *meaA* or *icm* mutants in either carbohydrate-based or oil-based media. Thus, in this mutant, *meaA* and *icm* together appear to be crucial for the monensin production in the tested media. As predicted, the monensin A-to-monensin B ratio and overall monensin titers were restored in the *icm meaA* mutant by plasmid-based expression of *meaA* (this experiment, like that with the WM2 strain, led to slightly higher levels of monensin B relative to those of monensin A).

Plasmid-based overexpression of *A. mediterrenei mutAB* in *S. cinnamonensis* mutants. MCM is a well-studied enzyme and has long been thought to play an important role in providing methylmalony-CoA for polyketide formation in many organisms, including the erythromycin-producing *S. erythraea* (21), rifamycin-producing *A. mediterranei* (49), and monensin-producing *S. cinnamonensis* (6). Indeed, overexpression of MCM-encoding genes *mutAB* in *S. cinnamonensis* wild-type strains has recently been found to increase both the overall monensin titers and the amount of monensin B relative to that of monensin A (49). The same phenomenon was observed in this study with plasmid-based overexpression of the *A. mediterranei mutAB* genes in the *meaA* mutant (Fig. 4). In this case, the monensin titers were increased almost twofold (relative to those of either the C730.1 or *meaA* mutant) and the monensin A-to-monensin B ratio switched from 4:1 to 1:5. The overexpression of *mutAB* genes in the mutant WD2 (which produced only 10% of the monensin titers of the C730.1 or WM2 strain) led to a remarkable 20-fold increase in monensin titers. As in the case of the *meaA* mutant, plasmid-based expression of *mutAB* in the WD2 strain led to a significant decrease in the monensin A-to-monensin B ratio. Thus, in either the WM2 or WD2 strain, the shift toward the monensin A analog caused by insertional inactivation of *meaA* can be reversed by plasmid-based expression of either *meaA* or *mutAB*. Furthermore, expression of either of these genes can be used to restore monensin production in a *meaA icm* mutant (WD2) to at least wild-type levels. These observations are consistent with MeaA playing a major role in methylmalonyl-CoA formation.

Effect of valine feeding on monensin production in the WD2 strain. Isotope labeling experiments with both polyether- and macrolide-producing streptomycetes have shown that valine can be catabolized to isobutyryl-CoA, and subsequently to methylmalonyl-CoA, for antibiotic biosynthesis (23, 30) (Fig. 1). Media supplemented with valine and isoleucine can sometimes stimulate macrolide antibiotic production, while increasing NH_4^+ concentrations can have negative effects on both macrolide production and valine dehydrogenase activity (31, 32), all suggesting that branched-chain amino acid catabolism may be an important source of building blocks for macrolide biosynthesis. Valine at 0.1 M was fed to fermentations of the *meaA icm* mutant in three separate aliquots (at 24, 48, and 72 h of fermentation in the production media). This experiment was also carried out using valine at a final concentration of 0.8 M. Feeding with 0.1 M valine had no significant effect on the monensin production by the WD2 mutant, while feeding with 0.8 M valine led to only moderate increases in the monensin titers (25% increase) (Fig. 4). In the latter case, the monensin titers were 40% of the level achieved by the wild-type C730.1 strain and the monensin A-to-monensin B ratios were essentially equivalent (1:1). Thus, the valine catabolic pathway in the *meaA icm S. cinnamonensis* mutant does not appear to be efficient process for producing methylmalonyl-CoA.

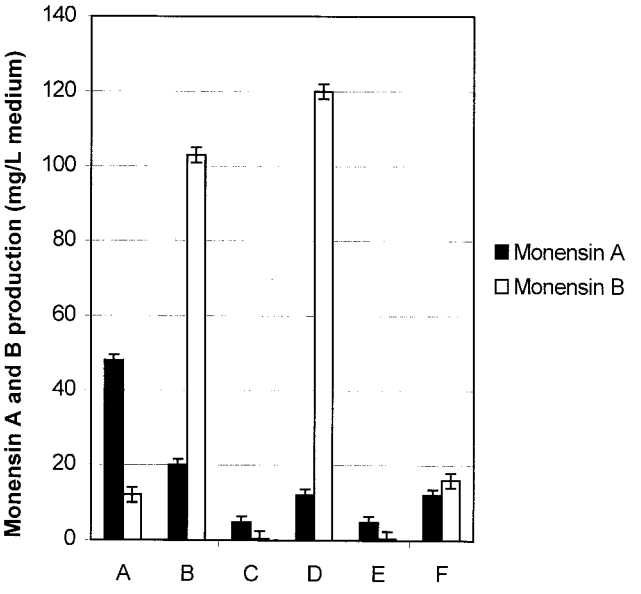

FIG. 4. Enhancement of monensin titers by either pZC32 plasmid-based expression of *mutAB* or addition of exogenous DL-valine. A, *meaA* mutant (WM2); B, WM2/pZC32; C, *meaA icm* mutant (WD2); D, WD2/pZC32; E, WD2 cells grown in the presence of 0.1 M DL-valine; F, WD2 cells grown with 0.8 M DL-valine.

Incorporation of [3,4-$^{13}C_2$]acetoacetate into monensin A in the *meaA* mutant. Biosynthetic studies in *S. cinnamonensis* have previously demonstrated that dual-labeled [1,3-$^{13}C_2$]acetoacetyl-CoA (generated by carrying out fermentations in the presence of the corresponding labeled ethyl acetoacetate) can be converted into [1,2-$^{13}C_2$]methylmalonyl-CoA in the absence of either ICM or MCM (44). The pathway by which this intact conversion, which requires a carbon skeleton rearrangement, occurs was undetermined. It was, however, suggested that MeaA might play a role in this pathway (44). To investigate this possibility, ethyl [3,4-$^{13}C_2$]acetoacetate was added to fermentations of C730.1 and the *meaA* mutant (WM2). Strong ^{13}C doublets surrounding the natural abundance signal for both C-32 and C-33 of monensin A were observed (Fig. 5) for the WM2 mutant, which indicated the simultaneous presence of a ^{13}C label at both carbons. The size of these doublets was substantially greater than the natural abundance signal and indicated an approximate 10-fold enrichment of ^{13}C at these carbons due to the intact utilization of the ^{13}C-labeled aceto-

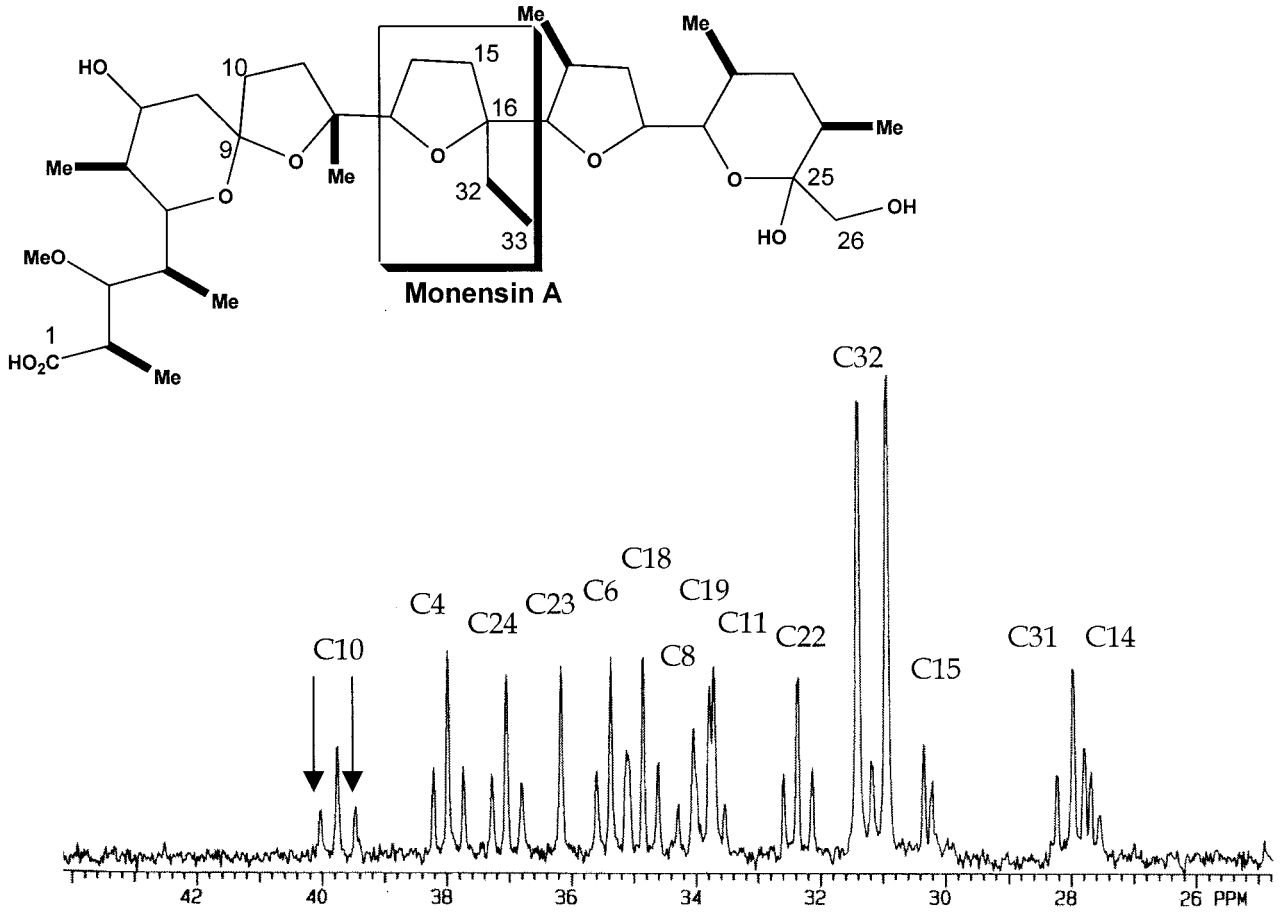

FIG. 5. A portion of the proton decoupled-^{13}C nuclear magnetic resonance spectrum of monensin A isolated after feeding ethyl [3,4-$^{13}C_2$] acetoacetate to a *meaA* mutant of *S. cinnamonensis* WM2. Enriched doublets surrounding the natural abundance signals for positions C-2, C-4, C-6, C-12, C-18, C-22, and C-24 and the corresponding methyl substituents were observed, consistent with intact incorporation of [2,3-$^{13}C_2$] methylmalonyl-CoA. Substantially larger enriched doublets were observed at C-32 and C-33 of monensin A, consistent with the intact incorporation of [3,4-$^{13}C_2$]ethylmalonyl-CoA. Arrows indicate the enriched doublet for the malonyl-CoA-derived C-10 position. No significant doulets were observed for monensin A carbons derived from C-1 of methylmalonyl-CoA. Carbon-carbon bonds in monensin A labeled intact from C-3 and C-4 of methylmalonyl-CoA are shown in bold. A similar pattern of labeling was observed for the wild-type C730.1 strain and the WD2/pZC32 strain.

acetate. The natural abundance ^{13}C signals for all of the carbons of monensin A derived from C-2 and C-3 of methylmalonyl-CoA were similarly surrounded by enriched doublets. In this case, however, there was only about a twofold enrichment of ^{13}C at these positions due to the intact utilization of the ^{13}C-labeled acetoacetate. Indistinguishable labeling patterns were observed for monensin A isolated from the C730.1 strain grown under identical conditions. Thus, insertional inactivation of *meaA* affects the pool of methylmalonyl-CoA but does not seem to significantly affect the amount generated from acetoacetyl-CoA or related metabolites. These data combined with data from the previous study (44) indicate that acetoacetyl-CoA can be converted intact to methylmalonyl-CoA in *S. cinnamonensis* in the absence of either ICM, MCM, or MeaA. A similar study was also carried out with the *icm meaA* double mutant (WD2). In order to generate sufficient monensin A for nuclear magnetic resonance analysis, this experiment was carried out using the WD2/pZC32 strain (plasmid-based expression of *mutAB*). Similar low levels of intact labeling of the methymalonyl-CoA-derived positions of monensin from labeled ethyl acetoacetate were also seen in this experiment (data not shown), indicating that conversion of acetoacetyl-CoA to methylmalonyl-CoA can proceed in the absence of both ICM and MeaA.

Fatty acid analyses. It has previously been shown that alterations in the levels of butyryl-CoA or isobutyryl-CoA pool can directly alter the types and amounts of branched-chain and straight-chain fatty acids in streptomycetes (45). This present study clearly demonstrated that disruption and overexpression of *meaA* affected the levels of the related metabolites and the methylmalonyl-CoA and ethylmalonyl-CoA pool and raised the possibility that changes in the fatty acid profiles might also be observed. Fatty acid profiles for the *S. cinnamonensis* wild-type C730.1 strain and *meaA* and *icm meaA* mutants were determined and shown to be essentially indistinguishable.

Fatty acid analyses were also carried out for these strains and the *icm* mutant grown in the presence of perdeuterated valine. It has previously been shown that approximately 60% of the isopalmitate in streptomycetes grown in the presence of perdeuterated valine is generated from use of the corresponding perdeuterated isobutyryl-CoA catabolite. In these experiments, the action of ICM on the labeled isobutyryl-CoA pro-

vides perdeuterated butyryl-CoA which can label intact as much as 10% of the palmitate pool. In the present experiments, only labeled palmitate was observed for the C730.1 and MeaA mutants grown in the presence of 200 mM perdeuterated valine. No labeled palmitate was observed for either the *icm* or *meaA icm* mutant, while labeled isopalmitate was observed in all four strains (data not shown). These observations are strongly suggestive of the absence of any significant in vivo ICM activity in mutants with an insertional inactivation of the *icm* gene under our test conditions.

DISCUSSION

Role of methylmalonyl-CoA in polyketide biosynthesis. The identification of numerous gene clusters involved in polyketide antibiotics has led to a better understanding of the genetic and biochemical elements governing the biosynthesis of these compounds (2, 20). By contrast, less information is available on the enzymes and pathways that provide the required biosynthetic precursors for these processes. Perhaps the best example is methylmalonyl-CoA, one of the most common precursors used in polyketide biosynthesis. Several different pathways have been proposed to be involved in the generation of this precursor, yet the relative importance of each of these for methylmalonyl-CoA production within different polyketide-producing organisms is unknown. Furthermore, it is not yet clear if there are additional pathways for producing methylmalonyl-CoA beyond those known to date. Substrate availability can clearly affect the type of polyketide analogs made in a fermentation (24, 38) and in some cases can be a limiting factor controlling overall titers. Knowledge of the enzymes and pathways involved in the precursor supply thus offers an opportunity for rational approaches for increasing fermentation titers. In this study, we reported the cloning and characterization of *meaA*, which encodes a novel coenzyme B_{12}-dependent mutase in monensin A-producing *S. cinnamonensis*. This gene has been shown to be necessary in the methanol and ethanol assimilation in *M. extorquens* AM1 and to be important in growth of *S. collinus* on acetate (9, 15, 37). The present study provides evidence that MeaA has a significant role in providing methylmalonyl-CoA for the biosynthesis of the monensin polyketide.

MeaA, a putative coenzyme B_{12}-dependent mutase. Sequence analysis predicts that *S. cinnamonensis meaA* encodes a 74-kDa protein with a perfectly conserved coenzyme B_{12} binding motif at the C terminus and 36 to 40% amino acid sequence identity to the two known coenzyme B_{12}-dependent mutases (MCM and ICM) (6, 27, 47). Isobutyryl-CoA and methylmalonyl-CoA (substrates for ICM and MCM, respectively) differ only in the oxidation state of one carbon. The two enzymes show significant amino acid sequence homology, particularly in the region shown from the crystal structure of the *P. freundenreichii* subsp. *shermanii* MCM shown to be involved in substrate binding (26). Most of these amino acids are also conserved in MeaA (data not shown). In MCM, the Tyr^{A89} conserved in all MCM sequences and Arg^{A208} are located near the bottom of the substrate binding hole and appear to form hydrogen bonds with the carboxyl group of the methylmalonyl-CoA substrate. These residues are not conserved in ICM (the substrate in this case contains the reduced methyl carbon substituent) but are conserved in the predicted amino acid sequences of all known MeaA proteins. Thus, the MeaA substrate might also contain two oxidized and possibly carboxylated substituents, with one activated as a CoA thioester.

Role of MeaA in providing methylmalonyl-CoA. The role of *meaA* in providing methylmalonyl-CoA for monensin biosynthesis was studied by two approaches. Firstly, *meaA* was overexpressed from a multicopy plasmid using the strong constitutive *ermE* promoter. This system has been used successfully in the past for overexpression of the *ccr* gene in *S. cinnamonensis* (24). Overexpression of the *meaA* gene resulted in a significant increase of methylmalonyl-CoA-derived monensin B compared to the ethylmalonyl-CoA-derived monensin A in the C730.1 type strain and *mutAB*-blocked mutants. These observations are consistent with an increased pool of methylmalonyl-CoA relative to that of ethylmalonyl-CoA and suggest that MeaA is able to generate methylmalonyl-CoA. The same conclusion was reached by generating a *meaA* mutant of *S. cinnamonensis* and observing that it made significantly higher ratios of monensin A compared to monensin B. No significant change in total monensin titers was observed in either of these experiments, indicating that under these conditions, another process provides the methylmalonyl-CoA for monensin biosynthesis. This observation was not made for the *meaA icm* mutant that produced less than 10% of the monensin titers than the corresponding control strains. Evidence that this decrease was due to a limitation in methylmalonyl-CoA was provided by the observation that plasmid-based expression of either *mutAB* or *meaA* restored monensin production at least to the same levels as those seen in the control. The reason that inactivation of insertional inactivation of *meaA* has a more pronounced effect on the *icm* mutant than on the C730.1 strain is unclear at present. It is interesting to note that only in the *icm* mutant did plasmid-based overexpression of *meaA* not lead to an increase in the monensin A-to-monensin B ratio. Regardless, these experiments have clearly indicated that alterations in the levels of MeaA in *S. cinnamonensis* can lead to significant changes in the amounts of methylmalonyl-CoA available for monensin biosynthesis and that its role can be efficiently replaced by MCM. Previous studies of MeaA in *M. extorquens* AM1 (9, 37) and *S. collinus* (15) have indicated that MeaA is neither an MCM nor an ICM. Thus, it seems likely that under the tested conditions, this methylmalonyl-CoA is being produced by a pathway involving a different type of coenzyme B_{12}-dependent mutase.

Role of valine degradation in providing methylmalonyl-CoA. Valine catabolism as a source for methylmalonyl-CoA has been well studied for some streptomycetes strains, including *S. coelicolor* (41), *Streptomyces ambofaciens*, and *Streptomyces fradiae* (42). Labeling studies, including those carried out with *S. cinnamonensis* cells, all indicate that the most likely pathway in a complex fermentation medium involves direct oxidation of one of the methyl groups of valine catabolite isobutyryl-CoA to yield methylmalonyl-CoA (Fig. 1) (34, 44). The decreased monensin titers in the *meaA icm* mutant clearly demonstrate that valine catabolism involving direct oxidation of isobutyryl-CoA is not a major contributor to methylmalonyl-CoA production (this process does not involve a carbon skeleton rearrangement and thus is not predicted to require a coenzyme B_{12}-dependent mutase). A partial restora-

tion of monensin titers with high levels of exogenously supplied valine indicates that such a B_{12}-independent process can provide at least some of the methylmalonyl-CoA. Nonetheless, significantly higher monensin titers could be achieved in the *icm meaA* mutant strain by introduction of either MeaA or MCM, suggesting that processes using these enzymes are more efficient at the generation of methylmalonyl-CoA.

A multitude of labeling studies carried out with isobutyrate, butyrate, and ethyl acetoacetate, in this and previous studies of *S. cinnamonensis*, have consistently shown significantly greater labeling of the butyryl-CoA (ethylmalonyl-CoA)-derived position of monensin A than the methylmalonyl-CoA-derived positions. These observations would also be consistent with the valine catabolite isobutyryl-CoA not being an intermediate in the major pathway for methylmalonyl-CoA production. A similar conclusion can be drawn concerning the role of valine catabolism in *Streptomyces avermitilis*. A *bkd* mutation of this strain is blocked completely in valine degradation, resulting in a complete lack of the isobutyryl-CoA starter unit required for biosynthesis of the polyketide avermectin (10). When alternative starter units, such as cyclohexanecarboxylic acid, are provided to this strain, novel avermectins requiring methylmalonyl-CoA precursors as extender units can be made, despite the lack of any isobutyryl-CoA (10). More recently, we have described that addition of labeled isobutyric acid to this strain results in production of avermectin B1b labeled 16-fold more efficiently at the isobutyryl-CoA-derived position than the methylmalonyl-CoA-derived positions (50). Thus, it is quite clear that under the tested conditions, isobutyryl-CoA derived from valine catabolism does not play a significant role in providing methylmalonyl-CoA for monensin or avermectin biosynthesis. These observations do not preclude the possibility that under certain conditions or with different organisms, valine catabolism may be playing a more significant role in providing methylmalonyl-CoA.

Multiple pathways linking acetoacetyl-CoA or butyryl-CoA and methylmalonyl-CoA. The intact conversion of [1,3-$^{13}C_2$] acetoacetyl-CoA into the methylmalonyl-CoA-derived positions of monensin has long been considered to be indicative of a pathway (Fig. 1) involving reduction to butyryl-CoA, isomerization to isobutyryl-CoA, and subsequent oxidation (38, 44). The retention of all three deuteriums of [$^{13}CD_3$] acetate into the methyl group of methylmalonyl-CoA in *S. cinnamonensis* is also consistent with such a process and inconsistent with other pathways involving MCM or PCC and MMT (Fig. 1) The recent observation that intact conversion of labeled acetoacetate into methylmalonyl-CoA was not affected by insertional in activation of the *icm* gene was surprising and suggested that another enzyme-catalyzed process must account for this conversion. In the present study, the same low levels of intact conversion of acetoacetyl-CoA into methylmalonyl-CoA were observed for *meaA* and *meaA icm* mutants and a wild-type strain, suggesting that *meaA* is not involved in converting acetoacetyl-CoA or related metabolites (butyryl-CoA) to methylmalonyl-CoA. Furthermore, it is evident from these data and a previous analysis (44) that a process not involving the cloned *icm, mcm*, or *meaA* genes is involved in this conversion (Fig. 1). One possible explanation might be the presence of a second copy of *icm* expressed under certain conditions. Recently, a putative second *icm* gene was discovered in the genome of *S. coelicolor* (accession no. CAB71920 and CAB71846 for the large and small subunits in the NCBI GenBank database, respectively). This gene contains two ORFs (designated the *mutB* and *icmA* genes, respectively) encoding 531 and 159 residues, respectively, which are similar in size to IcmA and IcmB of both *S. cinnamonensis* and *S. coelicolor* (33, 48). *mutB* encodes a protein with 51% identity to IcmB, while *icmA* encodes a protein with 68% identity to IcmA of *S. coelicolor*. Unlike the first *icmAB* genes characterized (33, 48), these two ORFs are separated by only 40 bp, suggesting that they may be translationally coupled. An alignment of this putative ICM with the MCM of *P. freundenreichii* subsp. *shermanii* (data not shown) revealed that many of the same amino acids lining the active site of MCM are conserved. The Tyr^{A89} and Arg^{207} present in MCM and in MeaA are absent in both this putative *S. coelicolor* ICM as well as the *S. cinnamonensis* ICM (data not shown). Thus, sequence data suggest, but do not prove, that there may be two copies of the genes encoding ICM in *S. coelicolor*. Such a possibility cannot be completely ruled out for *S. cinnamonensis*. However, it has been demonstrated that there is no detectable ICM activity in the *S. cinnamonensis icm* mutant (48). Furthermore, the fatty acid analyses carried out in the presence of valine in the present study showed that there is no detectable in vivo conversion of isobutyryl-CoA to butyryl-CoA in either the *icm* or *icm meaA* mutants using a minimal medium. We were unable to make this determination in a complex fermentation medium because only trace amounts of the straight-chain fatty acids required for this analysis were produced. The manner in which butyryl-CoA and acetoacetyl-CoA are converted into methylmalonyl-CoA in fermentations of these mutants thus remains uncertain.

Conclusion. In conclusion, MeaA plays an important role via an unknown pathway in providing methylmalonyl-CoA precursors for monensin biosynthesis in *S. cinnamonensis*. Increased methylmalonyl-CoA pools can be accomplished by plasmid-based expression of *meaA*. Conversely, these pools can be decreased by insertional inactivation of *meaA*. In the case of an *icm meaA* mutant, these decreased pools significantly reduce monensin titers. Restoration of monensin titers can be accomplished by plasmid-based expression of genes encoding either MeaA or MCM. By comparison, isobutyryl-CoA obtained either from valine catabolism or by ICM-catalyzed isomerization of butyryl-CoA plays a more minor role in providing methylmalonyl-CoA for monensin biosynthesis. Finally, an enzyme other than those encoded by the cloned *icm meaA* and *mcm* genes of *S. cinnamonensis* can catalyze the intact conversion of acetoacetyl-CoA to methylmalonyl-CoA.

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REFERENCES

1. **Alting-Mess, A. A., and J. M. Short.** 1989. pBluescript II: gene mapping vectors. Nucleic Acids Res. **17:**9494.

2. **August, P. R., L. Tang, Y. J. Yoon, S. Ning, R. Muller, T. W. Yu, M. Taylor, D. Hoffmann, C. G. Kim, X. Zhang, C. R. Hutchinson, and H. G. Floss.** 1998. Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amycolatopsis mediterranei* S699. Chem. Biol. **5:**69–79.
3. **Beran, M., and J. Zima.** 1993. Determination of monensin A and B in the fermentation broth of *Streptomyces cinnamonensis* by high performance liquid chromatograph. Chromatographia **35:**206–208.
4. **Bibb, M. J., and S. N. Cohen.** 1982. Gene expression in Streptomyces: construction and application of promoter-probe plasmid vectors in *Streptomyces lividans* Mol. Gen. Genet. **187:**265–277.
5. **Bierman, M., R. Logan, K. O. Brien, E. T. Seno, R. N. Rao, and B. E. Schoner.** 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces spp.* Gene **116:**43–49.
6. **Birch, A., A. Leiser, and J. A. Robinson.** 1993. Cloning, sequencing, and expression of the gene encoding methylmalonyl-coenzyme A mutase from *Streptomyces cinnamonensis*. J. Bacteriol. **175:**3511–3519.
7. **Bramwell, H., I. S. Hunter, J. R. Coggins, and H. G. Nimmo.** 1999. Propionyl-CoA carboxylase from *Streptomyces coelicolor* A3(2): cloning of the gene encoding the biotin-containing subunit. Microbiology **142:**649–655.
8. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**367–379.
9. **Chistoserdova, L. V., and M. E. Lidstorm.** 1996. Molecular characterization of a chromosomal region involved in the oxidation of acetyl-CoA to glyoxylate in the isocitrate-lyase-negative methylotroph *Methylobacterium extorquens* AM1. Microbiology **142:**1459–1468.
10. **Cropp, T. A., A. A. Smogowicz, E. W. Hafner, C. D. Denoya, H. A. I. McArthur, and K. A. Reynolds.** 2000. Fatty-acid biosynthesis in a branched-chain alpha-keto acid dehydrogenase mutant of *Streptomyces avermitilis*. Can. J. Microbiol. **46:**506–514.
11. **Donadio, S., M. J. Staver, J. B. McAlpine, S. J. Swanson, and L. Katz.** 1991. Modular organization of genes required for complex polyketide biosynthesis. Science **252:**675–679.
12. **Donadio, S., M. J. Staver, and L. Katz.** 1996. Erythromycin production in *Saccharopolyspora erythraea* does not require a functional propionyl-CoA carboxylase. Mol. Microbiol. **19:**977–984.
13. **Drennan, C. L., S. Huang, J. T. Drummond, R. G. Matthews, and M. L. Lidwig.** 1994. How a protein binds B12: a 3.0 A X-ray structure of B_{12}-binding domains of methionine synthase. Science **266:**1669–1674.
14. **Gorman, M., J. W. Chamberlin, and R. L. Hamill.** 1967. Monensin, a new biologically active compound. V. Compounds related to monensin. Antimicrob. Agents Chemother. **7:**363–368.
15. **Han, L., and K. A. Reynolds.** 1997. A novel alternate anaplerotic pathway to the glyoxylate cycle in *Streptomyces*. J. Bacteriol. **179:**5157–5164.
16. **Han, L., K. Yang, K. Kulowski, E. Wendt-Pienkowski, C. R. Hutchinson, and L. C. Vining.** 2000. An acyl-coenzyme A carboxylase encoding gene associated with jadomycin biosynthesis in *Streptomyces venezuelae* ISP5230. Microbiology **146:**903–910.
17. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
18. **Hector, M. L., and R. R. Fall.** 1976. Multiple acyl-coenzyme A carboxylases in *Pseudomonas citronellolis*. Biochemistry **15:**3465–3471.
19. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. John Innes Foundation, Norwich, England.
20. **Hopwood, D. A.** 1997. Genetic contributions to understanding polyketide synthases. Chem. Rev. **97:**2465–2497.
21. **Hunaiti, A. R., and P. E. Kolattukudy.** 1984. Source of methylmalonyl-coenzyme A for erythromycin synthesis: methylmalonyl-coenzyme A mutase from *Streptomyces erythreus*. Antimicrob. Agents Chemother. **25:**173–178.
22. **Hunaiti, A. R., and P. E. Kolattukudy.** 1982. Isolation and characterization of an acyl-coenzyme A carboxylase from an erythromycin-producing *Streptomyces erythreus*. Arch. Biochem. Biophys. **216:**362–371.
23. **Leiser, A. A. Birch, and J. A. Robinson.** 1996. Cloning, sequencing, overexpression in *Escherichia coli*, and inactivation of the valine dehydrogenase gene in the polyether antibiotic producer *Streptomyces cinnamonensis*. Gene **177:**217–222.
24. **Liu, H., and K. A. Reynolds.** 1999. Role of crotonyl coenzyme A reductase in determining the ratio of polyketides monensin A and monensin B produced by *Streptomyces cinnamonensis*. J. Bacteriol. **181:**6806–6813.
25. **MacNeil, D. J., K. M. Gewain, C. L. Ruby, G. Dezeny, P. H. Gibbons, and T. MacNeil.** 1992. Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel intergration vector. Gene **111:**61–68.
26. **Mancia, F., N. H. Keep, A. Nakagawa, P. F. Leadlay, S. McSweeney, B. Rasmussen, P. Bosecke, O. Dia, and P. R. Evans.** 1996. How coenzyme B_{12} radicals are generated: the crystal structure of methylmalonyl-coenzyme A mutase at 2 Å resolution. Structure **4:**339–350.
27. **Marsh, E. N., N. McKie, N. K. Davis, and P. F. Leadlay.** 1989. Cloning and structural characterization of the genes coding for adenosylcobalamin-dependent methylmalonyl-CoA mutase from *Propionibacterium shermanii*. Biochem. J. **260:**345–352.
28. **Marsh, E. N.** 1999. Coenzyme B_{12} (cobalamin)-dependent enzymes. Essays Biochem. **34:**139–154.
29. **Oh, S., and K. F. Chater.** 1997. Denaturation of circular or linear DNA facilitates targeted intergrative transformation of *Streptomyces coelicolor* A3(2): possible relevance to other organisms. J. Bacteriol. **179:**122–127.
30. **Omura, S., K. Tsuzuki, Y. Tanaka, H. Sakakibara, M. Aizawa, and G. Lukacs.** 1983. Valine as a precursor of n-butyrate unit in the biosynthesis of macrolide aglycone. J. Antibiot. (Tokyo) **36:**614–616.
31. **Omura, S., A. Taki, K. Matsuda, and Y. Tanaka.** 1984. Ammonium ions suppress the amino acid metabolism involved in the biosynthesis of protylonolide in a mutant of *Streptomyces fradiae*. J. Antibiot. (Tokyo) **37:**1362–1369.
32. **Omura, S., Y. Tanaka, H. Mamada, and R. Masuma.** 1984. Effect of ammonium ion, inorganic phosphate and amino acids on the biosynthesis of protylonolide, a precursor of tylosin aglycone. J. Antibiot. (Tokyo) **37:**494–502.
33. **Ratnatilleke, A., J. W. Vrijbloed, and J. A. Robinson.** 1999. Cloning and sequencing of the coenzyme B_{12}-binding domain of isobutyryl-CoA mutase from *Streptomyces cinnamonensis*, reconstitution of mutase activity, and characterization of the recombinant enzyme produced in *Escherichia coli*. J. Biol. Chem. **274:**31679–31685.
34. **Reynolds, K. A., D. O'Hagan, D. Gani, and J. A. Robinson.** 1988. Butyrate metabolism in streptomycetes. Characterization of an intramolecular vicinal interchange rearrangement linking isobutyrate and n-butyrate in *Streptomyces cinnamonensis*. J. Chem. Soc. Perkin. Trans. I **1988:**3197–3207.
35. **Rodriguez, E., and H. Gramajo.** 1999. Genetic and biochemical characterization of the alpha and beta components of a propionyl-CoA carboxylase complex of *Streptomyces coelicolor* A3(2). Microbiology **145:**3109–3119.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Smith, L. M., W. G. Meijer, L. Dijkhuizen, and P. M. Goodwin.** 1996. A protein having similarity with methylmalonyl-CoA mutase is required for the assimilation of methanol and ethanol by *Methylobacterium extorquens* AMI. Microbiology **142:**675–684.
38. **Sohling, B., and G. Gottschalk.** 1996. Molecular analysis of the anaerobic succinate degradation pathway in *Clostridium kluyveri*. J. Bacteriol. **178:**871–880.
39. **Stassi, D. L., S. J. Kakavas, K. A. Reynolds, G. Gunawardana, S. Swanson, D. Zeidner, M. Jackson, H. Liu, A. Buko, and L. Katz.** 1998. Ethyl-substituted erythromycin derivatives produced by directed metabolic engineering. Proc. Natl. Acad. Sci. USA **95:**7305–7309.
40. **Strohl, W. R.** 1992. Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. Nucleic Acids Res. **20:**961–974.
41. **Tang, L., and C. R. Hutchinson.** 1993. Sequence, transcriptional, and functional analyses of the valine (branched-chain amino acid) dehydrogenase gene of *Streptomyces coelicolor*. J. Bacteriol. **175:**4176–4185.
42. **Tang, L., Y. X. Zhang, and C. R. Hutchinson.** 1994. Amino acid catabolism and antibiotic synthesis: valine is a source of precursors for macrolide biosynthesis in *Streptomyces ambofaciens* and *Streptomyces fradiae*. J. Bacteriol. **176:**6107–6119.
43. **Thoma, N. H., and P. F. Leadlay.** 1998. Mechanistic and structural studies on methylmalonyl-CoA mutase. Biochem. Soc. Trans. **26:**293–298.
44. **Vrijbloed, J. W., K. Zerbe-Burkhardt, A. Ratnatilleke, A. Grubelink-Leiser, and J. A. Robinson.** 1999. Insertional inactivation of methylmalonyl coenzyme A (CoA) mutase and isobutyryl-CoA mutase genes in *Streptomyces cinnamonensis*: influence on polyketide antibiotic biosynthesis. J. Bacteriol. **181:**5600–5605.
45. **Wallace, K. K., B. Zhao, H. A. I. McArther, and K. A. Reynolds.** 1995. In vivo analysis of straight-chain and branched-chain fatty acid biosynthesis in three actinomycetes. FEMS Microbiol. Lett. **131:**227–234.
46. **Weber, J. M., B. Schoner, and R. Losick.** 1989. Identification of a gene required for the terminal step in erythromycin A biosynthesis in *Saccharopolyspora erythraea* (*Streptomyces erythreus*). Gene **75:**235–241.
47. **Wilkemeyer, M. F., A. M. Crane, and F. D. Ledley.** 1990. Primary structure and activity of mouse methylmalonyl-CoA mutase. Biochem. J. **271:**449–455.
48. **Zerbe-Burkhardt, K., A. Ratnatilleke, N. Philippon, A. Birch, A. Leiser, J. W. Vrijbloed, D. Hess, P. Hunziker, and J. A. Robinson.** 1998. Cloning, sequencing, expression and insertional inactivation of the gene for the large subunit of the coenzyme B_{12}-dependent isobutyryl-CoA mutase from *Streptomyces cinnamonensis*. J. Biol. Chem. **273:**6508–6517.
49. **Zhang, W., L. Yang, W. Jiang, G. Zhao, Y. Yang, and J. S. Chiao.** 1999. Molecular analysis and heterologous expression of the gene encoding methylmalonyl-CoA mutase from a rifamycin SV-producing *Amycolatopsis mediterranei* U32. Appl. Biochem. Biotechnol. **82:**209–225.
50. **Zhang, Y. X., C. D. Denoya, D. D. Skinner, R. W. Fedechko, H. A. I. McArthur, M. R. Morgenstern, R. A. Davies, S. Lobo, K. A. Reynolds, and C. R. Hutchinson.** 1999. Cloning and characterization of genes encoding acyl-CoA dehydrogenase (AcdH) homologs from *Streptomyces coelicolor* and *Streptomyces avermitilis*. Microbiology **145:**2323–2334.
51. **Zhou, C., Y. Yang, and A. Y. Jong.** 1990. Miniprep in 10 minutes. BioTechniques **8:**172–173.