Journal of Industrial Microbiology & Biotechnology (2001) 27, 368–377

www.nature.com/jim

Genetic approaches for controlling ratios of related polyketide products in fermentation processes

A Cropp, S Chen, H Liu, W Zhang and KA Reynolds

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

Simple acyl thioesters are used as precursors for both the initiation and elongation steps in polyketide biosynthetic processes. Several structurally related polyketide products are sometimes made in these processes. These analogs are typically generated by a combination of two factors: availability of structurally similar biosynthetic precursors, and biosynthetic enzymes unable to effectively discriminate between them. Often, only one polyketide product is desired from a fermentation process, requiring a method to control the ratio of these different analogs. Preferential production of one desired analog is accomplished using random mutagenesis and manipulation of fermentation conditions. A genetic enzymatic understanding of polyketide biosynthesis, as well as the pathways that provide the relevant precursors, allows for a rational and more contemporary approach for control of analogs produced in fermentation processes. This approach involves genetic manipulation of either the pathways that provide pools of the acyl CoA thioester precursors, or the function/specificity of the appropriate biosynthetic enzymes. Reviewed herein are three such examples where these approaches have been carried out successfully with polyketide biosynthetic processes. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 368–377.

Keywords: monensin; pikromycin; doramectin; polyketide synthase; *Streptomyces*; methylmalonyl CoA

Introduction

Modular polyketide synthases (PKSs) are responsible for generating the macrolide core of a diverse range of polyketide products with pharmaceutical, veterinary and agricultural applications [16]. These PKSs are comprised of numerous, large, multifunctional enzymes containing one or more discrete modules that catalyze the successive condensation of different extender units such as malonyl CoA and methylmalonyl CoA onto a starter unit [27]. Each module contains an acyltransferase (AT) domain responsible for loading the appropriate extender unit onto an acyl carrier protein (ACP) domain [27]. A thioesterase (TE) domain is often located at the C-terminus of the last module and is responsible for cleaving the acyl group from the adjacent ACP domain and is presumed to catalyze cyclization to the macrolactone.

A commercial process typically requires either exclusive or predominant production of one polyketide product. While the modular nature of a polyketide synthase permits controlled biosynthesis of a single chemical entity, a significant number of PKSs generate two or more related products [8,17,31]. One mechanism for generating related polyketides is the use of different starter units, which in the case of *Streptomyces avermitilis* fermentations gives rise to production of different antiparasitic avermectin compounds [8]. An ability to use more than one precursor at a specific stage during polyketide chain elongation also provides multiple polyketide products, as in the case of production of polyether ionophore antibiotics monensins A and B by *S. cinnamonensis* [17]. Finally, premature termination of production polyketide chain extension can give rise to different products of carbon chain lengths, as seen in the production of 12- and 14-membered ring ketolide antibiotics by *S. venezuelae* [39,40]. As described below, we have used these three organisms as examples to show the that genetic methods can be used to alter the relative levels of the related polyketide products in a fermentation process.

Correspondence: KA Reynolds, ISBDD, Suite 212, Virginia Commonwealth University, 800 East Leigh Street, Richmond, VA 23219, USA
Received 1 March 2001; accepted 8 August 2001

Genetic engineering of a *S. avermitilis* to produce the novel avermectin analog, doramectin

S. avermitilis normally uses isobutyryl CoA and 2-methylbutyryl CoA as starter units to produce avermectins, a family of related antiparasitic polyketide products (Figure 1) [8]. The products generated from isobutyryl CoA are the avermectin "b" components, while those derived from 2-methylbutyryl CoA are the avermectin "a" components. The major pathway for production of these acyl CoA precursors is degradation of the branched-chain amino acids, valine and isoleucine. A number of years ago, researchers at Pfizer described the generation of a *bkd* mutant of *Streptomcyes avermitilis*, in which the α-ketoacid dehydrogenase responsible for catalyzing the oxidative decarboxylation step in this pathway was inactive [8,11]. This mutant was originally isolated after random mutagenesis and colony screening using a radioactive enzyme assay. Subsequently, the appropriate *bkd* genes were cloned and a new mutant generated by deletion of the 5′ region of *bkdF* (proposed to encode the E1α subunit of this multifunctional enzyme complex) [8,11]. These *bkd* mutants were unable to produce the "a" and "b" avermectins, unless they were grown in the presence of 2-methylbutyric acid and isobutyric acid. These short, branched-chain fatty acids presumably cross the cell wall, are activated to the corresponding coenzyme A thioester, and used to prime the loading domain of the avermectin PKS. Addition of just one of these substrates allows the selective formation of either the "a" or "b" avermectin analogs.

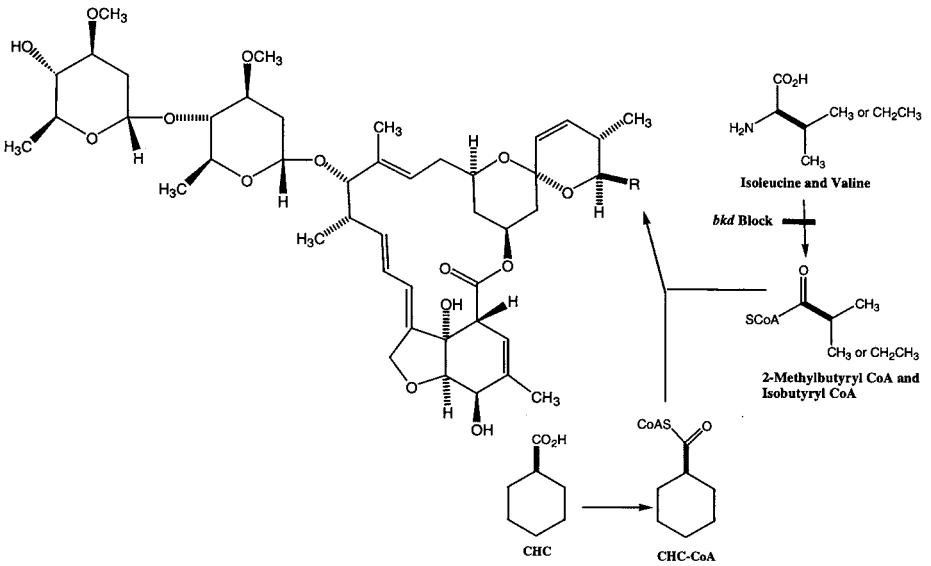

Figure 1 The role of precursors in the biosynthesis of avermectin and doramectin in *S. avermitilis*. Avermectin B1a and B1b are generated using the starter units 2-methylbutyryl CoA and isobutyryl CoA, respectively. In the *bkd* mutant of *S. avermitilis*, the formation of these starter units from branched-chain amino acid degradation is blocked. Doramectin B1 is generated using CHC CoA as a starter unit. This starter unit can be obtained either by addition of CHC to fermentations of the *S. avermitilis bkd* mutant or by introduction of pAC12.

More significantly, it was shown that a wide variety of avermectin analogs could be generated by providing different branched, short-chain fatty acids to the *bkd* mutant [9,20]. In these precursor-directed experiments, over 800 different precursors were fed and more than 60 were effective in generating novel avermectin analogs [20]. These experiments demonstrated that the loading domain of the avermectin PKS has relaxed substrate specificity and that manipulation of the branched-chain acyl CoA precursor pools through a combination of mutagenesis and directed biosynthesis (mutasynthesis) allows production of different avermectin analogs. These precursor-directed biosynthetic studies eventually led to the development of doramectin, produced by addition of cyclohexanecarboxylic acid (CHC) to the *S. avermitilis bkd* mutant (Figure 1) [20]. The antiparasitic efficacy and pharmacokinetic properties of doramectin were shown to have advantages over the existing commercial avermectin product, ivermectin [33]. Doramectin is sold under the trademark Dectomax and is reported to be the first commercially successful application of mutasynthesis [20].

We considered the possibility that a pathway for generating the unusual coenzyme A activated form of CHC (CHC CoA) could be engineered into the *S. avermitilis bkd* mutant. We reasoned that if the desired doramectin starter unit could be generated endogenously from primary metabolic intermediates, a strain should be capable of producing doramectin without CHC supplementation. The natural polyketide product ansatrienin A produced by *S. collinus* contains a side chain derived from CHC CoA and alanine (Figure 2) [21,30,37]. The CHC CoA is derived from shikimic acid in an unusual nine-step pathway (Figure 2) [21]. 1-Cyclohexenylcarbonyl CoA reductase (ChcA) catalyzes *in vitro* reductive steps in the pathway to CHC CoA [25]. The corresponding *chcA* gene located within the putative ansatrienin biosynthetic gene cluster is essential for CHC CoA biosynthesis [36]. Sequence analysis of four ORFs (*ansJ–ansM*) adjacent to *chcA* has suggested that they are also involved in this pathway and that all these five genes might encode enzymes for all steps in CHC CoA biosynthesis, with the exception of the penultimate step [4]. This step involves conversion of 2-cyclohexenylcarbonyl CoA to 1-cyclohexenylcarbonyl CoA. We recently purified a Δ^3, Δ^2-enoyl CoA isomerase (ChcB), which catalyzes this reaction, and have cloned and sequenced the corresponding *chcB* gene [22]. The *chcB* gene is not located within the putative ansatrienin biosynthetic gene cluster, and biochemical and genetic evidence suggests that *chcB* is present in many streptomycetes [22]. These results suggested that expression of the five genes (*ansJ–ansM* and *chcA*) in an *S. avermitilis bkd* mutant might be sufficient to allow the doramectin CHC CoA starter unit to be produced from the endogenous pool of shikimic acid.

A putative CHC biosynthetic gene cassette containing these five genes cloned downstream of the constitutive *ermE** promoter in the *Escherichia coli–Streptomyces* shuttle vector pSE34 [7]. The resulting pAC12 plasmid was transformed into a heterologous host, *S. lividans* 1326. Plasmid-based expression of *chcA* in *S. lividans* 1326/pAC12 was verified by ChcA assays using 1-cyclohexenylcarbonyl CoA as substrate (no detectable levels of ChcA activity were observed for extracts generated of *S. lividans* 1326 carrying the pSE34 plasmid without an insert). The observed ChcA activity demonstrated significant expression of *chcA*, the fourth gene downstream of the *ermE** promoter in pAC12, consistent with all five putative CHC biosynthetic genes being expressed. A series of fatty acid analyses revealed that *S. lividans* 1326/pAC12 produced ω-cyclohexyl fatty acids while the strain carrying pSE34 did not. These ω-cyclohexyl fatty acids generated using a CHC CoA starter unit indicated an intact CHC CoA pathway in the *S. lividans* 1326/pAC12 strain. The success of these studies prompted us to transform an *S. avermitilis bkd* mutant with pAC12 [7]. Fermentations of the resulting *S. avermitilis bkd*/pAC12 under standard doramectin production conditions resulted in production of doramectin without CHC supplementation. In contrast, doramectin production in the *S. avermitilis bkd* strain required CHC supplementation.

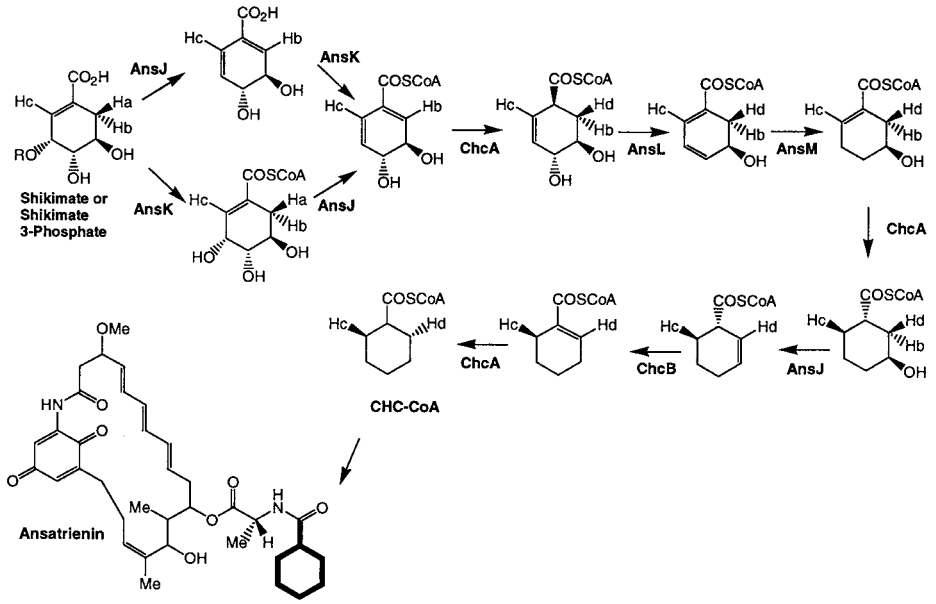

Figure 2 The CHC CoA biosynthetic pathway and its role in ansatrienin biosynthesis in *S. collinus*. Proposed roles of the proteins (AnsJ–AnsM) encoded by the CHC CoA biosynthetic genes are shown. The roles of ChcA and ChcB have been demonstrated by *in vivo* and *in vitro* analyses. The *chcB* gene encoding ChcB is not located with the *S. collinus* CHC CoA biosynthetic genes.

In conclusion, we have, in collaboration with our colleagues at Pfizer, used a rational approach to genetically engineer a strain of *S. avermitilis* that specifically generates doramectin, a commercial novel avermectin analog, under standard fermentation conditions. This strain has been obtained by inactivation of the pathway that provides the normal avermectin biosynthetic starter units (isobutyryl CoA and 2-methylbutyryl CoA) and introduction of a pathway that provides the CHC CoA required for initiating doramectin biosynthesis.

Genetic approaches for manipulation of the ratio of monensins A and B products made by *S. cinnamonensis*

Common precursors used for the elongation steps in the biosynthesis of natural products such as avermectin, erythromycin, pikromycin and rapamycin are malonyl CoA and methylmalonyl CoA [1,14,16,40]. The malonyl CoA is likely derived from the carboxylation of acetyl CoA, while a variety of different pathways provide methylmalonyl CoA (Figure 3) [17]. A number of streptomycete polyketide synthases, such as those involved in monensin, FK520, tylosin and niddamycin, also utilize ethylmalonyl CoA at a specific stage in the polyketide chain assembly [10,15,17,31]. This ethylmalonyl CoA is likely derived from carboxylation of butyryl CoA (Figure 3). In cases such as monensin and FK520, either methylmalonyl CoA or ethylmalonyl CoA can be utilized at the same stage in elongation, and reflects relaxed substrate specificity for the corresponding acyl transferase (AT) domain of the PKS [17]. Thus, fermentations of *S. cinnamonensis* produce a mixture of monensins A and B analogs (Figure 4) in a ratio presumably dependent upon the relative concentrations of ethylmalonyl CoA and methylmalonyl CoA. Manipulation of these precursor pools by changing the amounts of enzymes involved in their production should lead to predictable changes in the ratio of the two analogs. Furthermore, under some fermentation conditions, the absolute levels of these precursors presumably limit total monensin titers.

Ethylmalonyl CoA

A desire to both increase monensin titers and control the ratio of monensin analogs in a fermentation process led us to investigate the different pathways which contribute to production of ethylmalonyl CoA and methylmalonyl CoA [17,42]. Our initial interest lay in the pathways that lead to butyryl CoA-derived ethylmalonyl CoA. Experiments involving table isotope incorporation have indicated the presence of at least two pathways for butyryl CoA production in streptomycetes. One pathway involves isomerization of the valine catabolite, isobutyryl CoA, to form butyryl CoA and is catalyzed by the coenzyme B_{12}-dependent isobutyryl CoA mutase (ICM) [24,41] (Figure 3). The second pathway involves a condensation of two acetate units and is thought to culminate in a reduction of crotonyl CoA to butyryl CoA, catalyzed by crotonyl CoA reductase (CCR) (Figure 3) [35]. This enzyme was first purified from *S. collinus*, and the corresponding *ccr* gene was shown to be located within a set of primary metabolic genes involved in acetate assimilation in *S. collinus* [35]. A similar set of genes has recently been identified from sequencing the *S. coelicolor* chromosome. Subsequently, *ccr* homologues have been observed within the biosynthetic gene clusters of tylosin, niddamycin and coronafacic acid — all natural products that utilize an ethylmalonyl CoA precursor [10,15,23]. Although *ccr* homologues were found in these organisms, their role in providing the ethylmalonyl CoA precursor was not established.

We have recently cloned a *ccr* gene from the C730.1 *S. cinnamonensis* strain and have shown that it is located within a conserved set of primary metabolic genes [17]. A *S. cinnamonensis* mutant strain (L1) was constructed by inserting the *hyg* resistance gene into a unique *Bgl*II site within the *ccr* coding region [17].

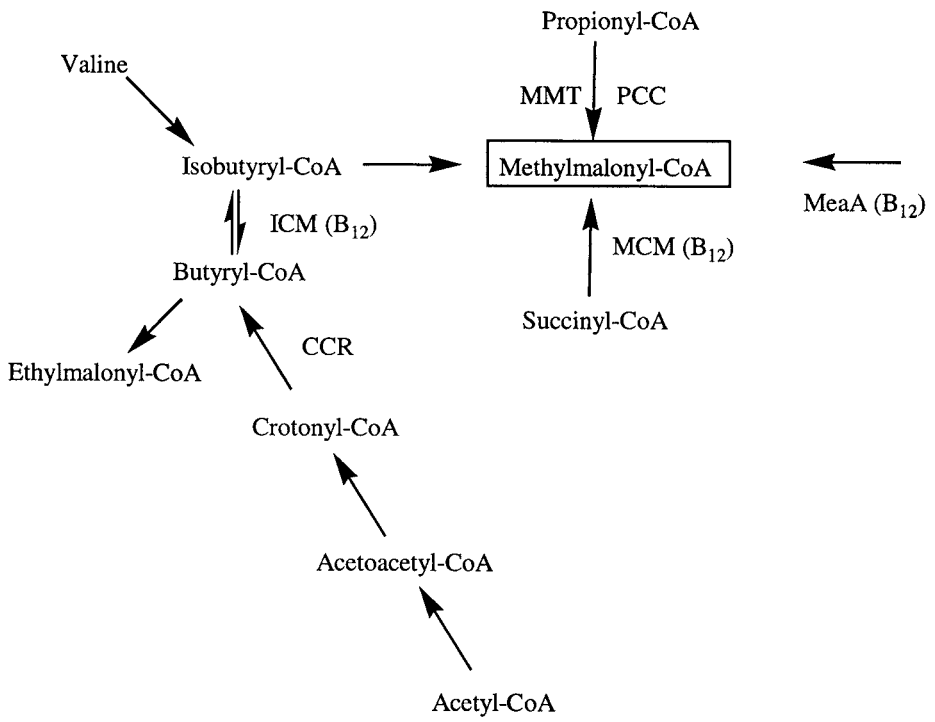

Figure 3 Proposed pathways for methylmalonyl CoA formation in *S. cinnamonensis*. The substrate for MeaA is unknown. Ethylmalonyl CoA is used for monensin A biosynthesis, while methylmalonyl CoA is used for both monensin A and B biosynthesis (see Figure 6). B_{12} indicates known or putative coenzyme B_{12}-dependent mutase. MCM: methylmalonyl CoA mutase, ICM: isobutyryl CoA mutase, MMT: methylmalonyl CoA transcarboxylase, PPC: propionyl CoA carboxylase, CCR: crotonyl CoA reductase.

CCR activity in the L1 mutant decreased by more than 90% in both yeast extract malt extract medium and a complex fermentation medium used for monensin production, as compared to the C730.1 strain. In the latter medium, *S. cinnamonensis* L1 produced monensins A and B in a percent ratio of 12:88, dramatically lower than the 50:50 ratio observed for both C730.1 (Figure 5). No overall change in monensin titers was observed. Using the *ermE** promoter, the *S. collinus ccr* gene was expressed from a plasmid in *S. cinnamonensis* C730.1/pHL18 and L1/pHL18. Plasmid-based (pHL18) expression of the *S. collinus ccr* gene in the L1 mutant restored the monensin A-to-monensin B ratio to approximately that observed in the C730.1 strain. The C730.1

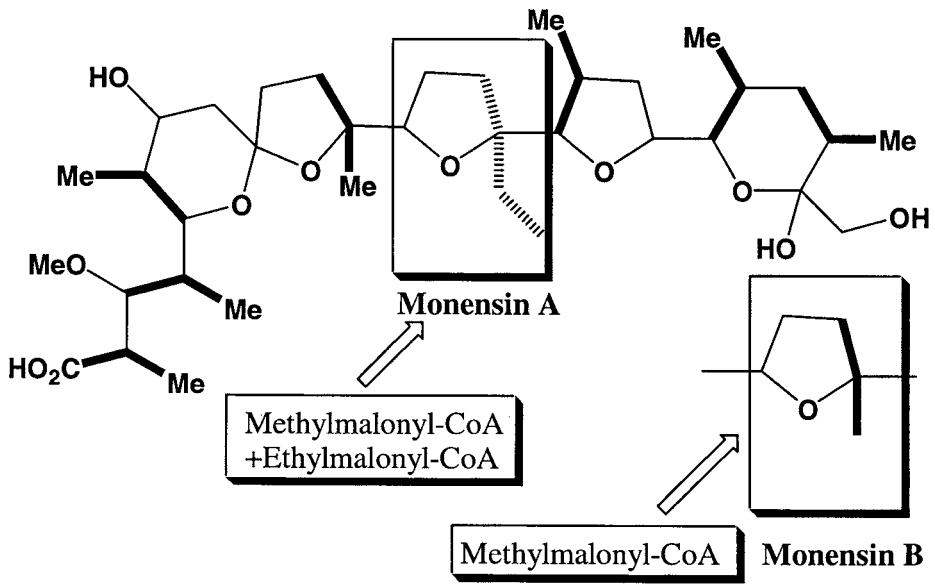

Figure 4 The structures of monensins A and B. Methylmalonyl CoA-derived positions in both monensins A and B are marked in bold. The ethylmalonyl CoA-derived position in monensin A is marked with a hashed bond.

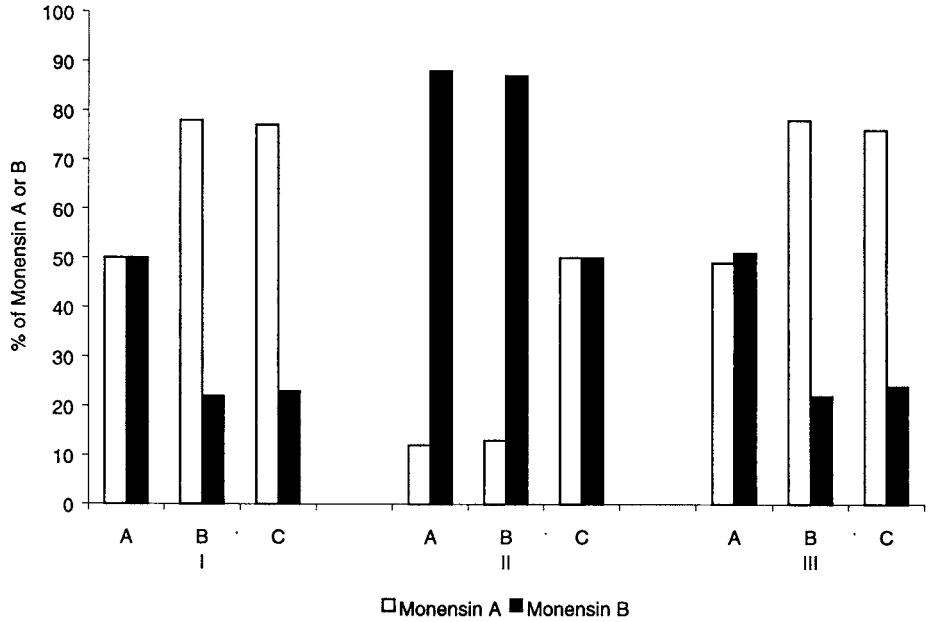

Figure 5 Relative amounts of monensins A and B produced by *S. cinnamonensis* C730.1 (I), L1 (II) and C730.1/pHL18 (III) grown in a complex fermentation medium (A), and media supplemented with crotonic acid (B) and butyric acid (C).

strain carrying pHL18 produced monensins A and B in the same ratio as its parent, despite having significantly higher levels of CCR activity (Figure 5).

These results indicated that CCR plays a significant role in providing butyryl CoA for monensin A biosynthesis, but is present in *S. cinnamonensis* C730.1 at a sufficient level that some other factor is limiting. The hypothesis, that availability of the appropriate crotonyl CoA substrate is limiting, was tested by feeding either 15 mM crotonic acid or 15 mM butyric acid to fermentations of the L1, C730.1 and C730.1/pHL18 strains [17]. As expected, addition of either crotonic acid or butyric acid increased the amount of monensin A relative to monensin B for both the C730.1 and C730.1/pHL18 strains (Figure 5). An increase in the monensin A-to-monensin B ratio was also observed for addition of butyric acid, but not crotonic acid, to the L1 mutant, which is consistent with the lack of significant CCR activity (Figure 5). Overall, these experiments support the role of the enzyme CCR and the crotonyl CoA substrate as the major pathway for providing ethylmalonyl CoA for monensin A biosynthesis under our fermentation conditions [17]. We reached a similar conclusion regarding formation of ethylmalonyl CoA in the erythromycin producer, *Saccharopolyspora erythraea* [29]. In that collaboration with researchers at Abbott Laboratories, we demonstrated that expression of a *ccr* gene is necessary for production of an ethylmalonyl CoA-derived 6-desmethyl-6-ethylerythromycin product [29].

Methylmalonyl CoA

We have also been interested in the pathways that provide methylmalonyl CoA for monensin biosynthesis [42]. The first of three generally accepted routes to methylmalonyl CoA is isomerization of succinyl CoA, catalyzed by the coenzyme B_{12}-dependent methylmalonyl CoA mutase (MCM) [18,32]. The second route is carboxylation of propionyl CoA, catalyzed by either propionyl CoA carboxylase (PCC) [3,26] or methylmalonyl CoA transcarboxylase (MMT) [13]. The third route is a multistep oxidation of isobutyryl CoA [24] (Figure 3). The *mutAB* genes encoding MCM involved in the first of these pathways have been cloned from the monensin A-producing *S. cinnamonensis* [2], as well as from rifamycin SV-producing *Amycolatopsis mediterranei* U32 [43] and other prokaryotic and mammalian sources [19,38]. A *mut* disruption in *S. cinnamonensis* has been reported to have no effect on either total monensin production or the analog ratio [34]. Plasmid-based overexpression of the *mutAB* gene in *S. cinnamonensis*, on the other hand, has been reported to give both a slight increase in total monensin production and a decreased ratio of monensin A to monensin B [43]. These observations suggest, under these conditions, that 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 [42]. These observations suggest that other pathways, such as carboxylation of propionyl CoA or oxidation of isobutyryl CoA, might provide methylmalonyl CoA for monensin production. In this regard, the *icm* genes encoding an *S. cinnamonensis* ICM — an enzyme that catalyzes formation of isobutyryl CoA from butyryl CoA — were recently cloned and sequenced (Figure 3) [41]. Insertional inactivation of *icm* has been shown to have no detectable effect on monensin production [34]. 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. The major pathway that provides methylmalonyl CoA for monensin biosynthesis under the fermentation conditions used in these experiments thus remains unknown.

We have recently isolated a *meaA* gene from *S. cinnamonensis* and have shown that it potentially encodes a 74-kDa protein with significant amino acid sequence identity to methylmalonyl CoA mutase (MCM) (40%), isobutyryl CoA mutase (ICM) large subunit (36%) and small subunit (52%)

from the same organism [42]. The predicted C-terminus of MeaA contains structural features highly conserved in all coenzyme B_{12}-dependent mutases. The *meaA* gene has been previously identified from both *S. collinus* [12] and *Methylobacterium extorquens* AM1 [6,28] and shown to be involved in a pathway for acetate assimilation. While the exact catalytic function of MeaA is unknown, the similarity to ICM and MCM suggested a potential role in generating methylmalonyl CoA for monensin biosynthesis in *S. cinnamonensis*. This hypothesis was supported by an observation that plasmid-based expression of *meaA* from the *ermE** promoter in the *S. cinnamonensis* C730.1 strain decreased the ratio of monensin A to monensin B from 50:50 to 30:70 [42]. Conversely, this ratio increased to greater that 85:25 in a *meaA* mutant, *S. cinnamonensis* WM2 (generated from the C730.1 strain by insertional inactivation of *meaA* using the erythromycin resistance gene) (Figure 6). In both of these experiments, the overall monensin titers were not significantly affected. However, monesin titers did decrease by more than 90% in a *S. cinnamonensis* WD2 strain (an *icm*, *meaA* mutant) (Figure 6). Almost all of the monensins produced by this mutant were in the form of monensin A (consistent with decreased levels of methylmalonyl CoA relative to ethylmalonyl CoA). The reason why insertional inactivation of *meaA* has a more pronounced effect on the *icm* mutant than on the C730.1 strain is unclear at present. Nonetheless, monensin titers in the WD2 strain were restored to at least wild-type levels by plasmid-based expression of the *meaA* gene (Figure 6). In this case, the monensin ratio shifted in favor of the B analog, consistent with increased levels of methymalonyl CoA relative to ethylmalonyl CoA. A similar observation of restoration of monensin production and shift toward the B analog was observed with plasmid-based expression of the *A. mediterranei mutAB* gene (encoding MCM) in the WD2 strain [42]. These experiments clearly indicate that alterations in the levels of MeaA in *S. cinnamonensis* can lead to significant changes in amounts of methylmalonyl CoA available for monensin biosynthesis, and that its role can be efficiently replaced by MCM.

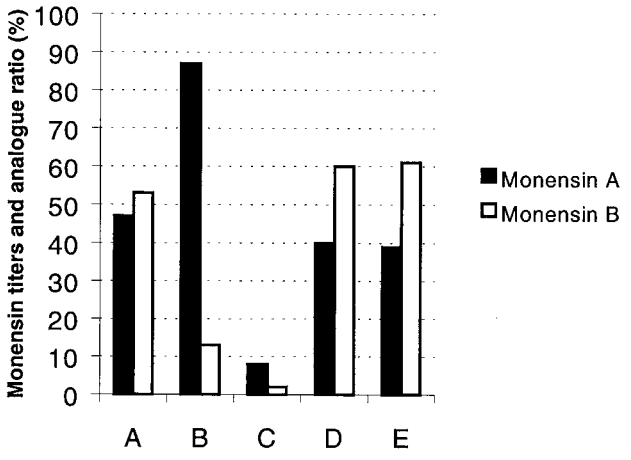

Figure 6 Monensin titers and analogue ratios for various *S. cinnamonensis* mutants in complex fermentation medium. (A) C730.1 (wild-type strain). (B) *meaA* mutant (WM2). (C) *icm*, *meaA* mutant (WD2). (D) WM2/pZR8 (plasmid-based expression of *meaA*). (E) WD2/pZR8. Total monensin titers (monensins A and B) are expressed as a percentage of that obtained using the C730.1 strain.

In conclusion, these studies have helped determine the major pathways and enzymes that generate methylmalonyl CoA and ethylmalonyl CoA for monensin biosynthesis under one set of fermentation conditions. The levels of enzymes in these pathways can be controlled at the genetic level, allowing for rational manipulation of the relative levels of these precursors and thus the monensin analogs. Similar approaches could be applied to other fermentation processes where the nonselective use of these precursors in extension steps in a polyketide biosynthesis gives rise to multiple polyketide products.

The use of heterologous ACP domains in the pikromycin polyketide synthase alters the ratio of 10-deoxymethynolide and narbonolide products

The pikromycin PKS of *S. venezuelae* is unusual in that it generates both 12- and 14-membered ring macrolactone products under different culture conditions [40]. This system generates a hexaketide product by using three polypeptides, PikAI, PikAII and PikAIII, to elongate a propionyl starter unit through five elongation steps (Figure 7). In PGM medium, PikAIV (containing just one module) catalyzes an additional elongation step using methylmalonyl CoA, and generates a heptaketide product [39]. This product is then released from PikAIV by a TE domain and cyclized to produce narbonolide, which is subsequently converted to narbomycin and pikromycin. In SCM media, polyketide chain extension is terminated at the end of PikAIII to generate 10-deoxymethynolide, which in turn is converted to methymycin and neomethymycin [39]. The use of SGGP media results in a mixture of both 12- and 14-membered ring macrolactone products. The premature chain termination is catalyzed by the TE domain at the C-terminus of PikAIV [39]. It has recently been shown that under these conditions, an alternative translational start codon 600 nucleotides downstream of the normal *pikAIV* start codon is used, giving rise to an N-terminal truncated PikAIV containing only half of the KS_6 domain that is unable to catalyze the final chain elongation step [39]. It has been proposed that partial loss of this domain allows the hexaketide product bound to the ACP domain of PikAIII to "skip" the last extension step and be cyclized to a 12-membered ring product by the TE domain of the truncated PikAIV (Figure 8) [39].

One of the most direct ways to alter the ratio of polyketide products generated by the pikromycin PKS under a standard set of fermentation conditions is by mutation of the alternative translation start codon so that only a full-length PikAIV is produced. Such an approach predictably leads to production of only narbonolide-derived products [39]. A similar mutational approach could be used to ensure that only the N-terminal-deleted PikAIV could be generated, allowing selective production of the 10-deoxymethynolide products. We have tried a less direct approach in which the ACP catalytic domain in PikAIV is exchanged with ACP domains from the rapamycin PKS (Figure 9) [5]. In this work, we first generated a strain of *S. venezuelae* (SC1022), which produces only the aglycone products, 10-deoxymethynolide and narbonolide (Figure 9). Under standard conditions, this strain (which contains the wild-type PikAIV) generated predominantly narbonolide (52 mg/l), with lower levels of 10-deoxymethynolide (12 mg/l). Strain SC1016 containing a hybrid PikAIV, in which the ACP domain was replaced with an ACP domain from module 14 of the rapamycin PKS, produced approximately 20% of the total

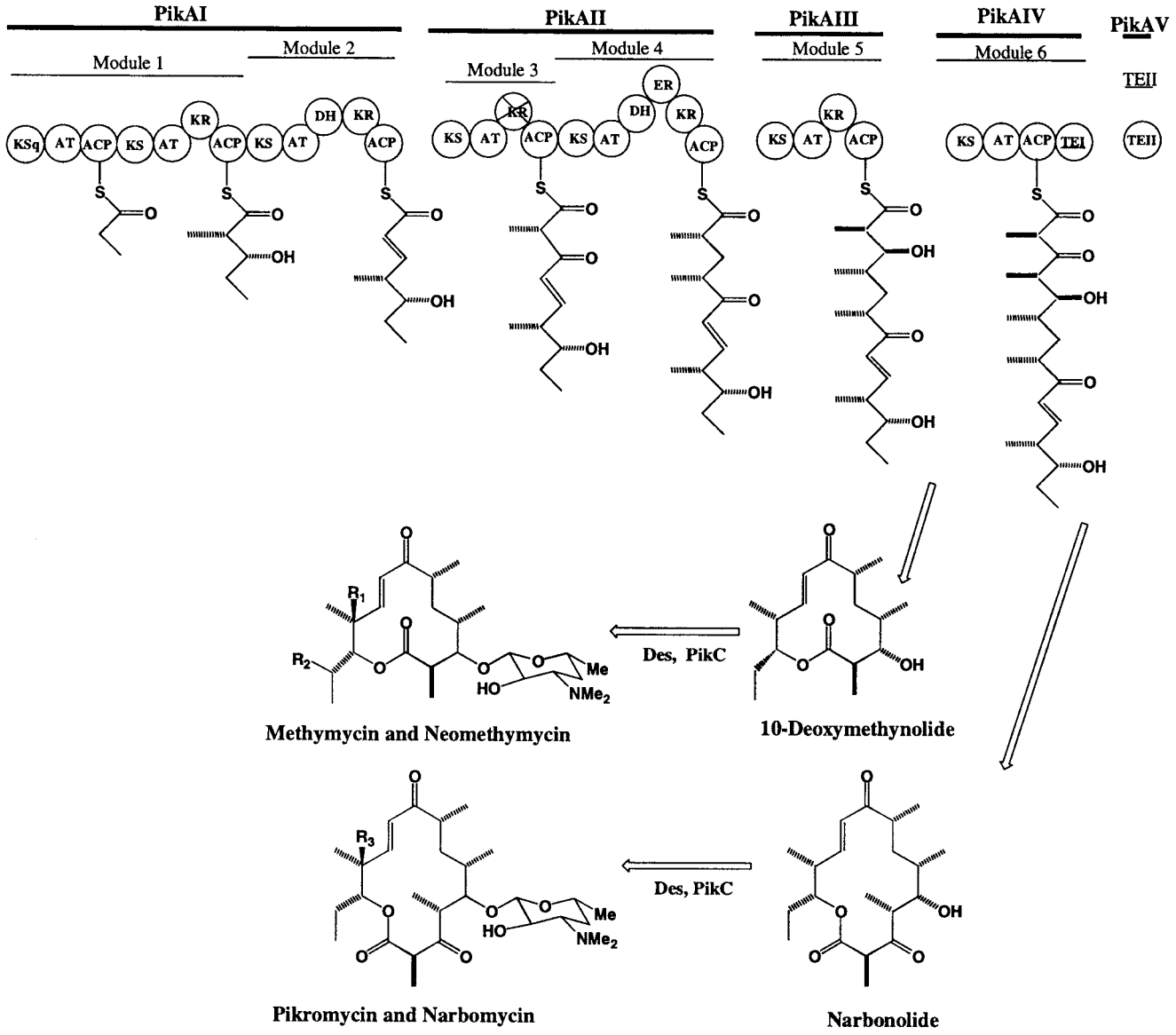

Figure 7 Biosynthesis of 10-deoxymethynolide and narbonolide by the PKS (adapted from Xue and Sherman [39]). The system is comprised of six modules for elongation and one loading module. Catalytic domains with the modules of the PKS are represented by circles (KS, ketosynthase; AT, acyl transferase; KR, ketoreductase; ACP, acyl carrier protein; DH, dehydratase, ER, enoyl reductase; KS^Q a methylmalonyl decarboxylase; TE, thioesterase). Final products generated by the action of the desosamine biosynthetic enzymes (Des) and a cytochrome *P*450 monooxygenase (PikC) are methymycin (R_1, OH; R_2, H), neomethymycin (R_1, H; R_2, OH), pikromycin (R_3, OH) and narbomycin (R_3, H).

polyketide products made by the SC1022 strain. In the SC1016 strain, all products were in the form of 10-deoxymethynolide (13 mg/l). Strain SC1015, in which the ACP domain in PikAIV was replaced with the ACP domain from module 13 of the rapamycin PKS, generated the same overall yield of polyketide products as SC1022, but with substantially more 10-deoxymethynolide (48 mg/l) than narbonolide (15 mg/l) [5].

The reason a hybrid PikAIV containing the $_{\mathrm{rap}}\mathrm{ACP}_{13}$ (SC1015), and not the $_{\mathrm{rap}}\mathrm{ACP}_{14}$ (SC1016), can catalyze elongation is unclear, but it may reflect a difference between the linker regions flanking the two ACPs. It is also puzzling that the hybrid PikAIV in SC1016 is less efficient at elongation, yet more efficient at chain termination at PikAIII, than the wild-type PikAIV. One possible explanation is that both the full-length and N-terminally truncated forms of PikAIV are produced and they rapidly associate with, and dissociate from, PikAIII [5]. In this model, a hybrid PikAIV that is less efficient at chain elongation would decrease narbonolide production, and provide greater opportunities for the truncated PikAIV to terminate elongation at PikAIII, and increase 10-deoxymethynolide production. An alternative explanation is that the elongated polyketide chain attached to the ACP domain of PikAIII is passed onto the ACP domain of PikAIV *before* being released by the cognate TE domain as 10-deoxymethynolide. The efficiency of the "skipping" step (10-deoxymethynolide production), relative to a normal PikAIV-catalyzed elongation step (narbonolide production), might change in strains that contain a hybrid PikAIV with a heterologous ACP domain.

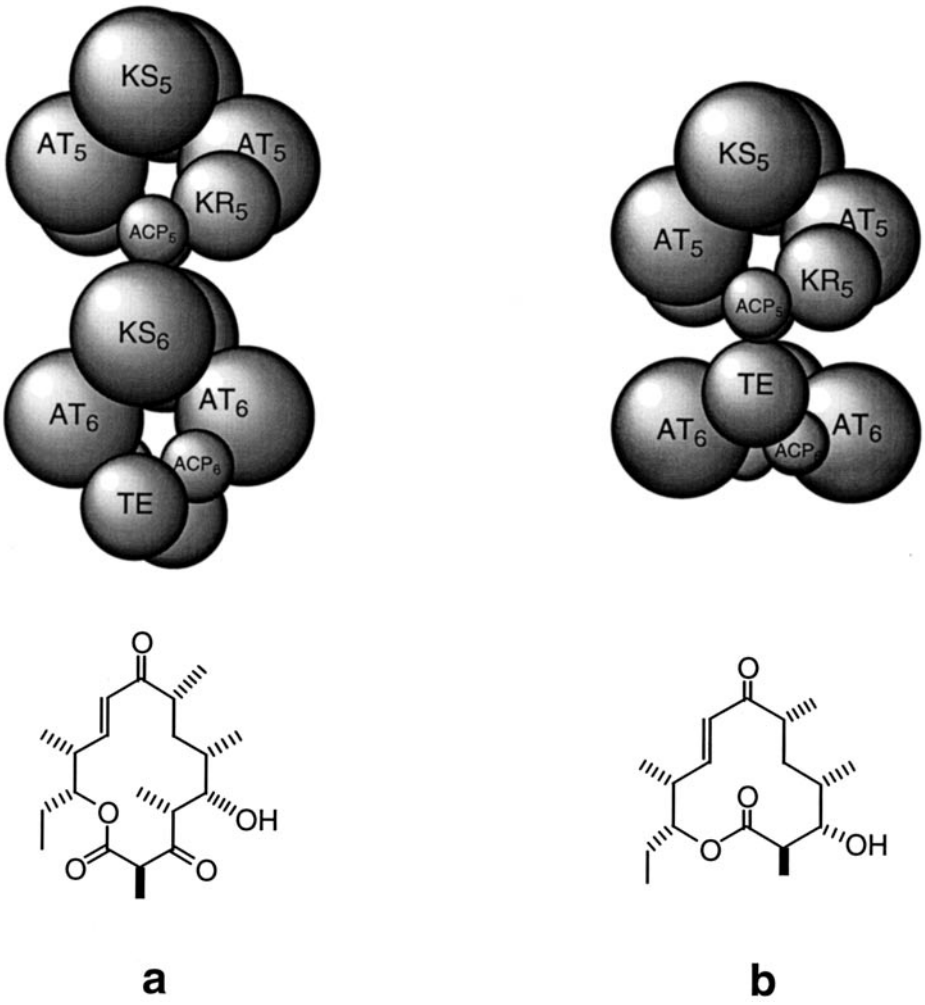

Figure 8 Proposed model for the role of PikAIV in production of narbonolide (a) and 10-deoxymethynolide (b) (adapted from Xue and Sherman [39]). Abbreviations for the catalytic domains (depicted as spheres) are provided in Figure 9.

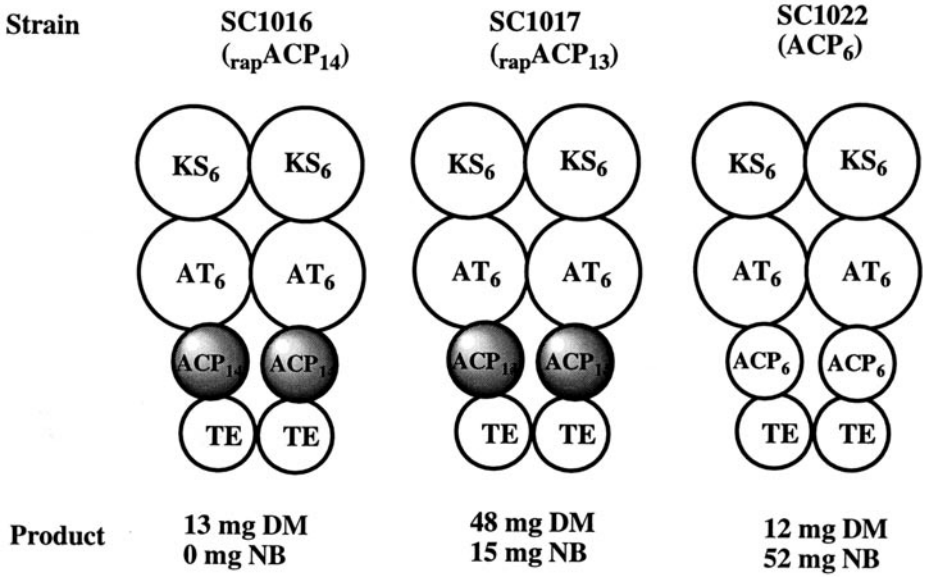

Figure 9 Yields of 10-deoxymethynolide (DM) and narbonolide (NB) aglycones from fermentations of *S. venezuelae* strains containing wild-type (SC1022) and hybrid PikAIV polypeptides, ACP_{13} and ACP_{14}, are catalytic domains from the corresponding modules of the rapamycin PKS.

While the exact mechanism by which 10-deoxymethynolide is generated by the TE domain of PikAIV remains to be determined, it is clear that several different genetic methods can be employed to alter the ratio of 10-deoxymethynolide and narbolide-derived products generated from an *S. venezuelae* fermentation.

Acknowledgements

This work was supported by grants from the National Institutes of Health (GM 50542 and GM 48562) and the National Science Foundation (MCB 941851). Our work on pikromycin PKS was carried out in collaboration with David Sherman and Alex Xue at the University of Minnesota. We also acknowledge financial support and a fruitful collaboration with Hamish McArthur and Ron Fedechcko in the Bioprocess Research Group, Pfizer.

References

1 Aparico JF, I Molnar, T Schwecke, A Konig, SF Haydock, LE Khaw, J Staunton and PF Leadley. 1996. Organization of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of the enzymatic domains in the modular polyketide synthase. *Gene* 169: 9–16.

2 Birch A, A Leiser and JA Robinson. 1993. Cloning, sequencing, and expression of the gene encoding methylmalonyl-coenzyme A mutase from *Streptomyces cinnamonensis*. *J Bacteriol* 175: 3511–3519.

3 Bramwell H, IS Hunter, JR Coggins and HG Nimmo. 1996. Propionyl CoA carboxylase from *Streptomyces coelicolor* A3(2): cloning of the gene encoding the biotin-containing subunit. *Microbiology* 142 (Pt 3): 649–655.

4 Chen S, D von Bamberg, V Hale, M Breuer, B Hardt, R Muller, HG Floss, KA Reynolds and E Leistner. 1999. Biosynthesis of ansatrienin (myocotrienin) and naphthomycin. Identification and analysis of two separate biosynthetic gene cluster in *Streptomyces collinus* Tu 1892. *Eur J Biochem* 261 (1): 98–107.

5 Chen S, Y Xue, DH Sherman and KA Reynolds. 2000. Mechanisms of molecular recognition in the pikromycin polyketide synthase. *Chem Biol* 7 (12): 907–918.

6 Chistoserdova LV and ME Lidstrom. 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.

7 Cropp TA, D Wilson and KA Reynolds. 2000. Combinatorial biosynthesis: Identification of a cyclohexylcarbonyl CoA biosynthetic gene cluster and application in the engineered production of the polyketide doramectin. *Nat Biotechnol* 18: 980–983.

8 Denoya CD, RW Fedechko, EW Hafner, HAI McArthur, MR Morgenstern, DD Skinner, K Stuztzman-Engwall, RG Wax and WC Wernau. 1995. A second branched-chain alpha-keto acid dehydrogenase gene cluster (bkdFGH) from *Streptomyces avermitilis*: Its relationship to avermectin biosynthesis and construction of a bkdF mutant suitable for production of novel antiparasitic avermectins. *J Bacteriol* 177: 3504–3511.

9 Dutton CJ, SP Gibson, AC Goudie, KS Holdom, MS Pacey, JC Ruddock, JD Bu'lock and MK Richards. 1991. Novel avermectins produced by mutational biosynthesis. *J Antibiot (Tokyo)* 44: 357–365.

10 Gandecha AR, SL Large and E Cundliffe. 1997. Analysis of four tylosin biosynthetic genes from the *tylM* region of the *Streptomyces fradiae* genome. *Gene* 184: 197–203.

11 Hafner EW, BW Holley, KS Holdom, SE Lee, RG Wax, D Beck, HA McArthur and WC Wernau. 1991. Branched-chain fatty acid requirement for avermectin production by a mutant of *Streptomyces avermitilis* lacking branched-chain 2-oxo acid dehydrogenase activity. *J Antibiot (Tokyo)* 44 (3): 349–356.

12 Han L and KA Reynolds. 1997. A novel alternate anaplerotic pathway to the glyoxylate cycle in streptomycetes. *J Bacteriol* 179: 5157–5164.

13 Hunaiti AR and PE Kolattukudy. 1982. Isolation and characterization of an acyl-coenzyme A carboxylase from an erythromycin-producing *Streptomyces erythreus*. *Arch Biochem Biophys* 216 (1): 362–371.

14 Ikeda H, T Nonomiya, M Usami, T Ohta and S Omura. 1999. Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*. *Proc Natl Acad Sci USA* 96 (17): 9509–9514.

15 Kakavas SJ, L Katz and D Stassi. 1997. Identification and characterization of the niddamycin polyketide synthase genes from *Streptomyces caelestis*. *J Bacteriol* 179: 7515–7522.

16 Katz L. 1997. Manipulation of modular polyketide synthases. *Chem Rev* 97: 2557–2575.

17 Liu H and KA 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 (21): 6806–6813.

18 Marsh EN. 1999. Coenzyme B12 (cobalamin)-dependent enzymes. *Essays Biochem* 34 (3): 139–154.

19 Marsh EN, N McKie, NK Davis and PF Leadlay. 1989. Cloning and structural characterization of the genes coding for adenosylcobalamin-dependent methylmalonyl CoA mutase from *Propionibacterium shermanii*. *Biochem J* 260: 339–352.

20 McArthur HAI. 1998. The novel avermectin, doramectin — a successful application of mutasynthesis. In: Hutchinson CR and J McAlpine (Eds.), Development in Industrial Microbiology – BMP'97, pp. 43–48.

21 Moore BS, H Cho, R Casati, E Kennedy, KA Reynolds, U Mocek, JM Beale and HG Floss. 1993. Biosynthetic an ansatrienin A. Formation of the cyclohexanecarboxylic acid moiety. *J Am Chem Soc* 115: 5254–5266.

22 Patton SM, T Ashton Cropp and KA Reynolds. 2000. A novel Δ^3, Δ^2 enoyl CoA isomerase involved in the biosynthesis of the cyclohexanecarboxylic acid-derived moiety of the polyketide ansatrienin A. *Biochemistry* 39: 7595–7604.

23 Rangaswamy V, R Mitchell, M Ullrich and C Bender. 1998. Analysis of genes involved in biosynthesis in coronafacic acid, the polyketide component of the phytotoxin coronatine. *J Bacteriol* 180: 3330–3338.

24 Reynolds K, D O'Hagan, D Gani and JA Robinson. 1988. Butyrate metabolism in *Streptomycetes*. Characterization of a vicinal interchange rearrangement linking isobutyrate and butyrate in *Streptomyces cinnamonensis*. *J Chem Soc, Perkin Trans 1* 3195–3207.

25 Reynolds KA, P Wang, KM Fox, S Lam, MK Speedie and HG Floss. 1992. Purification and characterization of a novel enoyl CoA reductase from *Streptomyces collinus*. *J Bacteriol* 174: 3850–3854.

26 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 (Pt 11) (4): 3109–3119.

27 Schwecke T, JF Aparico, I Molner, A Konig, LE Khaw, SF Haydock, M Oliynyk, P Caffrey, J Cortes, JB Lester, GA Bohm, J Staunton and PF Leadley. 1995. The biosynthetic gene cluster for the polyketide immunosuppresant rapamycin. *Proc Natl Acad Sci USA* 92: 7839–7843.

28 Smith LM, WG Meijer, L Dijkhuizen and P Goodwin. 1996. A protein having similarity with methylmalonyl CoA mutase is required for assimilation of methanol and ethanol by *Methylobacterium extorquens* AMI. *Microbiology* 142: 657–684.

29 Stassi DL, SJ Kakavas, KA Reynolds, G Gunawardana, S Swanson, D Zeidner, M Jackson, H Liu, A Buko and L Katz. 1988. Ethyl-substituted erythromycin derivatives produced by directed metabolic engineering. *Proc Natl Acad Sci USA* 95: 7305–7309.

30 Sugita M, Y Natori, N Sueda, K Furihata, H Seto and N Otake. 1982. Studies of mycotrienin antibiotics, a novel class of ansamycins: III. The isolation, characterization and structures of mycotrienols I and II. *J Antibiot* 35: 1474–1479.

31 Sun W-S, P Salmon, J Wilson and N Connors. 1998. Crotonic acid directed biosynthesis of the immunosuppressants produced by *Streptomyces hygroscopicus* var. *ascomyceticus*. *J Ferment Bioeng* 86: 261–265.

32 Thoma NH and PF Leadlay. 1998. Mechanistic and structural studies on methylmalonyl CoA mutase. *Biochem Soc Trans* 26 (3): 293–298.

33 Toutain PL, DW Upson, TN Terhune and ME Mckenzie. 1997. Comparative pharmacokinetics of doramectin and ivermectin in cattle. *Vet Parasitol* 72 (1): 3–8.

34 Vrijbloed JW, K Zerbe-Burkhardt, A Ratnatilleke, A Grubelnik-Leiser and JA 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 (18): 5600–5605.

35 Wallace KK, Z Bao, H Dai, R Digate, G Schuler, MK Speedie and KA Reynolds. 1995. Purification of crotonyl CoA reductase from *Streptomyces collinus* and cloning, sequencing and expression of the corresponding gene in *Escherichia coli*. *Eur J Biochem* 233: 954–962.

36 Wang P, CD Denoya, MR Morgenstern, DD Skinner, KK Wallace, R DiGate, S Patton, N Banavali, G Schuler, MK Speedie and KA Reynolds. 1996. Cloning and characterization of the gene encoding 1-cyclohexenylcarbonyl CoA reductase from *Streptomyces collinus*. *J Bacteriol* 178 (23): 6873–6881.

37 Weber W, H Zahner, M Damberg, P Russ and A Zeeck. 1981. Stoffwechselprodukte von Mikroorganismen. 201. Ansatrienin A und B, fungistatische Antibiotica aus *Streptomyces collinus*. *Zentralb Bakteriol, Mikrobiol Hyg, Abt 1, Orig C* 2: 122–124.

38 Wilkemeyer MF, AM Crane and FD Ledley. 1990. Primary structure and activity of mouse methylmalonyl CoA mutase. *Biochem J* 271 (2): 449–455.

39 Xue Y and DH Sherman. 2000. Alternative modular polyketide synthase expression controls macrolactone structure. *Nature* 403 (3): 571–574.

40 Xue Y, L Zhao, H-W Liu and DH Sherman. 1998. A gene cluster for macrolide antibiotic biosynthesis in *Streptomyces venezuelae*: Architecture of metabolic diversity. *Proc Natl Acad Sci USA* 95: 12111–12116.

41 Zerbe-Burkhardt K, A Ratnatilleke, N Philippon, A Birch, A Leiser, JW Vrijbloed, D Hess, P Hunziker and JA 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.

42 Zhang W and KA Reynolds. 2001. MeaA, a putative coenzyme B(12)-dependent mutase, provides methylmalonyl coenzyme A for monensin biosynthesis in *Streptomyces cinnamonensis*. *J Bacteriol* 183 (6): 2071–2080.

43 Zhang W, L Yang, W Jiang, G Zhao, Y Yang and J Chiao. 1999. Molecular analysis and heterologous expression of the gene encoding methylmalonyl-coenzyme A mutase from rifamycin SV-producing strain *Amycolatopsis mediterranei* U32. *Appl Biochem Biotechnol* 82 (3): 209–225.