6-Deoxyerythronolide B Analogue Production in *Escherichia coli* through Metabolic Pathway Engineering

Jonathan Kennedy,‡ Sumati Murli,‡ and James T. Kealey*

Kosan Biosciences, Inc., 3832 Bay Center Place, Hayward, California 94545

Received July 3, 2003

ABSTRACT: The erythromycin precursor polyketide 6-deoxyerythronolide B (6-dEB) is produced from one propionyl-CoA starter unit and six (2*S*)-methylmalonyl-CoA extender units. In vitro studies have previously demonstrated that the loading module of 6-deoxyerythronolide B synthase (DEBS) exhibits relaxed substrate specificity and is able to accept butyryl-CoA, leading to the production of polyketides with butyrate starter units. We have shown that we can produce butyryl-CoA at levels of up to 50% of the total CoA pool in *Escherichia coli* cells that overexpress the acetoacetyl-CoA:acetyl-CoA transferase, AtoAD (EC 2.8.3.8), in media supplemented with butyrate. The DEBS polyketide synthase (PKS) used butyryl-CoA and methylmalonyl-CoA supplied in vivo by the AtoAD and methylmalonyl-CoA mutase pathways, respectively, to produce 15-methyl-6-dEB. Priming DEBS with endogenous butyryl-CoA affords an alternative and more direct route to 15-Me-6-dEB than that provided by the chemobiosynthesis method [Jacobsen, J. R., et al. (1997) *Science 277*, 367−369], which relies on priming a mutant DEBS with an exogenously fed diketide thioester. The approach described here demonstrates the utility of metabolic engineering in *E. coli* to introduce precursor pathways for the production of novel polyketides.

The rise of antibiotic-resistant pathogenic bacteria has intensified the search for new and improved antibacterial agents. One approach for generating compounds that are effective against resistant organisms involves chemical modification of existing antibiotics to enhance and expand their activities. The antibacterial erythromycins, for example, can be chemically modified to produce novel antibiotics called ketolides [e.g., ABT773, telithromycin (Figure 1)], which are active against macrolide-resistant pathogenic bacteria (*1*). A promising new series of ketolide antibiotics is derived from 15-substituted erythromycins, which are generated by a combination of chemobiosynthesis (*2*, *3*) and biochemical transformations (*4*).

In *Saccharopolyspora erythraea*, the natural erythromycin producer, the erythromycin precursor polyketide 6-deoxyerythronolide B (6-dEB) is produced from one propionyl-CoA starter unit and six (2*S*)-methylmalonyl-CoA extender units, catalyzed by 6-deoxyerythronolide B synthase (DEBS) (*5*). 6-dEB is subsequently converted to erythromycin by a series of post-polyketide synthase oxidation, glycosylation, and methylation reactions. We are interested in the production of 15-substituted 6-dEBs for use as novel scaffold precursors for erythromycin analogues. Currently, the production of erythromycin analogues is accomplished by tedious chemical syntheses and biotransformations or by "chemobiosynthesis". The most practical of these, chemobiosynthesis, involves feeding a diketide thioester of *N*-propionylcysteamine (SNPC) to an organism that expresses DEBS with an inactive ketosynthase 1 (KS1°) domain (*2*). The SNPC diketide is incorporated at DEBS KS2 and is processed to produce 15-R-6-dEB, which is subsequently fed to a bioconversion strain of *S. erythraea* deficient in 6-dEB synthesis to convert the 15-R-6-dEB into the 15-R-erythromycin analogue (*4*). Using chemobiosynthesis, many 6-dEB analogues have been produced. Analogues of particular interest are derived from 15-Me-6-dEB, which is produced from feeding 3-hydroxy-2-methylhexanyl-SNPC to *Streptomyces coelicolor* expressing DEBS with an inactive KS1 (*3*).

In the present study, we developed an *Escherichia coli*-based system for 15-Me-6-dEB production which does not rely on chemobiosynthesis. The system takes advantage of the relaxed specificity of the wild-type DEBS loading domain for different acyl-CoA starter units. When DEBS is expressed in *S. coelicolor*, a mixture of propionyl-CoA-derived 6-dEB and acetyl-CoA-derived 14-nor-6-dEB is produced. In vitro studies with purified DEBS proteins have demonstrated that both butyryl-CoA and acetyl-CoA are accepted as starter units, although propionyl-CoA is the preferred substrate (*6*, *7*). These results led us to explore the possibility of producing 15-Me-6-dEB in *E. coli* by supplying butyryl-CoA as a starter unit in vivo.

Two strategies have been successfully used to supply precursors for 6-dEB biosynthesis in *E. coli* (*8*−*10*). In both cases the starter unit, propionyl-CoA, has been produced by feeding propionate to an *E. coli* strain overexpressing PrpE, a propionyl-CoA ligase. The two pathways used for extender unit supply are the propionyl-CoA carboxylase (PCC) pathway, in which (2*S*)-methylmalonyl-CoA is produced directly from carboxylation of propionyl-CoA (*8*), and the methylmalonyl-CoA mutase/epimerase pathway, in which (2*R*)-methylmalonyl-CoA is derived from the TCA cycle intermediate succinyl-CoA and converted to the 2*S* isomer by the action of the methylmalonyl-CoA epimerase (9). The

* Address correspondence to this author. Telephone: 510-731-5224. Fax: 510-732-8401. E-mail: kealey@kosan.com.

‡ These authors contributed equally to this work.

10.1021/bi035157t CCC: $25.00
Published on Web 11/13/2003

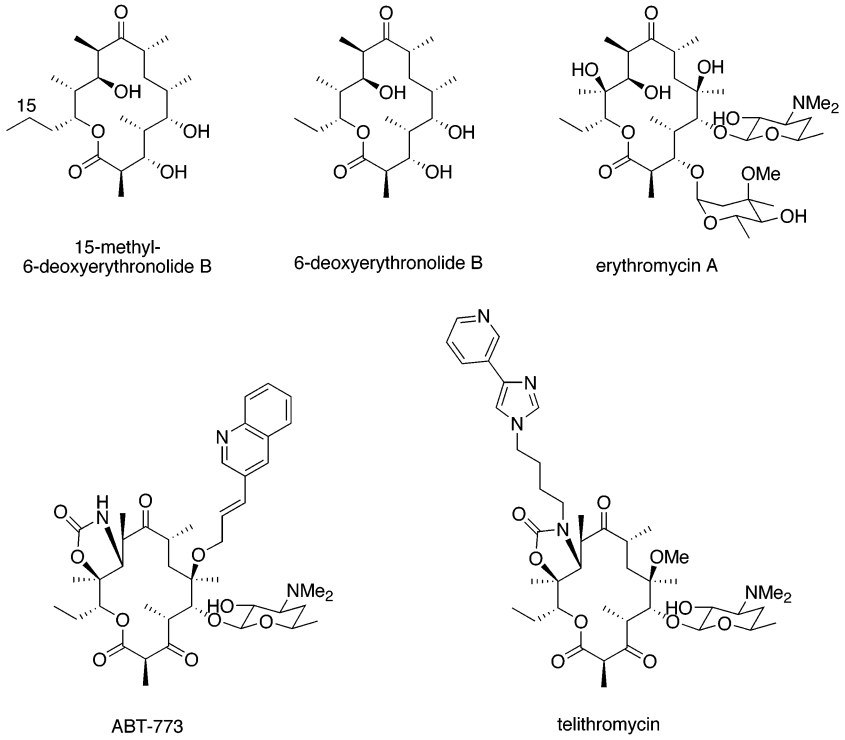

FIGURE 1: Structures of the erythromycin precursor polyketides 6-deoxyerythronolide B (6-dEB) and 15-methyl-6-dEB and the antibiotics erythromycin A and its semisynthetic ketolide derivatives ABT773 and telithromycin.

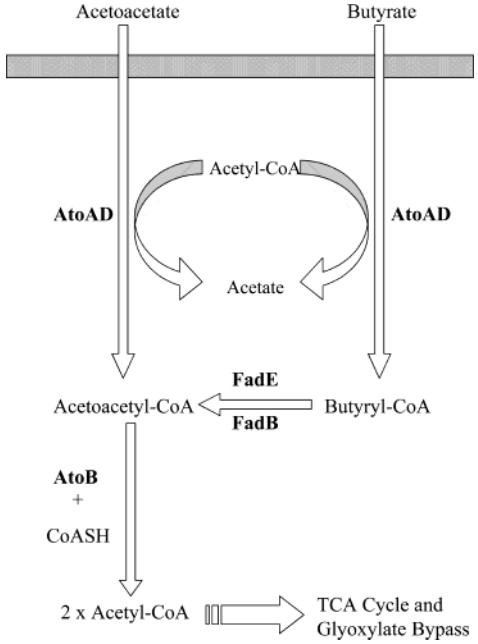

FIGURE 2: Ato pathway for utilization of acetoacetate and other four-carbon fatty acids in *E. coli*.

mutase/epimerase pathway can be used to supply the (2*S*)-methylmalonyl-CoA extender unit independently of the propionyl-CoA starter unit, thus enabling potential acyl-CoA starter unit analogues to be incorporated into the DEBS loading domain without competition from the natural starter unit, propionyl-CoA.

The *ato* operon in *E. coli* controls utilization of acetoacetate and short-chain fatty acids (Figure 2). The Ato pathway is normally induced by acetoacetate with expression controlled by AtoC, the positive transcriptional activator for the operon. Acetoacetate is activated by acetyl-CoA:acetoacetyl-CoA transferase, encoded by the *atoAD* genes (*11*). This enzyme also efficiently activates butyrate and a number of other short-chain fatty acids (A. Yeliseev et al., unpublished results) to their corresponding acyl-CoA derivatives. Mutant strains of *E. coli* capable of using butyrate as a sole carbon source constitutively express both *atoAD* and the fatty acid β-oxidation genes (*11*). Expression of the *ato* pathway alone should allow the accumulation of butyryl-CoA in the cell upon feeding butyrate.

In the background of an engineered *E. coli* strain that produces methylmalonyl-CoA by the methylmalonyl-CoA mutase pathway and harbors plasmids expressing DEBS, we overexpressed AtoC, the positive regulator of *atoAD*. Upon feeding butyrate, butyryl-CoA was produced in this strain and successfully incorporated as the starter unit into DEBS to form 15-Me-6-dEB.

MATERIALS AND METHODS

Chemicals. Antibiotics were used at the following concentrations: carbenicillin (carb), 100 μg/mL; kanamycin (kan), 50 μg/mL; streptomycin (strep), 20 μg/mL; tetracycline (tet), 7.5 μg/mL. [^{3}H]-β-Alanine (50 Ci/mmol) was purchased from American Radiolabeled Chemicals. Sodium propionate, sodium butyrate, monosodium glutamate, succinic acid, and hydroxocobalamin were purchased from Sigma and prepared as stock solutions, which were adjusted to pH 7.0−7.4. Glass beads (106 μm, acid washed) and acyl-CoA thioesters were purchased from Sigma. The CoA standard mix contained 0.5 mM CoA and 1.6 mM each of malonyl-, methylmalonyl-, succinyl-, acetyl-, propionyl-, and butyryl-CoA.

Strains. E. coli strains BL21(DE3) (Novagen, Inc.) and BAP1 (*8*), which was adapted for polyketide biosynthesis by deletion of the *prp* operon and introduction of the *prpE* and *sfp* genes under the control of the T7 promoter, have been previously described. K173-145 is a derivative of the

Table 1: *E. coli* Strains Used in This Study

strain	relevant genotype	source/reference
BL21(DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
BAP1	BL21(DE3) Δ*prpRBCD*::*T7*$_p$*sfp-T7*$_p$*prpE*	*8*
K173-145	BAP1 *panD*S25A	Kennedy et al., submitted
K207-3	K173-145 Δ*ygfG*::*T7*$_p$*accA1-T7*$_p$*pccB*	*10*
K214-037	K173-145 Δ*ygfG*::*T7*$_p$*mutAB-T7*$_p$*epi-T7*$_p$*atoC*	*10*

strain BAP1 with a mutation in the *panD* gene, *panD25A*, to allow CoA analysis through feeding of radiolabeled β-alanine (J. Kennedy et al., submitted for publication). *E. coli* strain K207-3, derived from K173-145, carries integrated copies of the *S. coelicolor* propionyl-CoA carboxylase genes, *pccB* and *accA1*, each under control of the T7 promoter and integrated at the *ygfG* locus (*10*). *E. coli* strain K214-037, also derived from K173-145, carries single copies of the *Propionibacterium shermanii* methylmalonyl-CoA mutase, *S. coelicolor* methylmalonyl-CoA epimerase, and *E. coli atoC* genes, each under control of the T7 promoter and integrated at the *ygfG* locus (*10*). See Table 1 for a list of *E. coli* strains used in this study.

Plasmids. The plasmids pBP130, expressing DEBS2 and DEBS3 under control of the T7 promoter in a plasmid backbone derived from pET21c ($carb^R$), and pKOS173-158, expressing DEBS1 under control of the T7 promoter in a backbone derived from pET28a (kan^R), have been described previously (*8*, *10*). The gene *atoC* was amplified by PCR from *E. coli* genomic DNA introducing an *Nde*I site at the start codon and an *Avr*II site downstream of the stop codon. After the sequence of the *atoC* gene was verified, the *atoC*-containing *Nde*I/*Avr*II fragment was cloned into pKOS116-95b (*9*), replacing the *mutAB*-containing *Nde*I/*Nhe*I fragment and creating plasmid pKOS149-49.A92, a pET22b derivative expressing *atoC*. The *atoAD* genes were amplified from *E. coli* genomic DNA by PCR. *Hin*dIII and *Avr*II sites were introduced upstream and downstream of the start and stop codons, respectively, and an *Nde*I site was incorporated at the ATG start codon. The 1.35 kbp product was digested with *Hin*dIII and *Avr*II and subcloned into Litmus28 (New England Biolabs) to give pKOS173-073. Following sequence verification, an *Nde*I/*Sac*I fragment containing the *atoAD* genes was subcloned from pKOS173-073 into pET26b to give pKOS173-091. The plasmid pKOS207-15a, expressing *mutAB*, *epi*, and *atoC* each under the control of independent T7 promoters in a backbone derived from pACYC184 (tet^R), has been described previously (*10*). The plasmid pKOS214-004 was derived from pKOS207-15a by digestion with *Xba*I and religation to remove the *mutAB* and *epi* genes, resulting in a pACYC184-derived plasmid with *atoC* under the control of the T7 promoter. The plasmid pKOS149-58b.A7 was made by cloning the *atoC*-containing *Nsi*I/*Avr*II fragment from pKOS149-52-45 (*10*) into the *Nsi*I/*Nhe*I sites of pKOS143-24-30 [essentially the same as pKOS143-35-50 (*9*) except the *P. shermanii* methylmalonyl-CoA epimerase is replaced by the *S. coelicolor* methylmalonyl-CoA epimerase]. pKOS149-58b.A7 has the same configuration as pKOS207-15a, *mutAB*, *epi*, and *atoC*, each under the control of independent T7 promoters in a backbone derived from pET22b ($carb^R$). For a list of relevant plasmids used in this study, see Table 2.

Acyl-CoA Analysis. The detection of ^{3}H-labeled acyl-CoAs by [^{3}H]-β-alanine feeding to *E. coli panD* strains has been described (*9*, *10*, *12*). Briefly, 20 μL of an overnight *E. coli* culture was used to inoculate 1 mL of Luria−Bertani (LB) medium containing 10 μCi of [^{3}H]-β-alanine (specific activity, 50 Ci/mmol). Media supplements, growth, and induction conditions paralleled those described in the Polyketide Analysis section below. Following growth at 22 °C, the cells were collected by centrifugation and were resuspended in 300 μL of cold (4 °C) 10% trichloroacetic acid (TCA) containing 5 μL of CoA standard mix, 0.15 mL of 100 μm diameter glass beads was added, and the samples were vortexed at 4 °C for 2 min. After centrifugation to remove precipitated protein, 100 μL of the supernatant was loaded onto an HPLC. HPLC was performed using a 150 × 4.6 mm 5μ ODS-3 Inertsil HPLC column (Ansys Technologies). HPLC buffer A contained 100 mM NaH_2PO_4 and 75 mM NaOAc, pH 4.6, and buffer B contained 40% buffer A and 60% methanol. The HPLC column was equilibrated with 95% buffer A/5% buffer B at a flow rate of 1 mL/ min. Following sample injection a linear gradient program consisting of the following steps was implemented: from 5% to 20% buffer B over 30 min, to 45% buffer B over 20 min, to 90% buffer B over 10 min, and to 5% buffer B over 1 min. The eluant was monitored at 260 nm and by an on-line radiometric detection Packard Instruments 500 TR flow scintillation analyzer.

Table 2: Plasmids Used in This Study

plasmid	origin	resistance	T7 regulated genes	reference
pBP130	ColE1	carb	*DEBS2, DEBS3*	*8*
pKOS173-158	ColE1	kan	*DEBS1*	*10*
pKOS149-49.A92	ColE1	carb	*atoC*	this work
pKOS173-091	ColE1	kan	*atoAD*	this work
pKOS207-15a	p15a	tet	*mutAB, epi, atoC*	*10*
pKOS214-004	p15a	tet	*atoC*	this work
pKOS149-58b.A7	ColE1	carb	*mutAB, epi, atoC*	this work

Polyketide Analysis. As described previously (*9*), overnight cultures of the polyketide production strains were grown in LB medium with the appropriate antibiotics. These overnight cultures were diluted 1:50 into 25 mL of LB medium with the appropriate antibiotics in a 250 mL Erlenmeyer flask and grown at 37 °C until the OD_{600} reached 0.4−0.5. The cultures were then cooled to 25 °C and induced with 0.5 mM IPTG, and media supplements were added to the following final concentrations: 5 μM hydroxocobalamin (added in the dark), 50 mM succinate, and 50 mM glutamate and propionate and butyrate as indicated. After 48 h at 22 °C, the OD_{600} of the cultures was determined, and the cells were collected by centrifugation. Cell-free supernatant (5 mL) was mixed with an equal volume of ethyl acetate to extract the polyketide products into the organic fraction (top layer). The organic fraction was dried under vacuum, and the precipitate was resuspended in 500 μL of methanol. The polyketide products were detected by LC-MS and quantified by evaporative light scattering detection (ELSD) as described (*9*). Polyketides

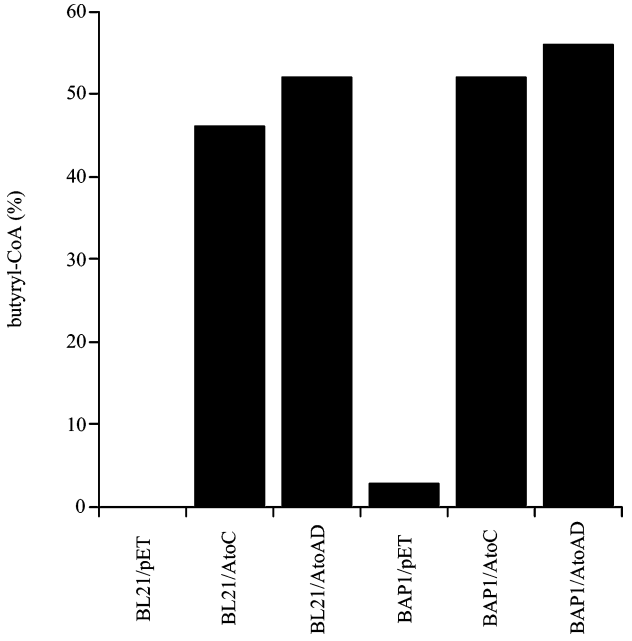

FIGURE 3: Butyryl-CoA production in *E. coli* overexpressing PrpE, AtoAD, or AtoC. *E. coli* strains BL21(DE3) or BAP1 (with an integrated copy of *prpE* under T7 promoter control) were transformed with the vector pET22b, a pET derivative overexpressing *atoC* (pKOS149-49.A92) or a pET derivative overexpressing *atoAD* (pKOS173-91), and the acyl-CoA profiles of the resulting strains were determined (see Materials and Methods). The levels of butyryl-CoA are expressed as a percentage of the total CoA pool.

were quantified by comparing the peak area from the ELSD with a standard curve of peak areas generated from an authentic sample. Polyketide titers are reported as the average with standard errors of duplicate or triplicate samples, derived from independent colonies of the strain analyzed.

RESULTS

Butyryl-CoA Production in E. coli. Production of 15-Me-6-dEB in *E. coli* required the expression of a pathway for the biosynthesis of the butyryl-CoA starter unit. The *ato* operon in *E. coli* controls utilization of acetoacetate and short-chain fatty acids (Figure 2) (*11*). To construct an *E. coli* strain capable of accumulating butyryl-CoA, two approaches were tested: (1) overexpression of the *atoAD* genes and (2) overexpression of *atoC*, the positive regulator of the *ato* operon. Production of soluble AtoAD and AtoC was verified by SDS−PAGE (data not shown). AtoAD collectively constituted approximately 36% of the total protein and AtoC constituted 22%. The levels of the protein bands corresponding to AtoAD were also raised upon overexpression of AtoC but to a lesser extent (10% of total protein for AtoA and AtoD, collectively). CoA analyses showed that butyryl-CoA constituted approximately 50% of the total CoA pool when either *atoAD* or *atoC* were overexpressed in the presence of 100 mM butyrate (Figure 3). The addition of 100 mM butyrate did not alter the growth characteristics of the strain. In our polyketide production strains, derived from *E. coli* strain BAP1 (*8*), PrpE, a propionyl-CoA ligase that has previously been shown to convert butyrate to butyryl-CoA (*13*), is also overproduced. However, although expression of PrpE does lead to the production of butyryl-CoA, significantly lower levels of butyryl-CoA (<3% of the total CoA pool) accumulate in the presence of PrpE alone, compared to that produced by strains also overexpressing *atoAD* or *atoC* (~50% of the total CoA pool; Figure 3). Thus, overexpression of *atoAD*, either directly or through overexpression of its positive regulator *atoC*, was necessary and sufficient to produce butyryl-CoA as the predominant acyl-CoA in *E. coli* cells grown in the presence of butyrate. For the experiments described below, overexpression of *atoC* was used to generate butyryl-CoA.

Propionyl-CoA Is the Preferred Starter Unit for DEBS in Vivo. The ability of butyryl-CoA to serve as a starter unit and prime 6-dEB biosynthesis in vivo was first tested using K207-3 (Table 1), a strain of *E. coli* in which the propionyl-CoA ligase gene and the propionyl-CoA carboxylase genes are integrated on the chromosome such that (2*S*)-methylmalonyl-CoA is generated from propionyl-CoA (*10*). The DEBS genes and *atoC* (for butyryl-CoA production) were expressed from plasmids pKOS173-158, pBP130, and pKOS214-004 (Table 2). The strain was grown in LB with the addition of the appropriate antibiotics, 5 mM propionate, and 0, 1, 5, 10, or 50 mM butyrate. The results show that although the butyrate-derived polyketide 15-Me-6-dEB was produced, more of the propionate-derived polyketide (6-dEB) was generated (Figure 4A). As the butyrate concentration was increased, the titer of 6-dEB decreased while the titer of 15-Me-6-dEB showed an initial increase, followed by a decrease. Analyses of acyl-CoA pools from these fermentations (Figure 4B) showed that as the concentration of butyrate increased, the relative butyryl-CoA level increased while the relative levels of (2*S*)-methylmalonyl-CoA and propionyl-CoA decreased. The reduction in overall polyketide titers reflected the reduction in (2*S*)-methylmalonyl-CoA and propionyl-CoA precursor levels as the butyrate concentration was increased. In conditions where the levels of butyryl-CoA and propionyl-CoA were equal (i.e., when both propionate and butyrate were fed at 5 mM), approximately 10-fold more 6-dEB was produced than 15-Me-6-dEB. Further analysis of these data, comparing the ratio of propionyl-CoA to butyryl-CoA to the ratio of 6-dEB to 15-Me-6-dEB revealed a strong linear correlation (Figure 4C; linear correlation coefficient, $R = 0.987$). These results demonstrated that in order to produce butyryl-CoA-derived 15-Me-6-dEB as the predominant polyketide species in engineered *E. coli*, propionyl-CoA must be eliminated or its intracellular concentration must be significantly reduced.

Methylmalonyl-CoA and Butyryl-CoA (in the Absence of Propionyl-CoA) Can Be Made in E. coli Expressing the Mutase and Ato Pathways. A pathway independent of propionyl-CoA has been developed for the production of methylmalonyl-CoA in *E. coli* (*9*). This pathway utilizes vitamin B_{12}-dependent methylmalonyl-CoA mutase, *mutAB*, which interconverts succinyl-CoA and (2*R*)-methylmalonyl-CoA, and methylmalonyl-CoA epimerase, *epi*, to generate the (2*S*)-methylmalonyl-CoA isomer, which is used exclusively by DEBS (*14*). To demonstrate that it was possible to produce (2*S*)-methylmalonyl-CoA and butyryl-CoA in the same *E. coli* cell, a plasmid (pKOS149-58b.A7) with *mutAB*, *epi*, and *atoC* under the control of the T7 promoter was introduced into BL21(DE3). Analysis of the acyl-CoA pool in this strain upon feeding 100 mM butyrate clearly demonstrated the presence of both butyryl-CoA (36% of total CoAs) and methylmalonyl-CoA (3% of total CoAs) and the

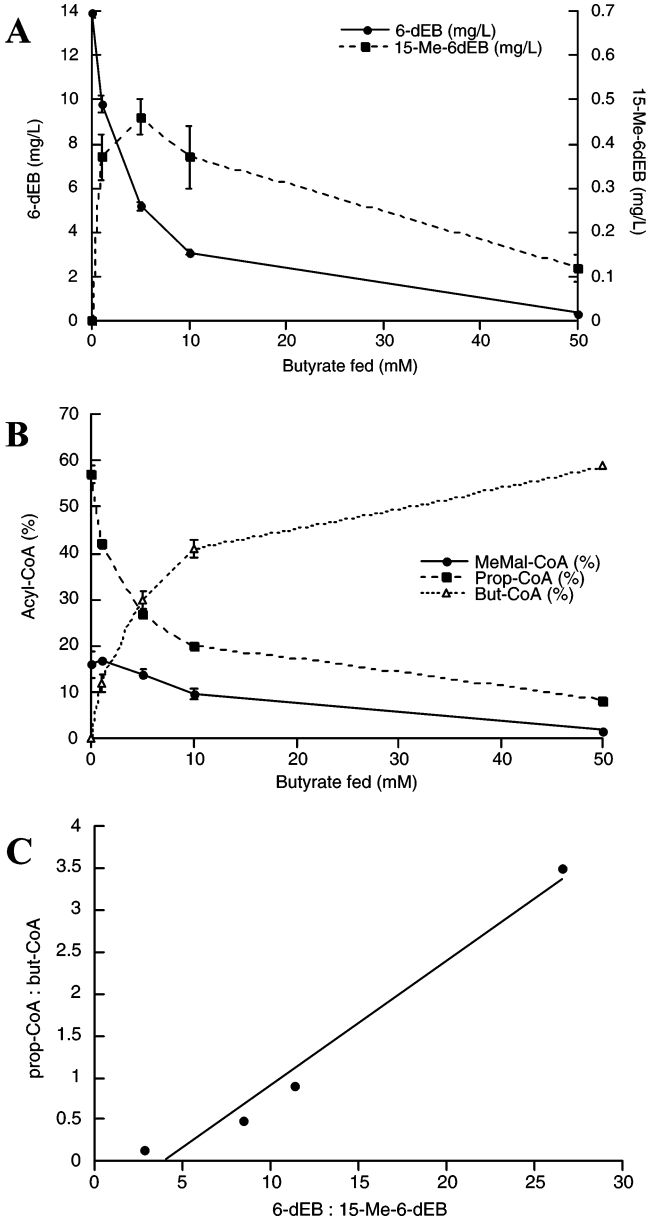

FIGURE 4: 6-dEB and 15-Me-6-dEB production using the propionyl-CoA carboxylase (PCC) pathway for (2*S*)-methylmalonyl-CoA production. *E. coli* strain K207-3 (with PCC genes integrated and under T7 promoter control) with plasmids pKOS214-004 (AtoC), pKOS173-158 (DEBS1), and pBP130 (DEBS2 and DEBS3) was grown as described in the Materials and Methods in the presence of 5 mM sodium propionate with sodium butyrate varied from 0 to 50 mM. Panel A shows titers of 6-dEB and 15-Me-6-dEB. Panel B shows levels of individual acyl-CoAs expressed as a percentage of the total acyl-CoA pool. Standard errors are shown for both plots. Panel C shows a plot of the propionyl-CoA:butyryl-CoA ratio vs the 6-dEB:15-Me-6-dEB ratio. A strong linear correlation can be seen ($R = 0.987$).

absence of significant amounts of propionyl-CoA (Figure 5).

Production of 15-Me-6-dEB Using the Ato and Mutase Pathways. Plasmids encoding the DEBS genes under the control of T7 promoters (pKOS173-158, pBP130) were introduced into the strain K173-145 with the plasmid pKOS207-15a, which expresses the *mutAB*, *epi*, and *atoC* genes for methylmalonyl-CoA and butyryl-CoA production. The strain was fermented in the presence of either 5 mM propionate or 5 mM butyrate for the production of 6-dEB or 15-Me-6-dEB, respectively (Figure 6). When supplemented with propionate, 0.8 ± 0.2 mg of 6-dEB/L was produced and no 15-Me-6-dEB was detectable. When butyrate alone was used, the major polyketide product was 15-Me-6-dEB (0.6 ± 0.1 mg/L), with 6-dEB (0.3 ± 0.1 mg/L) also produced. The propionate and butyrate additions had no noticeable impact on the growth characteristics of the strain.

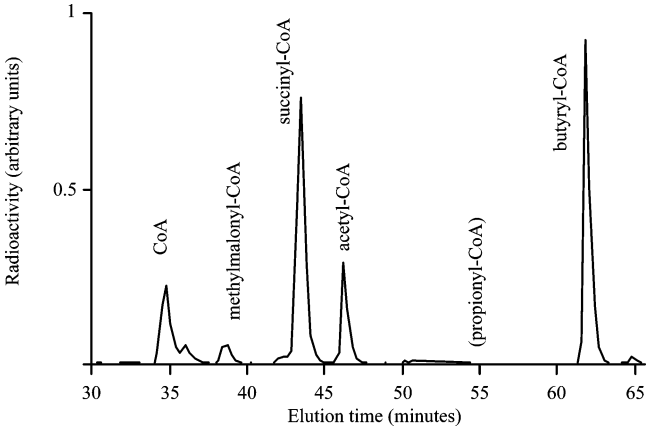

FIGURE 5: Chromatogram of an acyl-CoA profile showing production of butyryl-CoA and methylmalonyl-CoA upon overexpression of *atoC*, *mutAB*, and *epi* from plasmid pKOS149-58b.A7 in the *E. coli* strain BL21(DE3) and feeding 100 mM sodium butyrate. Relative levels of CoAs are as follows: CoA, 13%; methylmalonyl-CoA, 3%; succinyl-CoA, 36%; acetyl-CoA, 12%; butyryl-CoA, 36%; propionyl-CoA, not detected (propionyl-CoA elutes at a retention time of ~55 min, as indicated on the chromatogram).

The detection of 6-dEB without the addition of exogenous propionate suggests that propionyl-CoA is produced in the engineered strain. Propionyl-CoA could arise from trace amounts of propionate in LB, from degradation of L-threonine (*15*), or from the decarboxylation of methylmalonyl-CoA through the action of *ygfG*, a native *E. coli* decarboxylase. To examine whether YgfG-catalyzed decarboxylation of methylmalonyl-CoA was a significant source of propionyl-CoA and also to create a more stable strain with reduced plasmid load, the *mutAB*, *epi*, and *atoC* genes were integrated into the *E. coli* chromosome so as to disrupt the *ygfG* gene. This strain, K214-037, was then tested for 15-Me-6-dEB and 6-dEB production by introducing the DEBS genes on plasmids (pKOS173-158 and pBP130). In the absence of exogenous propionate or butyrate in this strain, $\ll$0.1 mg of 6-dEB/L was produced compared to 0.3 ± 0.05 mg of 6-dEB/L without disruption of *ygfG*. When 5 mM propionate was fed to K214-037 with the DEBS plasmids, 1.5 ± 0.1 mg of 6-dEB/L was produced and no 15-Me-6-dEB was detectable. When 5 mM butyrate was fed to this strain, titers of 15-Me-6-dEB were 0.9 ± 0.1 mg/L with only trace amounts ($<$0.1 mg/L) of 6-dEB observed. Integration of the acyl-CoA supply genes at the *ygfG* locus thus resulted in a 1.5-fold increase in polyketide titers and a reduction in background 6-dEB production when butyrate was fed to this strain.

DISCUSSION

The goal of this study was to engineer an *E. coli* host capable of producing 15-Me-6-dEB from butyryl-CoA and (2*S*)-methylmalonyl-CoA precursors. Production of 15-substituted 6-dEB analogues by direct fermentation is a novel alternative to chemobiosynthesis, which relies on feeding

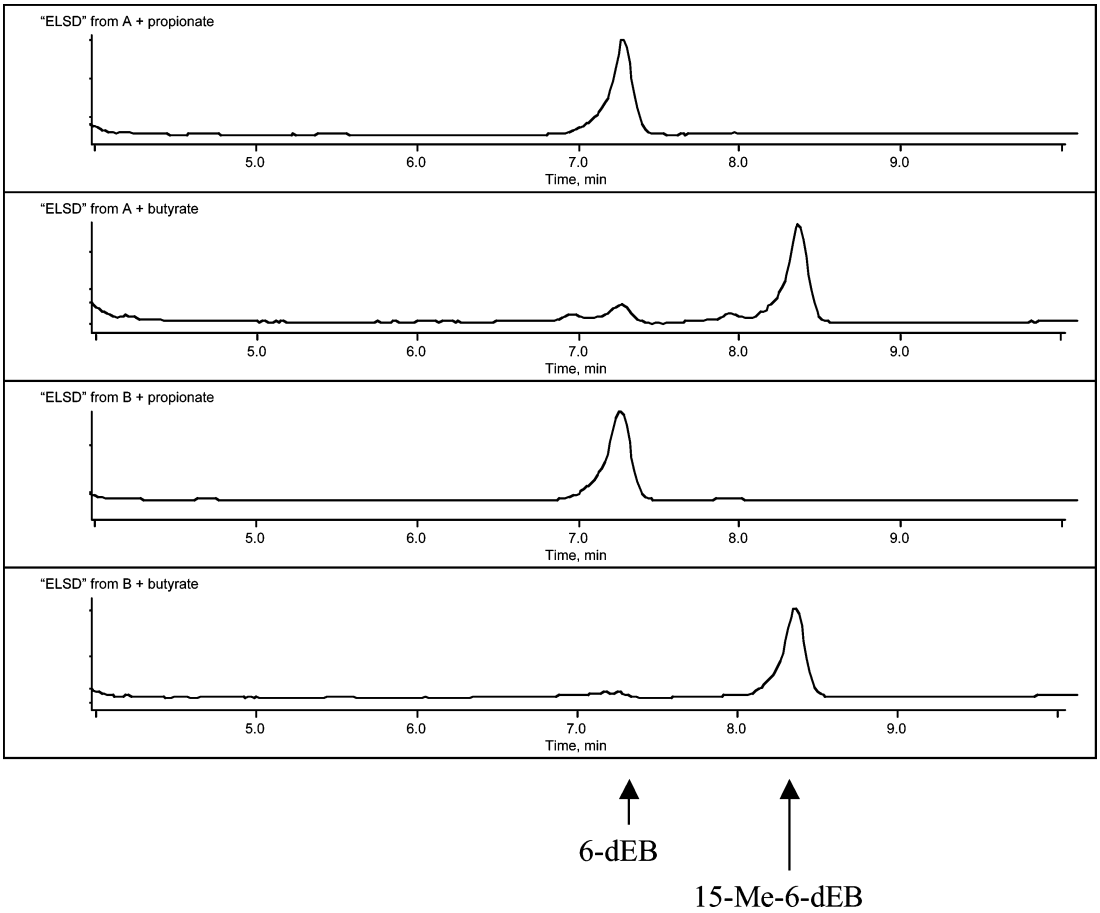

FIGURE 6: Chromatograms showing 6-dEB and 15-Me-6-dEB production in *E. coli*. Strain K173-145 with plasmids pKOS207-15a (*mutAB*/*epi*/*atoC*), pKOS173-158 (DEBS1), and pBP130 (DEBS2 and DEBS3) was fermented as described in the Materials and Methods in the presence of 5 mM sodium propionate (A + propionate) or 5 mM sodium butyrate (A + butyrate). Strain K214-037 (*mutAB*/*epi*/*atoC* integrated on chromosome) with plasmids pKOS173-158 (DEBS1) and pBP130 (DEBS2 and DEBS3) was similarly fermented in the presence of 5 mM sodium propionate (B + propionate) or 5 mM sodium butyrate (B + butyrate). The panels are HPLC chromatograms (ELSD detection) of extracts of these cultures. The 6-dEB and 15-Me-6-dEB peaks are indicated.

synthetic diketides to an *S. coelicolor* strain expressing DEBS deficient in the first ketosynthase domain (*3*).

The endogenous *E. coli* ato pathway was utilized in order to generate butyryl-CoA in vivo to serve as the starter unit for DEBS. Overexpression of either *atoAD* or *atoC* coupled with butyrate feeding was sufficient to generate intracellular levels of butyryl-CoA as high as 50% of the total CoA pool (see Figure 3). Moreover, the intracellular butyryl-CoA levels could be manipulated by altering the concentration of butyrate in the growth medium (see Figure 4).

The starter unit selectivity of DEBS was assessed in an *E. coli* strain expressing both *prpE* and *atoC* and fed both propionate and butyrate. This established an in vivo competition between the natural starter unit, propionyl-CoA, and butyryl-CoA for the DEBS loading domain. The polyketide product ratio (~10:1 6-dEB:15-Me-6-dEB) observed when the intracellular concentrations of propionyl-CoA and butyryl-CoA were equal showed that, in this system, DEBS preferred propionyl-CoA to butyryl-CoA by a factor of ~10. The selectivity of the DEBS loading domain that we observed in vivo is consistent with that measured in vitro for (a) a truncated version of DEBS consisting of DEBS1 plus the thioesterase domain (DEBS1-TE) (8-fold preference for propionyl-CoA over butyryl-CoA) (*7*) and (b) the isolated DEBS loading didomain, consisting of an acyltransferase (AT) and an acyl carrier protein (ACP) (~40-fold preference for propionyl-CoA over butyryl-CoA) (*16*). Hu et al. have shown that trans-acting factors can also influence starter unit processing and subsequent polyketide product ratios (*17*). In *S. coelicolor* fermentations, the acetyl-CoA-derived 14-nor-6-dEB can constitute 50% of the total polyketide produced. However, in the natural erythromycin producer, *S. erythraea*, only trace amounts of 14-norerythromycins are detected. The disruption of *ery-ORF5*, which encodes a thioesterase II (TEII), in *S. erythraea* causes a significant increase in the production of 14-norerythromycins (*17*). Similarly, coexpression of *ery-ORF5* with DEBS in *S. coelicolor* dramatically reduced 14-nor-6-dEB titers. The fact that 14-nor-6-dEB is not observed in *E. coli* fermentations is intriguing (and fortuitous), considering that acetyl-CoA is readily detectable in the *E. coli* strain used here (see Figure 5). The presence of an endogenous protein with activity similar to that of the *S. erythraea* TEII is a possible explanation for the absence of the acetyl-CoA-derived 14-nor-6-dEB in *E. coli* extracts.

The high relative specificity of DEBS for propionyl-CoA over butyryl-CoA underscored the need for a production host with clearly defined acyl-CoA pathways. Since the production system utilized wild-type DEBS for polyketide biosynthesis, it was necessary to eliminate sources of propionyl-CoA to prevent the preferred starter unit from competing with butyryl-CoA for the loading domain of DEBS. Hence,

we could not supply the (2*S*)-methylmalonyl-CoA extender unit via the propionyl-CoA-dependent PCC pathway, which we have previously shown supports higher polyketide titers than does the methylmalonyl-CoA mutase pathway (*10*). The use of the methylmalonyl-CoA mutase pathway enabled the production of 15-Me-6-dEB as the predominant polyketide product. However, the presence of detectable amounts of 6-dEB indicated that propionyl-CoA was still present. Disruption of the *ygfG* gene, encoding methylmalonyl-CoA decarboxylase, eliminated one potential source of propionyl-CoA and resulted in a reduction in the amount of 6-dEB produced when butyrate was fed to the *ygfG*-deficient strain.

Since we have now demonstrated direct production of 15-Me-6-dEB from acyl-CoA precursors in *E. coli*, further metabolic engineering, media development, and classical strain improvement are three well-established routes to increase polyketide productivity. It is likely that the supply of methylmalonyl-CoA via the methylmalonyl-CoA mutase pathway is limiting polyketide titers as we have consistently observed lower titers for 6-dEB production when methylmalonyl-CoA is supplied by the methylmalonyl-CoA mutase pathway rather than the PCC pathway (*10*). As methylmalonyl-CoA is derived from the TCA cycle intermediate, succinyl-CoA, strategies aimed at boosting the steady-state level of succinyl-CoA could increase methylmalonyl-CoA levels. In addition, methylmalonyl-CoA mutase requires the vitamin B_{12} cofactor which is not produced de novo by *E. coli*. To supply B_{12}, the vitamin B_{12} precursor, hydroxocobalamin, is added to the growth media, and the precursor is taken up by *E. coli* and converted into the mature cofactor (*9*, *18*). It is possible that intracellular levels of the vitamin B_{12} cofactor are insufficient for full activity of methylmalonyl-CoA mutase and that strategies aimed at boosting vitamin B_{12} levels could also increase the methylmalonyl-CoA pool.

The metabolic engineering approach described here may be difficult to replicate with natural polyketide producers such as *Streptomyces cinnamonensis* that have redundant pathways for the generation of starter and extender units (*19*, *20*). The advantage of using *E. coli* as a production host is that no such redundant pathways exist, since *E. coli* did not evolve to produce polyketides. Genetic engineering of the DEBS loading domain is an alternative and complementary strategy for increasing the specificity of the loading domain for starter unit analogues (*21*). A potential disadvantage of the genetic engineering approach, however, is that AT domain "swapping" and other engineered modifications to PKSs can impair enzyme function, leading to less productive strains. The metabolic engineering approach using wild-type DEBS has the advantage that no potentially deleterious changes to the DEBS PKS need to be made. The *E. coli* host system described here should also serve for the production of other 15-R-6-dEB analogues, as long as their corresponding acyl-CoA starter units can be generated in vivo and incorporated into DEBS.

ACKNOWLEDGMENT

We thank John Carney and Nina Viswanathan for polyketide analysis, Linda Dayem for acyl-CoA analysis, Zhihao Hu and Alexei Yeliseev for unpublished data, and C. Richard Hutchinson and Daniel V. Santi for helpful advice and critical reading of the manuscript.

REFERENCES

1. Ackermann, G., and Rodloff, A. C. (2003) *J. Antimicrob. Chemother. 51*, 497−511.
2. Jacobsen, J. R., Hutchinson, C. R., Cane, D. E., and Khosla, C. (1997) *Science 277*, 367−369.
3. Leaf, T., Cadapan, L., Carreras, C., Regentin, R., Ou, S., Woo, E., Ashley, G., and Licari, P. (2000) *Biotechnol. Prog. 16*, 553−556.
4. Carreras, C., Frykman, S., Ou, S., Cadapan, L., Zavala, S., Woo, E., Leaf, T., Carney, J., Burlingame, M., Patel, S., Ashley, G., and Licari, P. (2002) *J. Biotechnol. 92*, 217−228.
5. Rawlings, B. J. (2001) *Nat. Prod. Rep. 18*, 190−227.
6. Pieper, R., Luo, G., Cane, D. E., and Khosla, C. (1995) *Nature 378*, 263−266.
7. Pieper, R., Ebert-Khosla, S., Cane, D., and Khosla, C. (1996) *Biochemistry 35*, 2054−2060.
8. Pfeifer, B. A., Admiraal, S. J., Gramajo, H., Cane, D. E., and Khosla, C. (2001) *Science 291*, 1790−1792.
9. Dayem, L. C., Carney, J. R., Santi, D. V., Pfeifer, B. A., Khosla, C., and Kealey, J. T. (2002) *Biochemistry 41*, 5193−5201.
10. Murli, S., Kennedy, J., Dayem, L. C., Carney, J. R., and Kealey, J. T. *J. Ind. Microbiol. Biotechnol. 30*, 500−509.
11. Clark, D. P., and Cronan, J. E., Jr. (1996) in *Escherichia coli and Salmonella* (Neidhardt, F. C., Ed.) pp 343−357, ASM Press, Washington, DC.
12. Jackowski, S., and Rock, C. O. (1981) *J. Bacteriol. 148*, 926−932.
13. Valentin, H. E., Mitsky, T. A., Mahadeo, D. A., Tran, M., and Gruys, K. J. (2000) *Appl. Environ. Microbiol. 66*, 5253−5258.
14. Marsden, A. F., Caffrey, P., Aparicio, J. F., Loughran, M. S., Staunton, J., and Leadlay, P. F. (1994) *Science 263*, 378−380.
15. Hesslinger, C., Fairhurst, S. A., and Sawers, G. (1998) *Mol. Microbiol. 27*, 477−492.
16. Liou, G. F., Lau, J., Cane, D. E., and Khosla, C. (2003) *Biochemistry 42*, 200−207.
17. Hu, Z., Pfeifer, B., Chao, E., Murli, S., Kealey, J., Carney, J. R., Ashley, G., Khosla, C., and Hutchinson, C. R. (2003) *Microbiology 149*, 2213−2225.
18. Amaratunga, M., Fluhr, K., Jarrett, J. T., Drennan, C. L., Ludwig, M. L., Matthews, R. G., and Scholten, J. D. (1996) *Biochemistry 35*, 2453−2463.
19. Zhang, W., and Reynolds, K. A. (2001) *J. Bacteriol. 183*, 2071−2080.
20. Vrijbloed, J. W., Zerbe-Burkhardt, K., Ratnatilleke, A., Grubelnik-Leiser, A., and Robinson, J. A. (1999) *J. Bacteriol. 181*, 5600−5605.
21. Long, P. F., Wilkinson, C. J., Bisang, C. P., Cortes, J., Dunster, N., Oliynyk, M., McCormick, E., McArthur, H., Mendez, C., Salas, J. A., Staunton, J., and Leadlay, P. F. (2002) *Mol. Microbiol. 43*, 1215−1225.

BI035157T