Metabolic Engineering of a Methylmalonyl-CoA Mutase−Epimerase Pathway for Complex Polyketide Biosynthesis in *Escherichia coli*[†,‡]

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ABSTRACT: A barrier to heterologous production of complex polyketides in *Escherichia coli* is the lack of (2*S*)-methylmalonyl-CoA, a common extender substrate for the biosynthesis of complex polyketides by modular polyketide synthases. One biosynthetic route to (2*S*)-methylmalonyl-CoA involves the sequential actions of two enzymes, methylmalonyl-CoA mutase and methylmalonyl-CoA epimerase, which convert succinyl-CoA to (2*R*)- and then to (2*S*)-methylmalonyl-CoA. As reported [McKie, N., et al. (1990) *Biochem. J. 269*, 293−298; Haller, T., et al. (2000) *Biochemistry 39*, 4622−4629], when genes encoding coenzyme B_{12}-dependent methylmalonyl-CoA mutases were expressed in *E. coli*, the inactive apo-enzyme was produced. However, when cells harboring the mutase genes from *Propionibacterium shermanii* or *E. coli* were treated with the B12 precursor hydroxocobalamin, active holo-enzyme was isolated, and (2*R*)-methylmalonyl-CoA represented ∼10% of the intracellular CoA pool. When the *E. coli* BAP1 cell line [Pfeifer, B. A., et al. (2001) *Science 291*, 1790−1792] harboring plasmids that expressed *P. shermanii* methylmalonyl-CoA mutase, *Streptomyces coelicolor* methylmalonyl-CoA epimerase, and the polyketide synthase DEBS (6-deoxyerythronolide B synthase) was fed propionate and hydroxocobalamin, the polyketide 6-deoxyerythronolide B (6-dEB) was produced. Isotopic labeling studies using [^{13}C]propionate showed that the starter unit for polyketide synthesis was derived exclusively from exogenous propionate, while the extender units stemmed from methylmalonyl-CoA via the mutase−epimerase pathway. Thus, the introduction of an engineered mutase−epimerase pathway in *E. coli* enabled the uncoupling of carbon sources used to produce starter and extender units of polyketides.

Polyketides are complex natural products that are particularly abundant in soil microorganisms (*1*). Although fewer than 10 000 polyketides have been identified to date, they include a large number of major pharmaceuticals that span a broad range of therapeutic areas, including cancer (adriamycin), infectious disease (tetracyclines, erythromycin), cardiovascular (mevacor, lovastatin), and immunosuppression (rapamycin, tacrolimus).

Complex polyketides are produced by modular polyketide synthases (PKSs)[1]—large, multifunctional enzymes that are organized into multiple catalytic "modules", each containing a set of 3−6 functional domains that determine the identity of a 2-carbon unit of the polyketide. As illustrated in Figure 1 for the prototypical deoxyerythronolide B synthase (DEBS) (*2*), modules are linearly arranged, beginning with a starter module, followed by a number of extender modules, and terminating with a releasing domain. Each of the modules recognizes the acyl group of a specific acyl-CoA, catalyzes its condensation with an acyl-group tethered to the preceding module to form a nascent polyketide chain, and modifies the β-carbon atom of the growing chain. This extension/modification process occurs in an assembly line fashion through the entire sequence of modules, resulting in a one-to-one correlation between the constituent enzymatic activities of a PKS module and the corresponding 2-carbon ketide unit of a polyketide.

Owing to the modular nature of polyketide synthesis, genetic engineering can be used to create specific structural modifications of polyketides in a predictable fashion and to produce new libraries of these natural products. In theory, by modifying the genes that encode PKS modules, a specific 2-carbon unit of a polyketide may be changed to one of about 20 others, reflecting the combinations of chain extender units (malonate, methylmalonate, or rarer units), the fate of the incoming β-keto group at each step of chain extension (keto, hydroxyl, enoyl, or methylene), and the stereochemistry of methyl and hydroxyl branches. In practice, such modifications are complicated by several factors, including the facts

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[‡] The *P. shermanii* methylmalonyl-CoA epimerase DNA sequence has been deposited into the GenBank database under accession number AY046899.

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[1] Abbreviations: CoA, coenzyme A; PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography; DEBS, 6-deoxyerythronolide B synthase; IPTG, β-isopropyl-thiogalactoside; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; LB, Luria−Bertani medium; DTT, dithiothreitol; NADH, nicotinamide adenine dinucleotide; TLC, thin-layer chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TKL, (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid ∂-lactone (triketide lactone); 6-dEB, 6-deoxyerythronolide B; ELSD, evaporative light-scattering detector; ESI-TOF, electrospray ionization time-of-flight.

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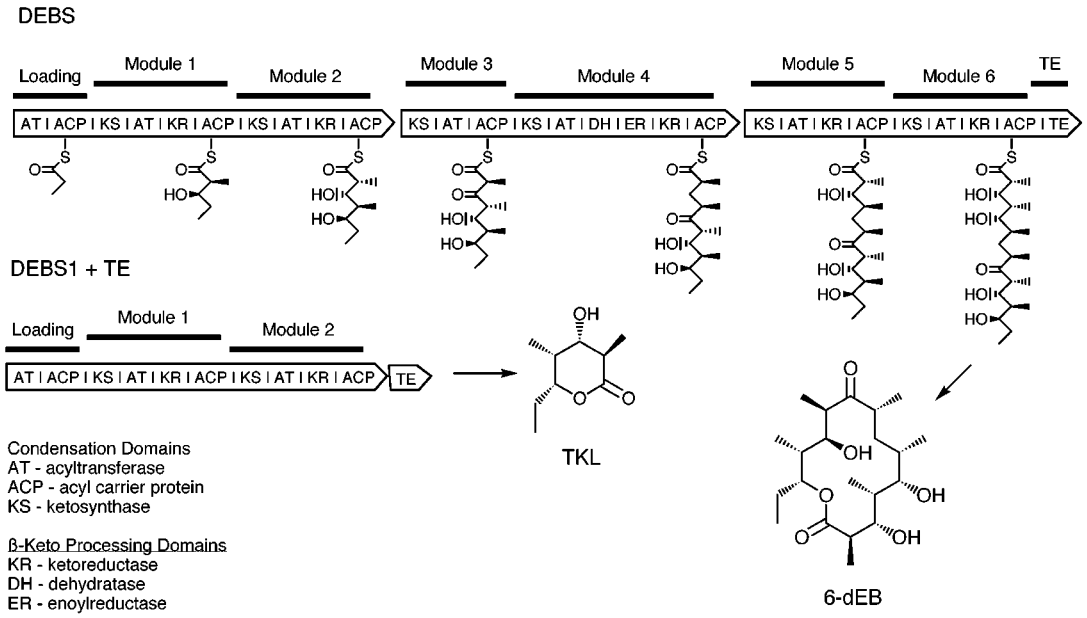

FIGURE 1: Biosynthesis of the complex polyketide 6-dEB and its truncated analogue TKL by the polyketide synthases DEBS and DEBS1+TE, respectively.

that most natural polyketide producers grow slowly, are poorly transformed by DNA, and are often intractable toward genetic manipulations. To exploit the molecular biological tools available in genetically "friendly" organisms, we are developing *Escherichia coli* as a general host for polyketide manipulation and production.

Heterologous production of polyketides in *E. coli* is problematic because of the lack of needed acyl-CoA precursors. For example, the erythromycin PKS DEBS requires propionyl-CoA as a starter unit and (2*S*)-methylmalonyl-CoA as an extender unit, neither of which is found in *E. coli* (*3*). Recently, however, *E. coli* was engineered to produce these metabolic intermediates (*4*). Propionyl-CoA was produced by propionate feeding of cells disrupted in propionyl-CoA catabolism and overexpressing propionyl-CoA ligase, the product of the *prpE* gene (*5*). In addition to providing the starter unit, propionyl-CoA served as a source of the extender unit, (2*S*)-methylmalonyl-CoA, via the coexpressed propionyl-CoA carboxylase (PCC). The metabolically engineered host supported the synthesis of a full-length polyketide (6-dEB), or alternatively a truncated triketide lactone (TKL) when only the starter and the first two extender modules of the PKS (DEBS1 + TE) were expressed (Figure 1).

In the present work, we describe the introduction into *E. coli* of the coenzyme B_{12}-dependent methylmalonyl-CoA mutase−epimerase pathway (*6*−*8*) (Figure 2), an important pathway for production of methylmalonyl-CoA in the polyketide-producing actinomycetes (*9*, *10*). In contrast to the PCC pathway, in which both the starter and extender units stem from propionyl-CoA, the mutase pathway produces the (2*S*)-methylmalonyl-CoA extender unit from the TCA cycle intermediate, succinyl-CoA. Thus, the polyketide extender unit is provided independently of the starter unit, making separate optimization feasible.

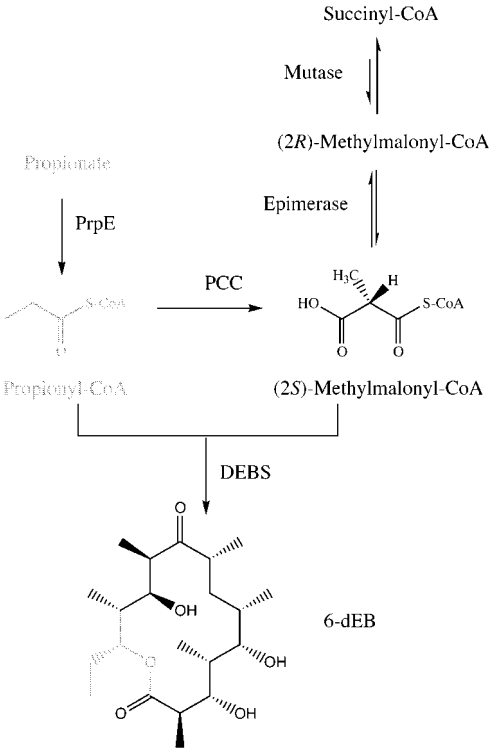

FIGURE 2: Biosynthetic routes to (2*S*)-methylmalonyl-CoA and production of 6-dEB. For the PCC pathway (middle), all atoms in 6-dEB are derived from propionate. For the mutase pathway (right), atoms shown in gray come from propionate while those shown in black arise from succinyl-CoA.

MATERIALS AND METHODS

Propionibacterium freudenreichii subsp. shermanii was obtained from NCIMB, Scotland (NCIMB #9885) and maintained as a stab in tomato juice agar (*11*). *Escherichia coli* BL21(DE3), *E. coli* XLI Blue, and pBluescript SK(+) were from Stratagene, and *E. coli* SJ16 (CGSC #6341) (*12*) was from the *E. coli* Genetic Stock Center, Yale University. The expression vectors pET-22b(+) and pET-16b were from Novagen. The vector pKOS116-63 is pET-22b with an altered linker containing restriction sites *Nde*I−*Hin*dIII−*Pac*I−*Nsi*I−*Nhe*I. Phage P1 cm clr (*13*) was provided by Bryan Julien, Kosan Biosciences. Transcarboxylase was

provided by the laboratory of H. G. Wood, Case Western Reserve University. BAP1 *E. coli* and plasmids pBP12, pBP130, and pBP144 are described in Pfeifer et al. (*4*). Strain k173-145, a *panD* version of BAP1, and plasmid pKOS173-158, a modified version of pBP144 in which the PCC genes were removed, were provided by Jonathan Kennedy, Kosan Biosciences (unpublished results). DNA sequencing was performed using a Beckman CEQ 2000 capillary sequencer. Mut medium consisted of M9 salts, glucose, thiamin, trace elements, and amino acids (*14*). Protease inhibitor cocktail tablets (Complete, Mini, EDTA-free) were obtained from Roche Molecular Biochemicals. [3-^{3}H]β-Alanine (50 Ci/mmol) and [1-^{14}C]propionate (56 mCi/mmol) were from American Radiolabeled Chemicals. [$^{13}C_3$]Propionate (99 atom %) was obtained from Aldrich. All other reagents were the purest available from commercial sources. Standard molecular biology techniques were as described (*15*). One unit (U) of enzyme activity is the amount of enzyme required for production of 1 μmol of product per minute.

E. coli BL21(DE3)/panD. *E. coli* SJ16 containing Tn10 (Tet^r) linked to *panD* was infected with P1 phage, and the lysate was used to infect *E. coli* BL21(DE3) (*13*). Following selection for Tet^r colonies, a strain (K117-60) was isolated that displayed a reduction in growth on mut medium, and a ~10-fold increase in incorporation of [^{3}H]β-alanine into acyl-CoAs.

Construction of Methylmalonyl-CoA Mutase Expression Vector. The translationally coupled *mutA* and *mutB* genes, encoding the β and α methylmalonyl-CoA mutase subunits [originally identified and sequenced by Leadlay and co-workers (*6*)], were amplified from *P. shermanii* genomic DNA by PCR in four fragments that were then joined. The PCR primers for the first fragment, mutA1, were 5′-CAC AG**T CTA GAC ATA TG**A GCA GCA CGG ATC AGG GGA CC-3′, containing introduced *Xba*I and *Nde*I sites (boldface type), and 5′-CAC AG*C TGC AG*G GCT GCG AAC GCG ATG GGA TCC-3′, containing a natural *Pst*I site (italics). For mutA2, the primers were 5′-CAC AG*C TGC AG*G GCA CCG AGC CGG ATC TGA CC-3′ (natural *Pst*I site, italics) and 5′-CAC AG**A AGC TT***G ATA TC*A AGG GTG GAG GAC-3′, introducing a *Hin*dIII site (boldface) at the 3′ end, adjacent to the naturally occurring *Eco*RV site (italics). In the mutB1 fragment, the start codon for the *mutB* gene was changed from GTG to **A**TG (*7*) using the PCR primer 5′-CAC AG**A CTA GT***G ATA TC*T TGG GAG TCG CGA A**A**T GAG CAC TCT GCC CCG TTT TGA TTC-3′, which also introduced a *Spe*I site (boldface) upstream of the natural *Eco*RV site (italics). The reverse primer for this fragment was 5′-CAC AG*C TGC AG*G AAC AGC TGG GTG TTA CGG GCG ATG C-3′, containing a natural *Pst*I site (italics). The primers for the mutB2 fragment were 5′-CAC AG*C TGC AG*C AGG AAT CGG GCA CGA CGC GCG TGA TC-3′ (natural *Pst*I site, italics) and 5′-CAC AG**A AGC TTC AAT TG**C TAG GCA TCG AGC GAA GCC C-3′, introducing *Mfe*I and *Hin*dIII sites (boldface) near the 3′ end of the fragment. Each fragment was cloned into an intermediate bluescript vector and sequenced. The fragments were excised from the intermediate vectors and pieced together: naturally occurring *Pst*I sites were used to join mutA1 and mutA2 (mutA), as well as mutB1 and mutB2 (mutB). The mutA and mutB fragments were combined at a naturally occurring *Eco*RV site to form the complete mutase gene, which was cloned into the vector pKOS116-63 between the restriction sites *Nde*I and *Hin*dIII, to form pKOS116-95B.

Expression of P. shermanii Holo-methylmalonyl-CoA Mutase. Operations were performed in a dark room with a safelight. A foil-wrapped 125 mL flask containing 25 mL of mut medium, 100 μg/mL carbenicillin, 5 μM β-alanine, and 5 μM hydroxocobalamin was inoculated with 250 μL of a starter culture of K117-60/pKOS116-95B (*mutAB*), which was grown at 27 °C for 20 h in the above medium, excluding hydroxocobalamin. After incubating overnight at 27 °C, cultures were induced with IPTG to 1 mM final concentration and incubated for an additional 5 h. Cells were transferred to a foil-wrapped conical tube, collected by centrifugation, and stored in the dark at −80 °C. For soluble extract preparation, the pellet was thawed, washed with buffer C (50 mM potassium phosphate buffer, pH 7.4, 5 mM EDTA, 10% glycerol, 1 protease inhibitor tablet per 10 mL of buffer), and resuspended in 0.5 mL of buffer C. Following sonication on ice, the extract was clarified by centrifugation.

Cloning and Expression of E. coli sbm. The gene for Sbm (Sleeping Beauty mutase) was amplified by PCR from *E. coli* K117-60 genomic DNA and cloned into pET-16b to introduce an N-terminal His_{10}-tag (pKOS143-49-2), as described (*16*). The gene was also cloned as an *Nde*I/*Sac*I fragment into pET-22b (pKOS143-40-39) to encode the native *sbm* sequence. Cultures of K117-60/pKOS143-49-2 (His_{10}-Sbm) were grown in LB with 100 μg/mL carbenicillin at 37 °C. At late-log phase, the cultures were induced with IPTG to 1 mM final concentration and grown for an additional 18 h at 22 °C. The cells were collected by centrifugation, and His_{10}-Sbm was purified by metal chelate chromatography (as specified by Qiagen). No attempt was made to purify native Sbm.

Acyl-CoA Analysis. HPLC was performed using a 150 × 4.6 mm 5 μm ODS-3 Inertsil HPLC column (MetaChem Technologies). HPLC buffer A contained 100 mM NaH_2PO_4, 75 mM NaOAc, pH 4.6, and buffer B contained 70% buffer A, 30% methanol. The HPLC column was equilibrated with 90% buffer A/10% buffer B at a flow rate of 1 mL/min. After sample injection, a linear gradient to 40% buffer B was formed over 35 min, followed by a linear gradient to 90% buffer B over 20 min; the eluant was monitored at 260 nm. For radioactive samples, 0.5 mL fractions were collected from 18 to 58 min, and the samples were counted in a liquid scintillation counter. The CoA standard mix contained 0.5 mM CoA and 1.6 mM each of malonyl-, methylmalonyl-, succinyl-, acetyl-, and propionyl-CoA.

Analysis of Intracellular Acyl-CoAs. Mut medium (1 mL) containing 100 μg/mL carbenicillin and 100 μM β-alanine was inoculated with single colonies of K117-60 harboring various mutase genes (pKOS116-95B, pKOS143-49-2, or pKOS143-40-39) or the vector control pET-22b. After overnight growth at 37 °C, cells were collected by centrifugation and washed 4 times with 1 mL of mut medium. The cells were suspended in 1 mL of mut medium containing 100 μg of carbenicillin, and grown for 4 h at 37 °C to deplete cells of β-alanine. Fresh mut medium (1 mL) containing 10 μCi of [^{3}H]β-alanine, 0.5 μM β-alanine, and 100 μg/mL carbenicillin was inoculated with 30 μL of the starved cells. After 3 h of growth at 37 °C, IPTG was added to each culture to a final concentration of 1 mM. The culture tubes were

wrapped in aluminum foil, hydroxocobalamin was added to 5 μM final concentration, and the cultures were incubated at 27 °C overnight. Cells were collected by centrifugation and washed twice with 1 mL of mut medium. The washed cell pellet was resuspended in 300 μL of cold 10% TCA containing 5 μL of CoA standard mix, and the suspension was sonicated on ice for 1 min at 5 W. Precipitants were removed by centrifugation, and 50 μL of the supernatant was analyzed for acyl-CoAs by HPLC as described above.

Methylmalonyl-CoA Mutase Assay. All operations for the mutase assay were performed in the dark or under a safelight. Enzyme assays (100 μL) contained 0.2 mM (2*RS*)-methylmalonyl-CoA and mutase extract in buffer C. For assays containing added coenzyme B_{12}, the mutase extract was preincubated with 0.01 mM coenzyme B_{12} in 75 μL of buffer C for 5 min at 30 °C for mutAB, or for 1 h at 4 °C with 2 mM dithiothreitol (*16*) for His_{10}-Sbm. (2*RS*)-Methylmalonyl-CoA was added, and after incubation at 30 °C for 2, 5, 10, or 20 min, reactions were quenched with 50 μL of 10% TCA and placed on ice for 10 min. After centrifugation, 100 μL of the supernatant was analyzed by HPLC to quantify conversion of methylmalonyl-CoA to succinyl-CoA.

His_{10}-Sbm was also assayed to determine which diastereomer of methylmalonyl-CoA it produced. Reaction mixtures (50 μL) contained 0.2 M potassium phosphate buffer, pH 6.9, 0.1 M NH_4SO_4, 5 mM sodium pyruvate, 2.4 mM succinyl-CoA, 0.0033 unit of transcarboxylase, and purified His_{10}-Sbm, with and without methylmalonyl-CoA epimerase. Reactions were initiated with succinyl-CoA, incubated in the dark at 30 °C for 10 min, and quenched as described above. Supernatants were analyzed by the HPLC system described above, which separated succinyl-CoA, methylmalonyl-CoA, and propionyl-CoA.

Methylmalonyl-CoA Epimerase Assay. Enzyme activity was assayed at 30 °C using a modification of a reported method (*8*). The epimerase (0.54 mg/mL) was first activated with 100 μM $CoCl_2$ at 4 °C for 1 h. Reaction mixtures (1.5 mL) contained 0.2 M potassium phosphate buffer, pH 6.9, 0.1 M NH_4SO_4, 5 mM sodium pyruvate, 0.08 mM (2*RS*)-methylmalonyl-CoA, 0.05 unit of transcarboxylase, 0.16 mM NADH, and 2.5 units of malate dehydrogenase. The reaction was initiated with (2*RS*)-methylmalonyl-CoA, and the decrease in A_{340} concomitant with consumption of the *S*-isomer was allowed to stabilize. Limiting epimerase was added to catalyze conversion of the remaining *R*- to *S*-isomer, and the initial rate and total decrease in A_{340} were monitored. For kinetic studies, ~1.2 nM epimerase was incubated with varying amounts of (2*RS*)-methylmalonyl-CoA (27−160 μM). The (2*R*)-methylmalonyl-CoA concentrations were assumed to be half of the concentration of (2*RS*)-methylmalonyl-CoA.

N-Terminal Sequencing of Methylmalonyl-CoA Epimerase from P. shermanii. A 10 L fermentation of *P. shermanii* was grown anaerobically at pH 6.85 (*11*). After 3 days, cells were collected by centrifugation, washed with water, and stored at −80 °C. Methylmalonyl-CoA epimerase was purified as described (*8*), followed by C-8 reversed-phase HPLC (*17*). The HPLC-purified epimerase was subjected to N-terminal amino acid sequencing, and also digested with 0.4 μg of *Achromobacter lyticus* protease (Lys-C) in 125 mM Tris/HCl, pH 8, 3.8 mM DTT for 16 h at 37 °C. The Lys-C peptides were separated by HPLC, and those showing 280 nm tryptophan absorbance were sequenced. We obtained 41 residues of the N-terminal sequence, and 6 residues of a Lys-C peptide.

P. shermanii Methylmalonyl-CoA Epimerase Gene. A cosmid library of *P. shermanii* was prepared as described (Stratagene). Degenerate PCR primers were designed based on the N-terminus and internal peptide sequences of the epimerase. The forward primer was 5′-CACAG**TCTAGA**ATHGAYCAYGTNGCNTAYGC-3′, and the reverse primer was 5′-CACAG**GGATCC**YTCRTCRTTNARNGGNGCYTT-3′, where the underlined sequences target the epimerase gene and the boldface type shows introduced *Xba*I (forward primer) and *Bam*HI (reverse primer) restriction sites. These primers were used to PCR-amplify from *P. shermanii* genomic DNA a 190 base pair fragment, which was used as a template to generate a digoxigenin (DIG)-labeled probe via PCR (Boehringer Mannheim). The labeled PCR product was used to probe the cosmid library by colony hybridization. Cosmids from positive colonies were screened for the epimerase gene by PCR, and several were subjected to DNA sequencing using primers specific to the epimerase sequence. The cosmid designated pKOS117-167-A7 contained the complete epimerase gene, the sequence of which has been deposited into the GenBank database under accession number AY046899.

The epimerase gene was amplified from pKOS117-167-A7 by PCR, using primers that introduced *Nde*I and *Bam*HI restriction sites at the 5′ end, and *Nhe*I and *Avr*II sites at the 3′ end. The forward primer also destroyed a natural *Nde*I site at nucleotides 41−46. The PCR product was cloned into an intermediate vector for sequencing, and then into the vector pKOS116-63 between *Nde*I and *Nhe*I sites, to give pKOS143-28-8.

S. coelicolor Methylmalonyl-CoA Epimerase Gene. The *P. shermanii* methylmalonyl-CoA epimerase DNA sequence was used to search the *S. coelicolor* genomic database (*www.sanger.ac.uk/Projects/S_coelicolor*) using the BLAST programs at the National Center for Biotechnology Information, NIH website (*18*), and *S. coelicolor* gene 8F4.02c was identified as a putative methylmalonyl-CoA epimerase. The gene was amplified by PCR from *S. coelicolor* genomic DNA, using the forward primer 5′-CAC AG**C ATA TG**C TGA CGC GAA TCG ACC-3′, which introduced a *Nde*I site at the 5′ end, and the reverse primer, 5′-CAC AG**A TGC AT**T CAG TGC TCA GGT GAC TCA ACG G-3′, which introduced an *Nsi*I site at the 3′ end. The PCR fragment was cloned into an intermediate vector and sequenced, and then into the vector pKOS116-63 between the restriction sites *Nde*I and *Nsi*I, to give pKOS117-174-A37.

Heterologous Expression and Purification of the Epimerases. E. coli BL21(DE3) harboring either pKOS143-28-8 (*P. shermanii* epimerase) or pKOS117-174-A37 (*S. coelicolor* epimerase) was grown in 750 mL of LB (100 μg/mL carbenicillin) at 37 °C until late-log phase, then induced with IPTG to 1 mM final concentration, and grown for an additional 3 h at 30 °C. The cells were collected by centrifugation, and the pellets were stored at −80 °C. The recombinant epimerase proteins were purified essentially as described for *P. shermanii* (*8*).

Coexpression of P. shermanii Mutase and Epimerase Genes. The methylmalonyl-CoA epimerase gene from *P. shermanii* was cloned as an *Nde*I/*Avr*II fragment downstream

of a T7 promoter in pKOS116-172a. The epimerase gene was excised along with the T7 promoter from pKOS116-172a as a *Pac*I/*Nhe*I fragment and cloned into pKOS133-9b, a pET plasmid containing *Pac*I−*Nhe*I sites directly downstream of the *mutAB* genes, and followed by a T7 transcriptional terminator. This provided pKOS143-35-50 with the configuration: [T7 promoter-*mutAB* genes]−[T7 promoter-epimerase gene]−[T7 terminator]. The *mutAB* and epimerase genes were also cloned in the same configuration into a tetracycline-resistant pACYC vector containing *atoC* to create pKOS207-15a (Sumati Murli, unpublished results).

TKL Analysis. E. coli BAP1 was transformed with pBP12 (DEBS1+TE) and either pKOS143-35-50 (*mutAB*/epimerase) or pKOS116-95b (*mutAB*). Individual transformants were inoculated into mut medium with 100 μg/mL carbenicillin and 50 μg/mL kanamycin, and the cultures were grown at 37 °C. Upon reaching late-log phase, the cultures were cooled at 22 °C for 3 min and centrifuged. The cell pellets were resuspended in 1 mL of the supernatant, 10 μCi of [^{14}C]propionate was added, and hydroxocobalamin and IPTG were added in the dark at final concentrations of 5 μM and 0.5 mM, respectively. The cultures were incubated in the dark at 22 °C for up to 43 h. Samples (100 μL) were removed from the cultures periodically, and supernatants were extracted twice with 300 μL of ethyl acetate. The extract was dried in vacuo and subjected to TLC analysis. Radioactive spots comigrating with authentic TKL were quantitated on a phosphorimager, and TKL concentrations were calculated using a standard curve of [^{14}C]propionate. Negative controls included cultures of BAP1/pBP12 (DEBS1+TE only) and BAP1/pBP12/pKOS143-35-50 (DEBS1+TE and *mutAB*/epimerase) without hydroxocobalamin.

6-dEB Analysis. LB medium (10 mL) was inoculated with *E. coli* strain k173-145 containing plasmids pKOS173-158 (DEBS1), pBP130 (DEBS2,3), and pKOS207-15a (*mutAB*/epimerase). As an alternative for the mutase−epimerase pathway, pKOS207-15a was substituted by pKOS143-189, a tetracycline-resistant pACYC vector containing the PCC genes behind a T7 promoter (Sumati Murli, unpublished results). Cultures were grown at 37 °C in LB medium with 100 μg/mL carbenicillin, 7.5 μg/mL tetracycline, and 50 μg/mL kanamycin. Upon reaching late-log phase, cultures were chilled on ice, either 5 mM propionate or 5 mM [$^{13}C_3$]-propionate was added, and the cultures were induced with IPTG to 0.5 mM final concentration. Hydroxocobalamin, succinate, and glutamate, to final concentrations of 5 μM, 50 mM, and 50 mM, respectively, were added to strains containing the mutase. The cultures were grown for 40 h at 22 °C; cells were collected by centrifugation, and the cell-free medium was extracted with an equal volume of EtOAc. The EtOAc extract (8 mL) was dried and resuspended in MeOH.

Extracts were analyzed by LC-MS on a system comprised of an Agilent 1100 HPLC and an Applied Biosystems Mariner time-of-flight mass spectrometer equipped with a Turbo IonSpray source (spray chamber temperature 400 °C; nozzle potential 110 V). The 6-dEB was adsorbed to a Metachem Inertsil ODS-3 column (5 μm, 2.1 × 150 mm) and eluted with a linear gradient from 35% to 100% MeCN (0.1% HOAc) at 300 μL/min over 10 min. The eluate was monitored by MS. Under these conditions, the retention time of 6-dEB was 7.9−8.1 min.

For quantitation, extracts were analyzed using a system consisting of a Beckman System Gold HPLC, an Alltech ELSD detector, and a PE SCIEX API100 LC MS-based detector configured with an atmospheric pressure chemical ionization source. The eluate from a Metachem Inertsil ODS-3 column (5 μm, 4.6 × 150 mm) of a linear gradient from 35 to 100% MeCN (0.1% HOAc) at 1 mL/min over 10 min was split 1:1 between the ELSD and MS detectors. Under these conditions, 6-dEB eluted at 7.4 min. Titers were determined from ELSD response by comparing the integrated area of 6-dEB from the cultures to standard curves generated from authentic 6-dEB.

RESULTS

PCR Amplification and Expression of Active Methylmalonyl-CoA Mutase. The translationally coupled genes (*mutAB*) for the α- and β- subunits of methylmalonyl-CoA mutase (EC 5.4.99.2) were amplified from *P. shermanii* genomic DNA, cloned, and sequenced. There were two discrepancies between the amino acid sequence predicted from the genes obtained here and the sequence reported in the database (accession no. X14965); we found $G_{990}C_{991}$ instead of $C_{990}G_{991}$, corresponding to $Glu_{330}Leu_{331}$ rather than $Asp_{330}Val_{331}$. Since our residue assignments corresponded with those in the crystallographic structure of the protein (*19*), we concluded that they are correct. To facilitate expression in *E. coli*, the naturally occurring GTG start codon for *mut*B was changed to ATG (*7*, *14*), and the *mutAB* genes were subcloned into an *E. coli* expression vector under the control of a T7 promoter.

We grew *E. coli* K117-60 harboring pKOS116-95b, a pET vector containing the *P. shermanii* mutase genes, at 27 °C in the defined mut medium with and without hydroxocobalamin (*14*). Mutase activity in cell extracts was monitored by the conversion of (2*R*)-methylmalonyl-CoA to succinyl-CoA by HPLC. As reported (*7*), in the absence of hydroxocobalamin, mutase activity was undetectable unless extracts were supplemented with coenzyme B_{12}, indicating exclusive expression of the apo-enzyme. However, when expression of the mutase was induced in cells grown in the presence of hydroxocobalamin, 0.038 unit/mg of mutase activity was observed in crude extracts. No succinyl-CoA formation was observed when (2*RS*)-methylmalonyl-CoA was incubated with extracts from strain K117-60 harboring the pET vector control.

The gene (*sbm*) for *E. coli* methylmalonyl-CoA mutase was cloned downstream of a T7 promoter with a His_{10}-tag, expressed in *E. coli*, purified, and reconstituted with coenzyme B_{12}. As reported (*16*), the reconstituted enzyme catalyzed the production of succinyl-CoA when incubated with (2*RS*)-methylmalonyl-CoA. To determine which isomer of methylmalonyl-CoA was used as the substrate, His_{10}-Sbm was incubated with succinyl-CoA to produce methylmalonyl-CoA, as well as with sodium pyruvate and transcarboxylase, which converts the (2*S*)- but not the (2*R*)-isomer to propionyl-CoA. HPLC analysis revealed that propionyl-CoA was not produced. However, when *P. shermanii* epimerase was added to the assay mixture to interconvert (2*R*)- and (2*S*)-methylmalonyl-CoA, propionyl-CoA was produced. This demonstrated that, as with other methylmalonyl-CoA mutases, Sbm catalyzes the stereospecific conversion of succinyl-CoA to (2*R*)-methylmalonyl-CoA.

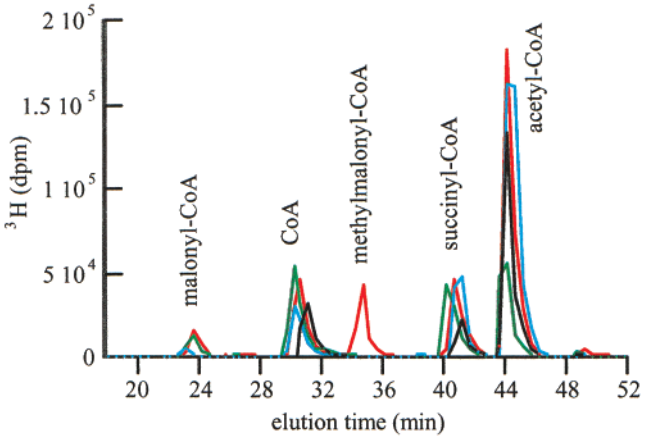

FIGURE 3: Intracellular acyl-CoA analysis in *E. coli*. Radioactivity in HPLC fractions of cell-free extracts from [^{3}H]β-alanine-fed *E. coli* harboring a pET vector containing the *P. shermanii* methylmalonyl-CoA mutase genes with (red) and without (green) hydroxocobalamin feeding, or harboring a pET vector control with (blue) and without (black) hydroxocobalamin feeding.

Production of Methylmalonyl-CoA in E. coli. Analysis of in vivo acyl-CoA pools is typically accomplished in *E. coli* strains deficient in the enzyme aspartate decarboxylase (PanD), which converts aspartate to β-alanine, a component of CoA. Such strains are "fed" [^{3}H]β-alanine, which is incorporated into CoA without isotopic dilution. The ^{3}H-labeled acyl-CoAs in crude lysates are separated by HPLC and analyzed by scintillation counting (*12*). To express the mutase in a *panD* background, we constructed a BL21(DE3)/*panD* strain (K117-60) by phage P1 transduction of the *panD* locus from SJ16 to BL21(DE3) (*12*). The resulting *panD* strain enabled detection of intracellular acyl-CoA pools at about 10-fold greater sensitivity compared with wild-type strains.

Cultures of *E. coli* K117-60 harboring various methylmalonyl-CoA mutase genes or the vector control pET were grown and induced in mut medium containing [^{3}H]β-alanine with and without hydroxocobalamin. Cells were lysed and extracts applied to an HPLC system along with CoA standards. Figure 3 shows in vivo acyl-CoA levels in cells with and without the *P. shermanii* mutAB. In cells expressing mutAB and grown with hydroxocobalamin, methylmalonyl-CoA comprised ~10% of the CoA pool, whereas in the cultures without mutAB and/or hydroxocobalamin, methylmalonyl-CoA was not detected. Similar levels of methylmalonyl-CoA were observed in cells grown with hydroxocobalamin and overexpressing Sbm and His_{10}-Sbm (data not shown). It has been reported that Sbm retained only 7% of its in vitro activity after cleavage of the N-terminal His_{10}-tag (*16*). However, we found that both versions of Sbm produced similar amounts of methylmalonyl-CoA in vivo. With the exception of methylmalonyl-CoA, the acyl-CoA pool composition was comparable to that observed for *E. coli panD* mutants grown on glucose (*3*). However, in cells overexpressing mutAB, malonyl-CoA levels were consistently elevated. In this minimal medium, we did not observe propionyl-CoA.

Methylmalonyl-CoA Epimerases. Methylmalonyl-CoA epimerase (EC 5.1.99.1) from *P. shermanii* was purified to homogeneity; peptide sequences were obtained and used to design degenerate PCR primers. A DIG-labeled probe was amplified by PCR and used to isolate the complete 447 bp epimerase gene from a *P. shermanii* cosmid library via colony hybridization. The predicted protein sequence of the *P. shermanii* epimerase was used to identify a putative methylmalonyl-CoA epimerase from the *S. coelicolor* genomic database; the two sequences are ~44% identical and ~64% similar. The genes for both epimerases were cloned into *E. coli* expression vectors, and the proteins were purified to homogeneity. We also cloned the *Bacillus subtilus yqjC*, identified as a putative methylmalonyl-CoA epimerase gene (*16*), into a pET expression vector containing a T7 promoter. However, the protein was expressed in *E. coli* as insoluble particles and not studied further.

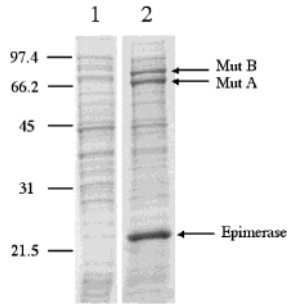

FIGURE 4: SDS−PAGE analysis of soluble protein from *E. coli* cells grown in the presence of IPTG and hydroxocobalamin. Lane 1, cells harboring a pET vector; lane 2, cells harboring a pET vector containing the *P. shermanii* mutase (*mutAB*) and epimerase genes. Arrows indicate the positions of MutA, MutB, and Epimerase. Molecular mass markers are indicated in kDa. (Lanes 1 and 2 are from the same gel, with intervening lanes removed for clarity of presentation.)

The calculated molecular masses of the epimerases are 16 716 Da for *P. shermanii* and 16 081 Da for *S. coelicolor*. SDS−PAGE analysis of the purified *P. shermanii* epimerase revealed a band at molecular mass ~23 kDa; however, ESI-TOF mass spectrometry confirmed the expected molecular mass of 16.7 kDa. The *S. coelicolor* epimerase had a molecular mass of 16.1 kDa by ESI-TOF mass spectrometry, and migrated on SDS−PAGE as a band of molecular mass ~18 kDa. The steady-state kinetic constants for the *P. shermanii* (K_m = 38 μM; k_{cat} = 150 s^{-1}) and *S. coelicolor* (K_m = 57 μM; k_{cat} = 75 s^{-1}) epimerases were comparable.

Production of Complex Polyketides via the Mutase Route. The gene encoding the methylmalonyl-CoA epimerase from *P. shermanii* was cloned under the control of a T7 promoter downstream of the *mutAB* genes, yielding the expression plasmid pKOS143-35-50. Analysis by SDS−PAGE of soluble protein from induced *E. coli* cells harboring the *mutAB* and epimerase genes showed that MutA, MutB, and the epimerase were overexpressed (Figure 4). To test the ability of the *P. shermanii* methylmalonyl-CoA mutase and epimerase to support polyketide biosynthesis in vivo, the recently described BAP1 *E. coli* cell line was transformed with pBP12 (DEBS1+TE) (*4*) and either pKOS143-35-50 (mutAB-epimerase) or pKOS116-95b (mutAB). The strains were grown in a minimal medium containing [^{14}C]propionate and hydroxocobalamin. Following IPTG induction, the cultures were sampled and evaluated by TLC to show that coexpression of mutAB and epimerase in the BAP1 cell line resulted in production of the truncated polyketide TKL (data not shown). In a control experiment omitting the epimerase gene, no TKL was detected; this is in accord with previous

work demonstrating that (2*R*)-methylmalonyl-CoA is not a substrate for DEBS1+TE (*20*, *21*).

Having demonstrated that the (2*S*)-methylmalonyl-CoA supplied via the engineered mutase−epimerase pathway supported synthesis of a triketide, we sought to produce the full-length polyketide, 6-dEB, and to show that the polyketide starter and extender units were derived from separate sources. The cell line k173-145 (BAP1/*pan*D) was transformed with the genes encoding DEBS (pKOS173-158 and pBP130) and either the mutase−epimerase genes (pKOS207-15a) or the PCC genes (pKOS143-189) (Figure 2). Cultures were supplemented with propionate, and following ethyl acetate extraction, the 6-dEB in supernatants was analyzed by LC/MS. In numerous experiments, average 6-dEB titers for this system were ∼1 mg/L for the mutase−epimerase pathway and ∼10 mg/L for the PCC pathway (unpublished results).

The origins of the carbon atoms were determined by LC/MS analysis of the 6-dEB product formed upon feeding propionate or [$^{13}C_3$]propionate. The mass spectrum of the 6-dEB produced when propionate was fed to the strain containing the mutase−epimerase pathway is shown in Figure 5a. When [$^{13}C_3$]propionate was fed to the same strain, all ions were shifted up by exactly 3 mass units (Figure 5b versus Figure 5a), indicating that 1 mol of exogenous propionate was incorporated per mole of 6-dEB. Considering the metabolic pathways engineered into the system, it was deduced that the [$^{13}C_3$]propionate was incorporated only as a starter unit and not as an extender unit. As expected for the PCC pathway, [$^{13}C_3$]propionate was incorporated at all 21 carbons of 6-dEB (Figure 5c). Under these conditions, 14-desmethyl 6-dEB was not observed, demonstrating that acetyl-CoA was not used as a starter unit for DEBS.

DISCUSSION

Production of polyketides in heterologous hosts requires PKS expression, posttranslational phosphopantetheinylation (*22*), and a supply of appropriate acyl-CoA precursors including acetyl-, malonyl-, propionyl-, and (2*S*)-methylmalonyl-CoA. Whereas acetyl- and malonyl-CoAs are ubiquitous metabolic intermediates (they are essential for fatty acid biosynthesis), propionyl- and (2*S*)-methylmalonyl-CoAs are not. Recently, *E. coli* was engineered to produce and accumulate sufficient propionyl- and (2*S*)-methylmalonyl-CoA to support complex polyketide biosynthesis (*4*). For propionyl-CoA accumulation, its catabolism was inhibited by interruption of the *prp* operon, and *prpE* was placed under independent control of a T7 promoter. For (2*S*)-methylmalonyl-CoA production, heterologous propionyl-CoA carboxylase (PCC) genes were introduced. In this system, propionyl-CoA served as the source of both starter and extender substrates for polyketide biosynthesis.

In the present work, we introduced the methylmalonyl-CoA mutase−epimerase pathway into *E. coli*. This pathway converts TCA cycle-derived succinyl-CoA to (2*R*)-methylmalonyl-CoA, which is then epimerized to the PKS extender substrate, (2*S*)-methylmalonyl-CoA (Figure 2). The methylmalonyl-CoA mutase−epimerase pathway provides several potential advantages over the PCC pathway for production of polyketides. First, if a starter acyl-CoA is not supplied, (2*S*)-methylmalonyl-CoA could potentially yield propionyl-CoA through decarboxylation by expression of a known

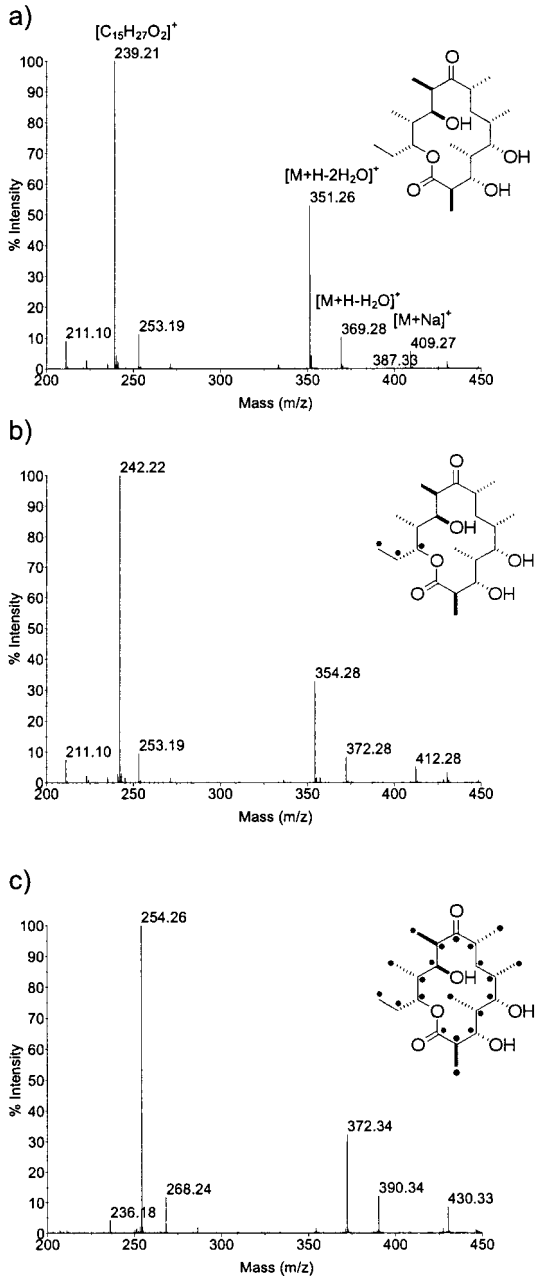

FIGURE 5: LC-MS spectra comparing (a) unlabeled, (b) partially labeled, and (c) fully labeled 6-dEB produced via the mutase−epimerase pathway with (a) propionate or (b) [$^{13}C_3$]propionate, and (c) via the PCC pathway with [$^{13}C_3$]propionate. The mass spectrum of unlabeled 6-dEB consists primarily of ions due to $[M+Na]^+$ (409.3), $[M+H-H_2O]^+$ (369.3), $[M+H-2H_2O]^+$ (351.3), and a 15 carbon fragment ($C_{15}H_{27}O_2$) that includes the carbons of the propionate starter unit (239.2); a pseudo-molecular ion $[M+H]^+$ is rarely observed. 6-dEB titers in this experiment (average of 2 samples) were (a) 0.73 mg/L, (b) 0.8 mg/L, and (c) 2.1 mg/L.

methylmalonyl-CoA decarboxylase (*16*), thereby permitting all of the carbon atoms of a polyketide to be derived from glucose. Second, if a starter acyl-CoA is supplied, the source of the starter and extender units will be decoupled, allowing unusual starter units to be incorporated into the polyketide.

We anticipated three potential problems in introducing an effective methylmalonyl-CoA mutase−epimerase pathway into *E. coli*: (1) expressing holo-methylmalonyl-CoA mutase, since *E. coli* does not contain coenzyme B_{12}; (2) effectively converting intracellular (2*R*)-methylmalonyl-CoA to the (2*S*)-isomer needed for polyketide synthesis; and (3) accumulation

of intracellular foreign acyl-CoAs in sufficient amounts without untoward toxicity. In this study, each of these issues was addressed.

Our first objective was to express holo-methylmalonyl-CoA mutase in E. coli and to demonstrate accumulation of methylmalonyl-CoA. Although *E. coli* possesses a gene (*sbm*) that encodes a putative methylmalonyl-CoA mutase, neither mutase activity nor methylmalonyl-CoA is detectable in cell extracts. The *P. shermanii* and *E. coli* methylmalonyl-CoA mutases have previously been expressed in *E. coli* as inactive apo-enzymes that require reconstitution with coenzyme B_{12} to form the active holo-enzyme (*7*, *16*). We confirmed these results and also showed that, as with the *P. shermanii* enzyme (*23*), the coenzyme B_{12}-reconstituted His_{10}-Sbm produced (2*R*)- rather than (2*S*)-methylmalonyl-CoA. Although *E. coli* does not produce coenzyme B_{12}, it possesses *btuR*, which encodes an enzyme that introduces the adenosyl moiety into mature coenzyme B_{12} precursors, such as hydroxocobalamin (*24*). Indeed, active B_{12}-dependent methionine synthase was expressed in *E. coli* by inclusion of hydroxocobalamin in a minimal growth medium (*14*). When *E. coli* cells expressing *P. shermanii* mutase were grown in minimal medium containing hydroxocobalamin, the mutase recovered from cell extracts was catalytically active. Furthermore, when *E. coli* harboring the *P. shermanii* or *E. coli* mutase genes (native or His_{10}) was grown in medium containing hydroxocobalamin, intracellular methylmalonyl-CoA comprised ~10% of the acyl-CoA pool (Figure 3), corresponding to ~40 μM (*3*). This exceeds the K_m of (2*S*)-methylmalonyl-CoA (~25 μM) for DEBS or DEBS1+TE (*25*, *26*) and should thus support polyketide synthesis.

Our next objective was to convert intracellular (2R)-methylmalonyl-CoA to the (2S)-isomer. To produce (2*S*)-methylmalonyl-CoA in vivo, coexpression of a functional epimerase is required to interconvert the (*R*)- and (*S*)-isomers of methylmalonyl-CoA (Figure 2). Although the cloning and sequencing of methylmalonyl-CoA epimerase from *P. shermanii* (*8*) have previously been reported (*27*), the gene sequence was neither available in the public domain nor available to us. Thus, we isolated the *P. shermanii* epimerase gene by screening a cosmid library of genomic DNA, using DNA probes derived from the sequence of the *P. shermanii* epimerase protein that we purified and sequenced. The *P. shermanii* epimerase sequence was also used to identify a methylmalonyl-CoA epimerase gene from the *S. coelicolor* genomic database. The *P. shermanii* and *S. coelicolor* epimerases were expressed in *E. coli* both individually and with the methylmalonyl-CoA mutases. The two epimerases examined were shown to have similar steady-state kinetic parameters.

Our final objective was to coexpress mutase, epimerase, and DEBS, and demonstrate polyketide biosynthesis. Ultimate proof of having engineered a functional mutase−epimerase pathway in *E. coli* hinged on production of a complex polyketide that required (2*S*)-methylmalonyl-CoA, the final product of the engineered pathway. We coexpressed the *P. shermanii* mutase and epimerase in *E. coli* k173-145 along with DEBS, a PKS that produces 6-dEB from propionyl- and (2*S*)-methylmalonyl-CoA, and demonstrated that 6-dEB was produced at ~1 mg/L. To ascertain the origin of the carbon atoms in the 6-dEB product, we fed cultures [$^{13}C_3$]-labeled propionate and determined the isotopic content of resulting 6-dEB by mass spectroscopy. All ions of 6-dEB were shifted exactly 3 mass units higher when [$^{13}C_3$]-propionate was used as the exogenous precursor (Figure 5a,b). Furthermore, no unlabeled product was detected, indicating that the exogenous [$^{13}C_3$]propionate was not diluted by endogenously derived propionate, as might arise from decarboxylation of the unlabeled methylmalonyl-CoA extender unit. Thus, exogenous propionate serves exclusively as the starter unit. As a control, we used the PCC system to produce (2*S*)-methylmalonyl-CoA from [$^{13}C_3$]propionyl-CoA, which generated a uniformly labeled 6-dEB product, indicating that both the starter and extender units stemmed from the fed propionate (Figure 5c).

Perspectives. The "chemobiosynthetic" method for incorporating unusual starter units into polyketides involves inactivating the first module of a PKS and feeding host cells a chemically synthesized diketide thiol ester; the diketide enters the biosynthetic pathway at the second module and is incorporated into the polyketide product (*28*). The present work presents an alternative method for producing polyketides with novel starter units, independent of diketide feeding. The approach exploits the mutase−epimerase pathway by enabling extender units to be generated independently of starter units. That is, the extender units are derived from endogenously produced intermediates—malonyl- and methylmalonyl-CoA—whereas the starter unit is derived from an exogenous source—in the present case, propionate—that is activated to an acyl-CoA foreign to the cell. By introducing a pathway to produce a foreign acyl-CoA from an exogenously supplied carboxylic precursor, it is possible to generate and accumulate novel acyl-CoAs in *E. coli*. Provided such foreign acyl-CoAs are accepted as substrates by the starting module of a PKS, they would be incorporated into the polyketide, affording an alternative to diketide feeding. The results of such studies will be the subject of a forthcoming report.

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