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Precursor-Directed Polyketide Biosynthesis in *Escherichia coli*

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Abstract—Precursor-directed polyketide biosynthesis was demonstrated in the heterologous host *Escherichia coli*. Several diketide and triketide substrates were fed to a recombinant *E. coli* strain containing a variant form of deoxyerythronolide B synthase (DEBS) from which the first elongation module was deleted resulting in successful macrolactone formation from the diketide, but not the triketide, substrates.

Polyketides are a group of natural products that possess a wide range of biological and medicinal properties. Examples include the antibiotic erythromycin, the antitumor agent epothilone, and the immunosuppressant FK506. Large modular megasynthases called polyketide synthases (PKSs) biosynthesize their polyketide products using simple building blocks such as acetyl coenzyme A (acetyl-CoA), propionyl-CoA, malonyl-CoA, and methylmalonyl-CoA.[1] The most extensively characterized modular polyketide synthase is the 6-deoxyerythronolide B synthase (DEBS) found within the soil bacterium *Saccharopolyspora erythraea*.[2,3] DEBS, an enzymatic complex containing three individual proteins (DEBS1, DEBS2, and DEBS3), catalyzes the formation of the erythromycin macrolide aglycone, 6-deoxyerythronolide B (6-dEB, **1**), from a propionyl-CoA primer and six methylmalonyl-CoA extender units (Fig. 1A). Each of the six rounds of polyketide chain condensation and functional group modification is mediated by a dedicated group of active sites organized into a functional unit known as a module (with two modules located on each of the DEBS proteins). Each multifunctional protein module is in turn made up of a set of catalytic domains of 100–400 amino acids each that are similar in both function and sequence to individual enzymes of fatty acid biosynthesis.

Precursor-directed biosynthesis is a promising approach to generating novel analogues of erythromycin and other polyketides.[4,5] This technique is based on the use of a mutant of a modular PKS in which one or more of the early-stage enzyme activities has been eliminated by inactivation or deletion. The resultant mutant is thus blocked in the biosynthesis of the natural polyketide product. For example, DEBS(KS1^0) is a mutant of DEBS that has been inactivated by site-directed mutagenesis of the β-ketoacyl-ACP synthase domain of module 1 (KS1). Because the KS1 domain would normally catalyze the first condensation step of 6-deoxyerythronolide B biosynthesis, the DEBS(KS1^0) mutant is incapable of carrying out the first round of polyketide chain elongation from the natural propionyl-CoA starter and methylmalonyl-CoA chain-elongation substrate and is therefore unable to catalyze the formation of the macrolide 6-dEB (**1**). Formation of the natural polyketide can be restored, however, by introduction of a synthetic derivative of the natural diketide intermediate as the *N*-acetyl cysteamine (SNAC) thioester **2** (Fig. 1B). We have described precursor-directed biosynthesis experiments in which **2** and various diketide and triketide analogues were tested as substrates for *Streptomyces coelicolor* CH999/pJRJ2, an engineered strain harboring DEBS(KS1^0).[4,6–8]

Notwithstanding earlier success with precursor-directed formation of macrolide analogues, the manipulation of modular PKSs is often hampered by the biological constraints posed by organisms (principally actinomycetes) in which complex polyketides have thus far been produced. To address this problem, an *Escherichia coli* host was recently engineered to produce complex polyketides.[9] Multiple changes were made to the *E. coli* genome

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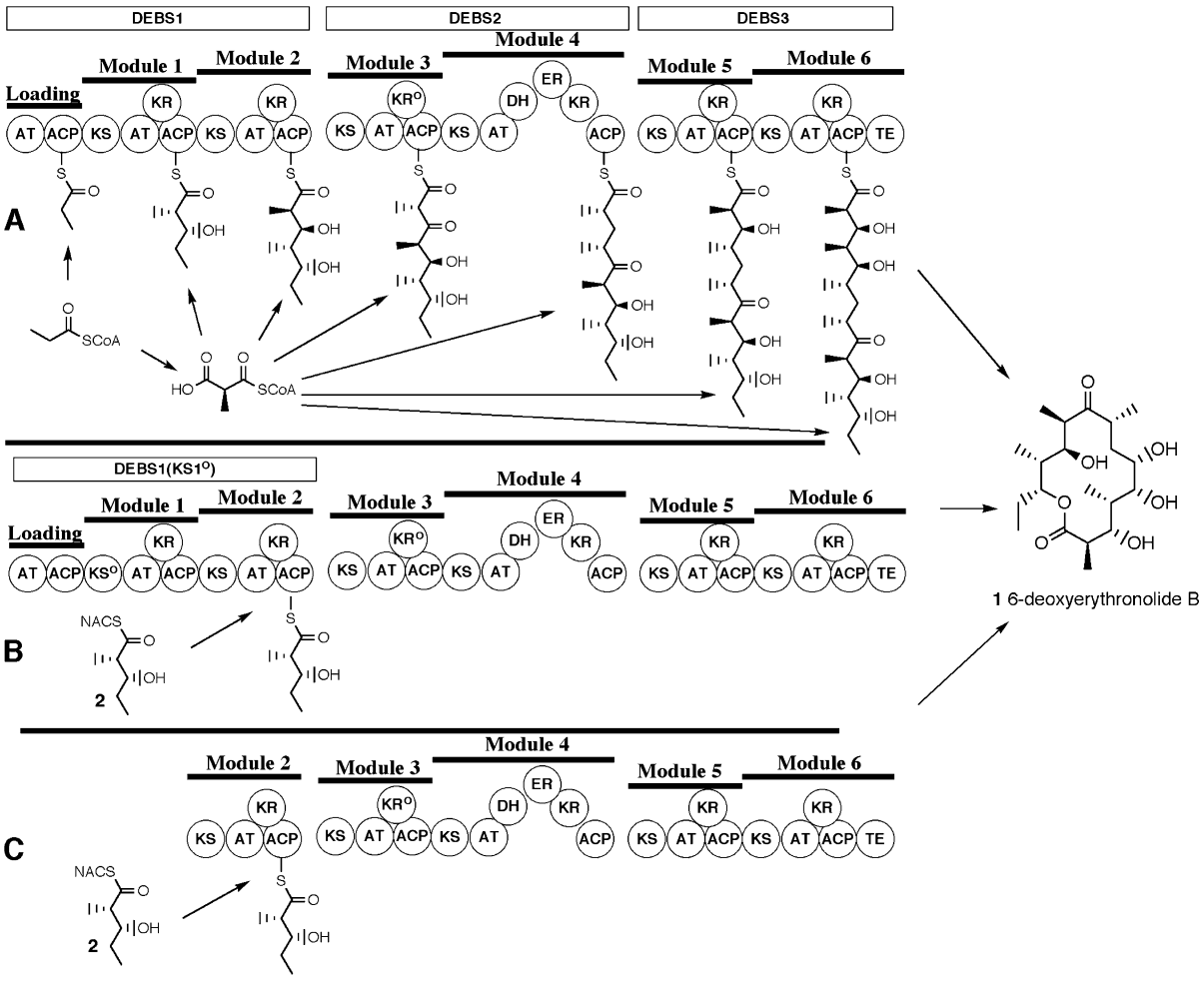
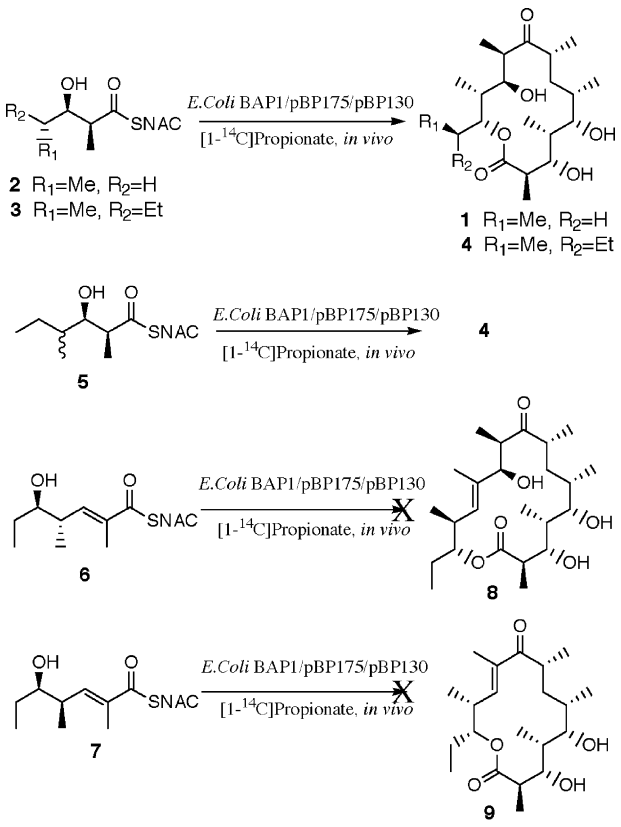


Figure 1. Modular organization and function of the DEBS and its intermediates. (A) Each module contains three core catalytic domains, a ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP), and a variable number of auxiliary domains, ketoreductase (KR), dehydratase (DH), and enoylreductase (ER). In addition to the six modules, there is a loading didomain to prime module 1 and a thioesterase (TE) domain at the C-terminus of module 6 that cyclizes the heptaketide and generates 6-dEB (**1**). (B) Formation of 6-dEB by DEBS(KS1^{O}) is blocked but can be restored by addition of the diketide-SNAC **2**. (C) Formation of 6-dEB by DEBS(module1Δ) is blocked but can be restored by addition of the diketide-SNAC **2**.

to support 6dEB production, including introduction of the three DEBS genes from *Sac. erythraea*, the *sfp* phosphopantetheinyl transferase gene from *Bacillus subtilis*,[10] and the genes encoding a heterodimeric propionyl-CoA carboxylase (*pcc*) from *S. coelicolor*.[11] When gene expression was coordinately induced, propionate could be converted into 6dEB by this metabolically engineered cellular catalyst. Indeed, when cultivated at high cell densities, this derivative of *E. coli* BAP1 harboring DEBS1, −2, and −3 could achieve titers of 6-dEB in excess of 200 mg/L.[12]

To evaluate further the potential for manipulating polyketide biosynthesis in *E. coli*, we have engineered an *E. coli* BAP1 strain in which DEBS module 2 is used in place of DEBS1(KS1^{0}), taking advantage of the enhanced expression of the smaller protein (subunit MW ~150 kDa) in *E. coli*. *E. coli* BAP1 DEBS(module1Δ) harbors DEBS module 2 plus *pcc* on plasmid pBP175 (carrying kanamycin resistance) and DEBS2 and DEBS3 on plasmid pBP130 (carbenicillin resistance), with gene expression controlled by the IPTG- (isopropyl-β-D-thiogalactopyranoside) inducible T7 RNA polymerase promoter.[13] To investigate precursor-directed biosynthesis using *E. coli* BAP1/pBP175/pBP130, LB medium cultures were grown at 37 °C to mid-log phase, then induced with 1 mM IPTG and supplemented with 1 mM synthetic natural diketide-SNAC thioester analogue **2** and 1 mM [1-^{14}C]propionate at 20 °C (Fig. 1C, Scheme 1). After an additional 24 h incubation, the culture supernatants were extracted with ethyl acetate and analyzed for the formation of the expected macrolactone 6-dEB (**1**) by analytical TLC and phosphoimaging (Fig. 2).[14]

The identity of the 6-dEB sample was confirmed by carrying out a preparative-scale incubation in the presence of **2** and [1-^{13}C]propionate. Analysis of the resultant labeled 6-dEB (**1**) by ^{13}C NMR revealed the expected pattern of 5 enhanced peaks, corresponding to enrichment of C1, C3, C5, C7, and C9.[15,16] The ^{1}H NMR spectrum of [1,3,5,7,9-$^{13}C_5$]-**1** was essentially identical to that of unlabeled **1** except for the expected additional ^{1}H–^{13}C couplings for the H-3, H-5, and H-7 signals. The time-course of 6-dEB formation was also monitored by TLC/phosphoimaging, with cultures



Scheme 1. Precursor-directed biosynthesis of 6-dEB (**1**) and analogues by incubation of diketide and triketide analogues with *E. coli* BAP1/pBP175/pBP130 [DEBS(module1Δ)].

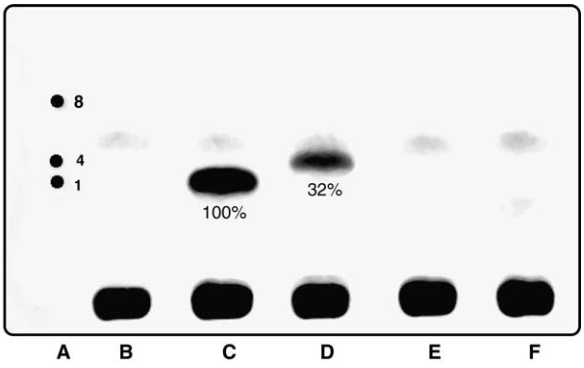


Figure 2. TLC/phosphoimaging to measure relative productivity of macrolide formation by incubation of diketide and triketide analogues plus [1-^{14}C]propionate with *E. coli* BAP1/pBP175/pBP130. Lane A: authentic samples of macrolides **1**, **4**, and **8**, lane B: negative control, lane C: **2**, lane D: **3**, lane E: **6**, lane F: **7**. Solvent system: 50% EtOAc/hexanes.

achieving a titer of up to 11.9 nmol/mL (~5 mg/L) of **1** within 72 h of IPTG induction and administration of diketide **2** (Fig. 3). These yields of macrolactone are within the titer range previously attained under similar culture conditions using the *E. coli* BAP1 strain harboring the complete set of DEBS genes.[9,12]

Having established that *E. coli* BAP1 DEBS(module1Δ) would support precursor-directed biosynthesis from the natural diketide **2**, we examined the ability of this system to tolerate variations in precursor structure (Scheme 1). The branched-chain diketide **3** has the same

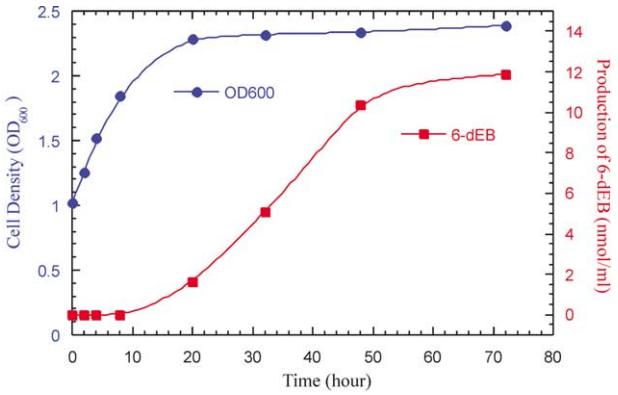


Figure 3. Time course of 6-dEB (**1**) production by administration of **2** to *E. coli* BAP1/pBP175/pBP130.

substitution pattern and stereochemistry at C-2 and C-3 as the preferred *syn*-diketide substrate **2**, but a chain length more like that of a triketide, with a (4*R*)-methyl substituent but no C-5 hydroxyl group. Indeed, we have previously found that **3** is an excellent surrogate substrate for DEBS module 2, with a k_{cat}/K_m 5.5× that of diketide **2**, and a K_m that is 9-fold lower.[17] (By contrast, the (4*S*)-diastereomer of **3**, has a k_{cat}/K_m that is 0.8× that of **2**.)[17] Administration of branched-chain diketide **3** to *E. coli* BAP1/pBP175/pBP130 in the presence of [1-^{14}C]propionate led to formation of the ^{14}C-labeled 6-dEB analogue **4**, (Scheme 1), with a relative productivity compared to diketide **2** of 32% (~1.5 mg/L), as determined by TLC/phosphoimaging (Fig. 2). The structure of macrolactone **4** was confirmed by a preparative scale incubation that for experimental convenience utilized **5**, a 3:2 (4*R*/4*S*) mixture of diastereomers of diketide **3**, in the presence of [1-^{13}C]propionate, giving rise exclusively to labeled **4** which exhibited 5 enhanced ^{13}C NMR peaks (C1, C3, C5, C7, and C9). Labeled **4** was analyzed by 1D NMR (^{1}H and ^{13}C NMR) and 2D NMR [^{13}C–^{1}H COSY and Heteronuclear Single Quantum Correlation (HSQC)] as well as HRFAB-MS and proved to have the same properties as authentic **4** previously obtained by feeding either diketide **3** or the diastereomeric diketide mixture **5** to *S. coelicolor* CH999/JRJ2.[16,17] (The formation of a single diastereomer of **4** is due to exclusive utilization by DEBS module 2 of the preferred diketide diastereomer **3**).[17] By contrast with the efficient utilization of diketides **2** and **3**, neither unsaturated triketide **6** nor **7** was converted by *E. coli* BAP1 DEBS(module1Δ) to the expected macrolactones **8** and **9**, respectively (Fig. 2, Scheme 1), the products that were formed when these anomalous substrates had been administered to the cultures of *S. coelicolor* CH999/pJRJ2 harboring DEBS1(KS1^0).[4,6,7] Addition of β-oxidation inhibitors such as 4-pentynoic acid or 3-(tetradecylthio)propionic acid or other supplements such as 2,6-*O*-dimethyl-β-cyclodextrin known to improve incorporation of polyketide-SNAC derivatives failed to stimulate macrolide analogue production from either unsaturated triketide **6** or **7**.

In summary, we have demonstrated the feasibility of precursor-directed biosynthesis of 6-dEB **1** and its

macrolactone analogue **4** by feeding diketides **2** and **3** to an engineered *E. coli* strain harboring DEBS modules 2 through 6. Thus *E. coli* BAP1/pBP175/pBP130 lacking DEBS module 1 and its attached loading domain can be used with the diketides examined to give the same macrolactones as were produced by feedings with *S. coelicolor* CH999/pJRJ2 harboring DEBS(KS1^0). The unsaturated triketides **6** and **7**, however, were not converted to the expected macrolactone products by the engineered *E. coli* strain, possibly reflecting uptake and/or metabolic differences between *E. coli* and *S. coelicolor*. Precursor-directed biosynthesis using *S. coelicolor* CH999/pJRJ2 has already proved to be powerful tool for rational synthesis of structural analogues of erythromycin and related macrolides. We believe that *E. coli* may offer many biological advantages over *S. coelicolor*, including metabolic rate, scale-up potential, and ease of metabolic engineering.

Acknowledgements

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13. The plasmid encoding the DEBS2 and DEBS3 genes (pBP130) has been reported earlier.[9] Plasmid BP175 (containing module 2 and the pcc) is a derivative of pBP144 (containing DEBS1 and the pcc[9]) with module 2 replacing DEBS1. The construction of module 2 has been described previously: Tsuji, S.; Cane, D. E.; Khosla, C. *Biochemistry* **2001**, *40*, 2326.
14. Small scale feeding using [1-^{14}C]propionate was performed as follows: an *E. coli* BAP1/pBP175/pBP130 transformant was inoculated into 1 mL LB medium in the presence of carbenicillin (100 mg/mL) and kanamycin (50 mg/mL) at 37 °C and 250 rpm. Cultures were grown to mid-log phase ($OD_{600} = 0.6$), cooled at 20 °C for 15 min, and then centrifuged. The cell pellets were resuspended in 1 mL of fresh LB media and induced with 1 mM IPTG. In addition, [1-^{14}C]propionate (56 mCi/mmol) and either diketides **2** or **3** or triketide analogues **6** and **7** were each administered at final concentrations of 1 mM. The culture was then shaken for an additional 24 h at 20 °C. At this point the culture was centrifuged and 1 mL of the supernatant was extracted with 2 mL of ethyl acetate. The extract was dried in vacuo and subjected to thin-layer chromatography (TLC) analysis. Negative controls included cultures without substrate.
15. Large-scale feeding using [1-^{13}C]propionate was performed as follows: A transformant of *E. coli* BAP1 DEBS/pBP175/pBP130 was used to start a 3-mL LB culture with carbenicillin (100 mg/mL) and kanamycin (50 mg/mL) at 37 °C and 250 rpm. Cultures were grown to mid-log phase ($OD_{600} = 0.7$) and then centrifuged at 3000 rpm. The cell pellets were resuspended in 3 mL of fresh LB media. This culture was used to inoculate 200 mL LB media with the same antibiotic concentrations as above. These cultures were grown at 250 rpm and 37 °C to mid-log phase ($OD_{600} = 0.5$–0.7), cooled for 15 min in a 20 °C bath, and induced with 1 mM IPTG. 1 mM [1-^{13}C]propionate and 0.5 mM substrate **2** or **5** were added at the same time, and the cultures were incubated at 20 °C for 48 h. The sample was then centrifuged and the supernatant extracted twice with 100 mL ethyl acetate (2×100 mL), and the derived macrolide products, **1** and **4**, respectively, were purified by SiO_2 gel column chromatography (ca. 1 g) from the concentrated extract. The products obtained were then analyzed by 1D (^{1}H and ^{13}C) and 2D NMR and by high-resolution mass spectrometry to confirm their individual structures.
16. **1**: ^{13}C NMR ($CDCl_3$, 100 MHz) δ 213.42 (C9), 178.36 (C1), 79.53 (C3), 76.52 (C5), 37.68 (C7) (enriched carbon atoms only); HRFAB-MS ($[M+Na]^+$, NBA/NaI): Calcd for ($^{12}C_{16}{}^{13}C_5H_{38}O_6$)$Na^+$: 414.2734, Found: 414.2754. **4**: ^{13}C NMR ($CDCl_3$, 400 MHz) δ 213.29 (C9), 178.28 (C1), 79.56 (C3), 76.46 (C5), 37.79 (C7) (enriched carbon atoms only); HRFAB-MS ($[M+Na]^+$, NBA/NaI): Calcd for ($^{12}_{18}{}^{13}C_5H_{42}O_6$)$Na^+$: 442.3095, Found: 442.3100.
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