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Article

Complete Biosynthesis of Erythromycin A and Designed Analogs Using *E. coli* as a Heterologous Host

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SUMMARY

Erythromycin A is a potent antibiotic long-recognized as a therapeutic option for bacterial infections. The soil-dwelling bacterium *Saccharopolyspora erythraea* natively produces erythromycin A from a 55 kb gene cluster composed of three large polyketide synthase genes (each ~10 kb) and 17 additional genes responsible for deoxysugar biosynthesis, macrolide tailoring, and resistance. In this study, the erythromycin A gene cluster was systematically transferred from *S. erythraea* to *E. coli* for reconstituted biosynthesis, with titers reaching 10 mg/l. Polyketide biosynthesis was then modified to allow the production of two erythromycin analogs. Success establishes *E. coli* as a viable option for the heterologous production of erythromycin A and more broadly as a platform for the directed production of erythromycin analogs.

INTRODUCTION

Erythromycin is an established antibiotic effective against a broad spectrum of Gram-positive bacterial pathogens and is natively produced from the soil-dwelling bacterium *Saccharopolyspora erythraea* (Schonfeld and Kirst, 2002). The genes responsible for erythromycin are found clustered within the *S. erythraea* chromosome (Cortes et al., 1990; Donadio et al., 1991; Oliynyk et al., 2007; Summers et al., 1997) and can be further subdivided into those responsible for (1) formation of the molecule's polyketide core, termed 6-deoxyerythronolide B (6dEB); (2) deoxysugar biosynthesis and attachment; and (3) additional tailoring and self-resistance (Figures 1A and 1B; see Figure S1 available online). Naturally produced erythromycin includes a number of variants, with erythromycin A being the most abundant and biologically active form (Figure 1B) (Kibwage et al., 1985; Majer et al., 1977).

The slow-growing, fastidious nature of *S. erythraea* coupled to the lack of sophisticated molecular biology protocols has hampered more directed efforts to either overproduce or alter erythromycin A biosynthesis. The situation spurred an interest in heterologous biosynthesis through more technically amenable microbial systems. Successful heterologous production would require coordinated expression of a minimum of 20 genes, including 2 cytochrome P450 monooxygenases and 3 type I modular polyketide synthase genes that encode for a large (2 MDa) $\alpha_2\beta_2\gamma_2$ multi-domain enzyme complex requiring 4′-phosphopantetheine posttranslational modification. The collective polyketide synthase is referred to as deoxyerythronolide B synthase (DEBS) and is composed of the DEBS1, 2, and 3 enzymes (Figure 1B). Furthermore, the system requires (2*S*)-methylmalonyl-CoA and propionyl-CoA substrates that may prompt separate metabolic engineering of the heterologous host to support eventual biosynthesis. Thus, the erythromycin case poses nearly every challenge that must be addressed in the quest for heterologous complex natural product biosynthesis. Erythromycin A was also particularly attractive because of the numerous opportunities to influence final compound structure through manipulation of either polyketide or tailoring biosynthesis. Supporting this view were the successful semisynthetic erythromycin analogs that extended the molecule's utility in the face of acquired pathogenic drug resistance (Denis et al., 1999; Ma et al., 2001). Heterologous biosynthesis provides an even greater opportunity for molecular diversification because the modular polyketide scheme offers seemingly limitless options to reprogram biosynthesis for analog formation (McDaniel et al., 1999).

Despite advances using *S. erythraea* or *Streptomyces* strains (Jacobsen et al., 1997; Kao et al., 1994; Xue et al., 1999), the knowledge base, molecular biology protocols, and growth kinetics of *E. coli* offered even greater potential in terms of heterologous biosynthesis. More broadly, the last 10–15 years have seen a growing commitment to the use of heterologous biosynthesis toward the production of antibiotic or similarly therapeutic natural products (Zhang et al., 2011). By engineering for substrate provision, DEBS posttranslational modification and activity, and coordinated gene expression, 6dEB production through *E. coli* was accomplished (Pfeifer et al., 2001). Efforts then began toward full biosynthesis using an analogous gene cluster from *Micromonospora megalomicea* or a hybrid pathway composed of genes from *S. erythraea*, *S. fradiae*, and *S. venezuelae*, which produced the intermediates erythromycin C and 6-deoxyerythromycin D, respectively (Lee and Khosla, 2007; Peiru et al., 2005).

These previous heterologous attempts with *E. coli*, while demonstrating the range of molecular biology options

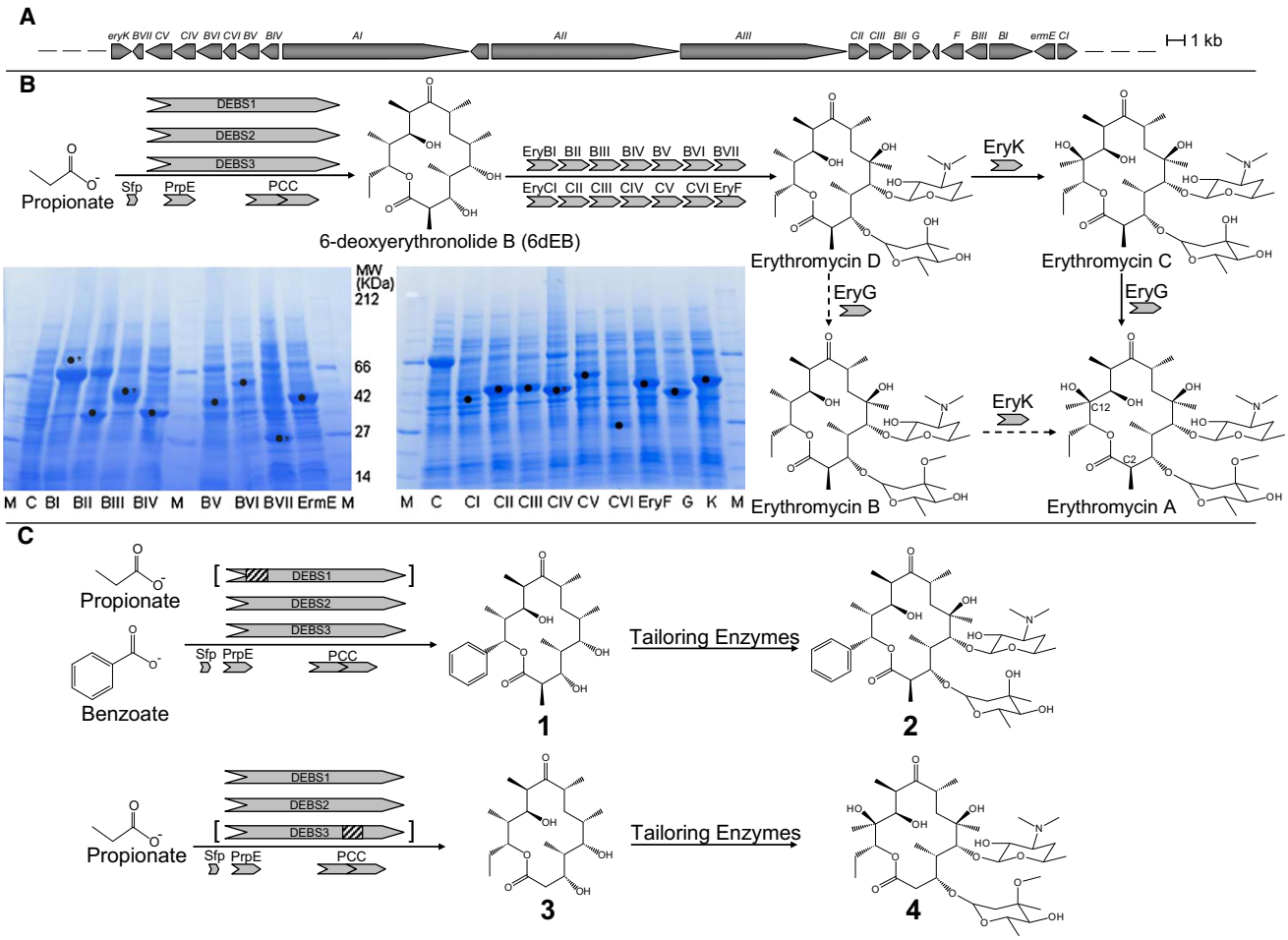

Figure 1. Heterologous *E. coli* Erythromycin A and Analog Production

(A) The erythromycin A gene cluster as organized in the *S. erythraea* chromosome.

(B) Simplified erythromycin A *E. coli* biosynthesis scheme including SDS-PAGE analysis of the tailoring biosynthetic enzymes. The Sfp enzyme is required for posttranslational modification of the DEBS1, 2, and 3 polyketide synthase enzymes; whereas, PrpE (propionyl-CoA synthetase) and PCC (propionyl-CoA carboxylase, composed of two protein subunits) are required to convert exogenous propionate to the starting precursors propionyl-CoA and (2*S*)-methylmalonyl-CoA (Pfeifer et al., 2001). Conversion of the 6dEB molecule to erythromycin A is accomplished by the deoxysugar biosynthesis and attachment, additional tailoring, and self-resistance enzymes (collectively referred to as the tailoring enzymes). The solid arrows present the expected preferred pathway from erythromycin D to erythromycin A (Lambalot et al., 1995; Paulus et al., 1990). Those genes (*eryBI*, *eryBIII*, *eryBVII*, *eryCIV*) expressed from pET28a have been denoted with an asterisk. The remaining genes were expressed from pET21c. M-protein marker; C-BL21(DE3) cellular lysate control; ErmE-erythromycin self-resistance enzyme.

(C) The polyketide and erythromycin analogs resulting from the indicated modifications to the DEBS1 and DEBS3 polyketide synthase enzymes.

See also Figure S1.

associated with the new host, have thus far failed to produce the most bioactive final form of erythromycin, erythromycin A. Here, we report the directed production of erythromycin A through the systematic reconstitution of the *S. erythraea* biosynthetic pathway in *E. coli*. The effort required the heterologous transfer of >50 kb of DNA and the coordinated expression of 26 genes, 23 of which were foreign. In addition, the erythromycin A modular polyketide biosynthetic scheme was altered to produce two rationally designed analogs. The results establish erythromycin A production using *E. coli* as a heterologous host and support future applied studies to continually modify the erythromycin biosynthetic pathway for new antibiotic compounds and activity.

RESULTS AND DISCUSSION

Tailoring Biosynthetic Gene Isolation and Individual Expression

We first used PCR to isolate the tailoring biosynthetic genes using *S. erythraea* chromosomal DNA as a template. Each gene was then placed into separate pET21c vectors to test individual gene expression. Although most genes expressed from pET21c (as assessed by SDS-PAGE) (Figure 1B), four proteins (EryBI, EryBIII, EryBVII, and EryCIV) were not observed when gene expression was induced from this vector. However, *eryBI*, *eryBIII*, *eryBVII*, and *eryCIV* were successfully expressed from pET28a. The pET28a vector includes an N-terminal sequence

(containing a 6 × histidine tag) preceding the original start codon of the gene to be expressed, and this addition may account for the positive impact upon final protein production. Convenient biosynthetic or phenotypic tests were then performed to confirm the activity of select enzymes. Specifically, *eryF* expression allowed conversion of 6dEB to erythronolide B, and *ermE* expression provided resistance to erythromycin (Figure S2). Because most polyketide products (including erythromycin A) derive from hosts exhibiting a high G+C genetic content, alternative approaches to improving expression have included designed gene synthesis to eliminate rare codon usage within a new host or commercially available hosts designed to accommodate G+C rich genes (Gouy and Gautier, 1982; Menzella et al., 2006). However, our system relied totally on the original gene sequences from *S. erythraea* and highlighted the native capabilities of *E. coli*, at least in this case, to accommodate significantly foreign genes and proteins. In addition, our approach was in contrast to earlier efforts, which used a combination of tailoring genes from multiple sources (*S. erythraea*, *S. fradiae*, and *S. venezuelae*) that each demonstrated sufficient heterologous expression within *E. coli* (Lee and Khosla, 2007). Here, we were able to directly reconstitute full activity from the original *S. erythraea* erythromycin A pathway.

Tailoring Biosynthetic Operon Design

With successful individual gene expression confirmed, our attention turned to packaging the tailoring and resistance genes for transfer to and coordinated expression within *E. coli*. Operons were designed using a strategy similar to earlier efforts at introducing modular polyketide and nonribosomal peptide pathways to heterologous hosts (Kao et al., 1994; Lee and Khosla, 2007; Peiru et al., 2005; Pfeifer et al., 2001, 2003; Watanabe et al., 2006). Namely, synthetic operons were constructed according to the diagram in Figure 2 (a more detailed diagram is provided in Figure S3). Here, the cassettes demonstrating successful individual expression were used to construct two operons of eight and nine genes with each operon driven by a preceding T7 promoter under the control of a *lac* operator. It should be noted that no specific biosynthetic activity has been assigned to EryBI, and the removal of *eryBI* has been shown to have negligible effect on native *S. erythraea* erythromycin A production (Gaisser et al., 1998). However, here, *eryBI* was included so as to retain any resulting beneficial activity within a heterologous environment. The *ermE* self-resistance gene was similarly included to protect the *E. coli* host from nascent erythromycin A activity. The final operons were subsequently used in the following experiments to generate *E. coli*-derived erythromycin A, and coordinated gene expression and enzymatic activity were confirmed in the context of those experiments.

E. coli Growth Phenotype and Plasmid Stability when Including the Tailoring Biosynthetic Operons

To characterize cell growth with the inclusion of the tailoring biosynthetic operons, strain BL21(DE3)(pHZT1/pHZT2) (including pGro7; please see below) was compared under different growth conditions (Figure S3). No obvious differences were observed with or without induction, implying that the tailoring plasmids were not overly burdensome to the cell during gene expression. However, as previously observed for those

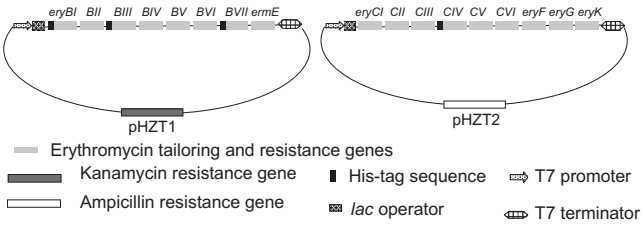

Figure 2. Operon Design for the pHZT1 and pHZT2 Plasmids
See also Figure S3.

plasmids harboring the DEBS genes (Pfeifer et al., 2001), plasmid instability was apparent for pHZT1 (61% retention 12 hr after induction) and pHZT2 (28% retention 12 hr after induction). This no doubt stems from both plasmids containing the same origin of replication, further supported by the 100% retention of pGro7 containing a different, compatible origin.

E. coli-Derived Erythromycin A Biosynthesis

Initial efforts at *E. coli* erythromycin A biosynthesis consisted of producing 6dEB from a strain containing the required DEBS1, 2, and 3 enzymes followed by feeding the extracted 6dEB product to a separate *E. coli* strain containing the plasmids harboring the newly constructed tailoring biosynthetic operons. No production was observed under these conditions. However, when plasmid pGro7 containing the genes for the *E. coli* GroEL/ES chaperone system was introduced to the strain containing the tailoring biosynthetic operons, production of erythromycin B and D was observed by LC-MS analysis (Figure S4). Similar approaches to aid protein folding and/or association have been needed during previous complex natural product biosynthetic attempts (Betancor et al., 2008; Lee and Khosla, 2007; Mutka et al., 2006; Peiru et al., 2005; Pfeifer et al., 2001), further highlighting the challenges associated with reconstituting such pathways. In this case, the accumulation of erythromycin B suggested to us that biosynthesis might be stalled at the step where the EryK P450 hydroxylase converts erythromycin D to erythromycin C (Figure 1B).

Given this status, we analyzed the positioning of *eryK* in our operon design. The *eryK* gene was the last of nine genes in one of the tailoring operons. As such, we hypothesized a lack of gene expression and supplemented expression by adding another plasmid expressing only *eryK*. This addition resulted in erythromycin C and A production at 2 and 10 mg/l, respectively, with 90% of the erythromycin product secreted to the surrounding medium; the erythromycin extract was further characterized by MS/MS analysis, and antibiotic activity was confirmed through a separate *Bacillus subtilis* growth inhibition assay (Figure 3; Figure S5). The initial erythromycin A titer is considered promising given the metabolic and process engineering tools available for *E. coli*. For example, *E. coli* fed-batch bioreactor protocols have previously been used to overproduce the 6dEB precursor to titers >1 g/l, a nearly 200-fold increase over original levels (Lau et al., 2004).

In an effort to consolidate the entire erythromycin A biosynthetic pathway to a single *E. coli* cell, we altered the resistance markers of the plasmids responsible for 6dEB biosynthesis such that one cell harbored all six plasmids required for both

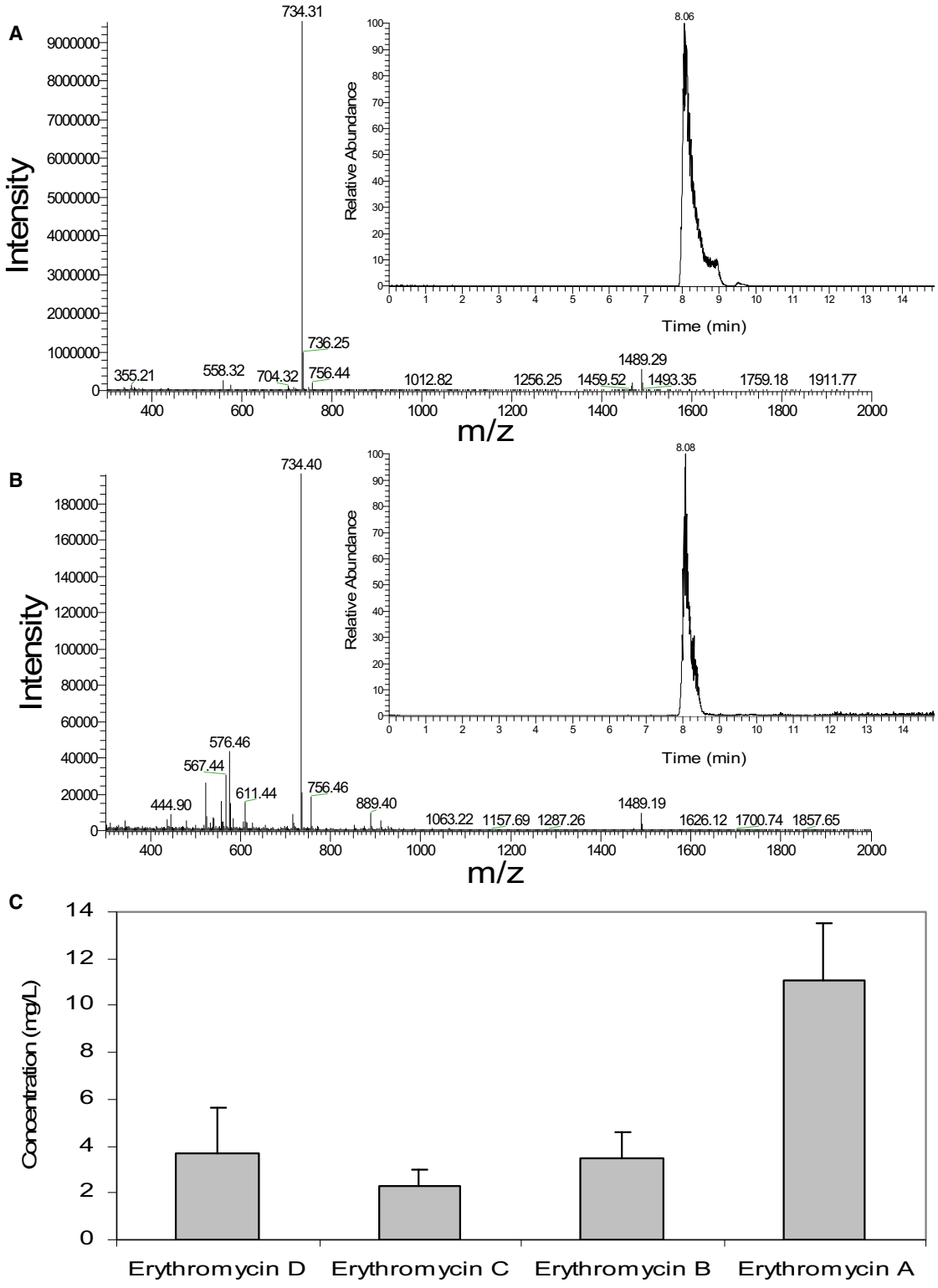

Figure 3. Confirmation of *E. coli* Erythromycin A Production from the Serial, Two-Cell Scheme

(A) Chromatogram and mass spectrum for erythromycin A standard.

(B) Chromatogram and mass spectrum for *E. coli*-derived erythromycin A.

(C) Final titer quantification (± standard deviation; N = 3).

See also Figure S5.

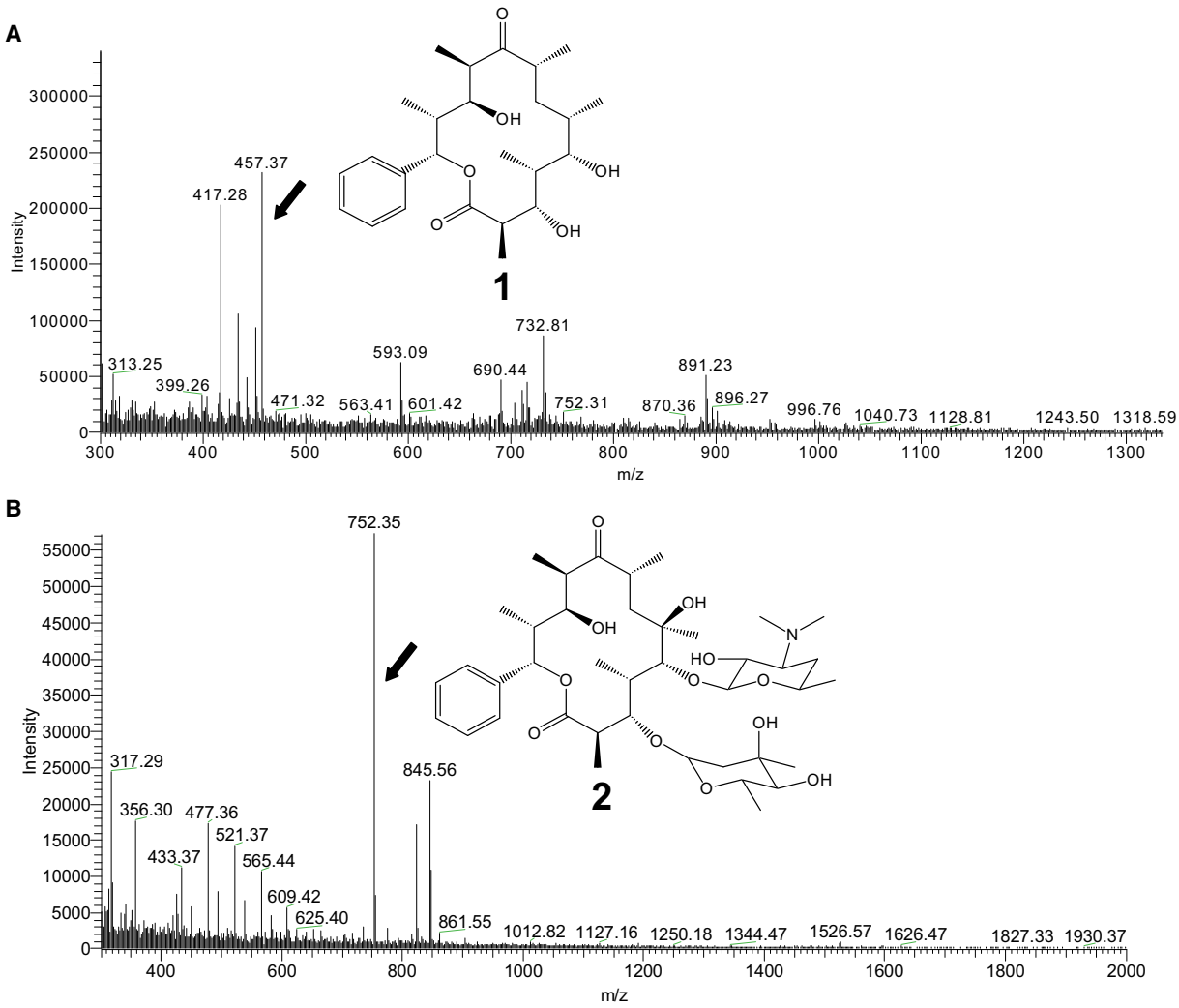

Figure 4. Erythromycin Analog 2 Production and Quantification

The mass spectra for *E. coli*-derived (A) **1** (457 m/z; sodium adduct) and (B) **2** (752 m/z) produced from the serial, two-cell scheme using BAP1(pBP130/pBP165) followed by BL21(DE3)(pHZT1/pHZT2/pHZT4/pGro7). The higher m/z peaks observed most likely represent co-eluting contaminants extracted from production medium or dimerized erythronolide products.

polyketide and tailoring biosynthesis. Doing so resulted in erythromycin A production at 0.6 mg/l (Figure S6), and even though the final titer was reduced when compared to the sequential production strategy, one-cell production confirmed the capability of *E. coli* to coordinately express the 26 genes required for final erythromycin A biosynthesis from an inexpensive three-carbon building block of propionate.

Erythromycin Analog Design and Biosynthesis

We next sought to extend the *E. coli* erythromycin production platform toward analog biosynthesis. As illustrated in Figure 1C, variations were made to the DEBS1 and DEBS3 enzymes in an effort to leverage the multi-catalytic capability of the modular polyketide synthase. Specifically, the loading module of DEBS1 was replaced with the loading module from the rifamycin polyketide synthase such that a benzoate starter unit could be accepted, producing a benzyl addition to the erythromycin structure (Admiraal et al., 2001; Pfeifer et al., 2001). Similarly, the second module of DEBS3 was altered to accept a malonyl-CoA extender unit, which would result in the lack of a methyl group at the C2 position when compared to the original erythromycin A compound (Liu et al., 1997). In this way, analogs of 6dEB and, subsequently, erythromycin were expected.

Figures 1C, 4, and 5 summarize the analogs produced by this approach. Compound **2**, an analog of erythromycin D with a designed benzyl unit, was produced at a titer of 0.49 ± 0.19 (standard deviation) mg/l; analog **4** was generated together with its corresponding B, C, and D derivatives at titers between 0.05 and 0.2 mg/l. For all erythromycin A and analog heterologous production experiments, intermediates such as erythronolide B and 3-mycarosyl-erythronolide B (or their corresponding analog equivalents) were also observed during final LC-MS analyses (Figure S1). We believe compound **2** results from the benzyl group hindering the activity of EryK. This would then

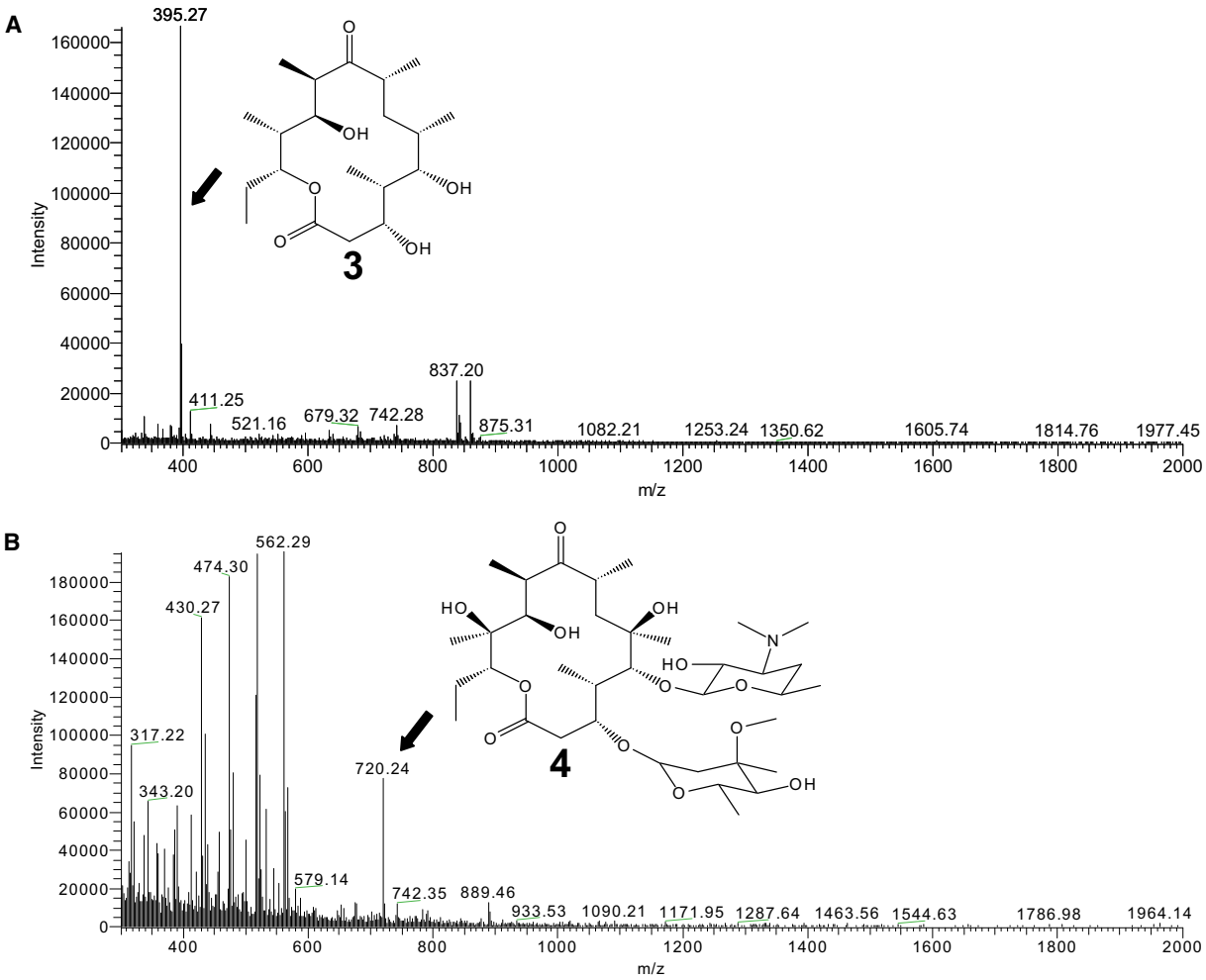

Figure 5. Erythromycin Analog 4 Production and Quantification

The mass spectra for *E. coli*-derived (A) **3** (395 m/z; sodium adduct) and (B) **4** (720 m/z) produced from the serial, two-cell scheme using BAP1(pBP144/pBP173) followed by BL21(DE3)(pHZT1/pHZT2/pHZT4/pGro7).

explain the lack of B, C, and A analog equivalents for compound **2** because the preferred pathway for biosynthesis begins with the activity of EryK. Interestingly, erythromycin A was also observed from the experiments to produce compounds **2** and **4**. For the case of compound **2**, this unexpected result could be explained by propionyl-CoA being loaded directly to the ketosynthase domain of DEBS1 (Long et al., 2002; Pereda et al., 1998; Weissman et al., 1998); however, for compound **4**, the reasons for erythromycin A coproduction are unclear. Finally, we note that analog production demonstrates a level of flexibility associated with the glycosyltransferase enzymes responsible for attaching the deoxysugar residues to the macrolactone polyketide core. This flexibility has been observed with other systems and identified as a key step in realizing the full potential of altered structural design (Borisova et al., 2006). Success in this case supports the capabilities of the native glycosyltransferase enzymes to accommodate similar modifications to the polyketide and tailoring steps anticipated in analog biosynthetic approaches.

In summary we present the complete biosynthesis of erythromycin A using *E. coli* as a heterologous host. Besides providing access to the most potent form of erythromycin, the *E. coli* production platform offers numerous next-generation engineering opportunities. Optimal protein levels and activity, availability of required cofactors and precursors, conversion of substrate to product, and scaled bioreactor production are just of a few of the challenges to be addressed with the established engineering tools available to *E. coli*. Aiding this effort will be the range of molecular biology strategies to manipulate native and heterologous metabolism. These same molecular engineering strategies will also offer extended erythromycin analog design and have been used here for the directed production of compounds **2** and **4**. However, the modular, multi-catalytic nature of the DEBS polyketide synthase, in addition to the engineering opportunities within tailoring biosynthesis, promises a wide range of new compounds. This potential then stands as an answer to the need for new antibiotics to combat the growing presence of pathogenic drug resistance (Fischbach and Walsh, 2009; Walsh, 2003).

SIGNIFICANCE

There is no doubt about the positive impact antibiotics and other natural products have had on modern medicine. However, beneath this medicinal value lie the technical challenges of ready access to complex natural compounds. Production from intractable environmental sources limits the speed, economy, and structural diversity possible from reconstituted biosynthesis in technically convenient heterologous hosts. Thus, heterologous biosynthesis offers the potential of rapid overproduction of original and analog forms of clinically relevant natural compounds. This report presents the complete production of erythromycin A using *E. coli* as a heterologous host. The work is significant for several reasons: (1) erythromycin A is an established antibiotic compound with a complex polyketide biosynthetic pathway; (2) the full heterologous production of this compound has not been reported, and the steps to do so required overcoming several challenges in heterologous gene transfer, coordinated gene expression, metabolic engineering, and final biosynthetic activity; (3) unoptimized product titers have reached 10 mg/l, a starting point considered promising given the range of metabolic and process-engineering options associated with *E. coli*; and (4) two designed analogs were produced through directed manipulation of polyketide biosynthesis; subsequent efforts offer the promise of generating a range of new bioactive analogs. The last point is of particular relevance given the balance between bacterial drug resistance to erythromycin (and other antibiotics) and the compound's modular biosynthetic pathway. Thus, successful reconstitution through *E. coli* offers an alternative route to the original compound and a platform for full manipulation of the biosynthetic pathway with the express purpose of expanding molecular diversity and antibiotic activity.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals

The reagents and chemicals used in this study were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma (St. Louis, MO, USA). All restriction enzymes and the Phusion High-Fidelity PCR Master Mix were purchased from New England Biolabs (Ipswich, MA, USA). PCR primers were synthesized by Operon (Huntsville, AL, USA). The chaperone plasmid kit and the pCDFDuet vector were purchased from Takara (Madison, WI, USA) and EMD Chemicals Inc. (Gibbstown, NJ, USA), respectively.

Cell Culture Media and Growth Conditions

All *E. coli* cell cultures for molecular biology and SDS-PAGE analysis were conducted in Luria-Bertani (LB) medium at 37°C and 250 rpm. Heterologous 6dEB and erythromycin biosynthesis were conducted in production medium at 22°C and 250 rpm. One liter of production medium contained 5 g yeast extract, 10 g tryptone, 15 g glycerol, 10 g sodium chloride, 3 ml 50% v/v Antifoam B, 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesuffonic acid (HEPES) buffer, and was adjusted to pH 7.6 by 5 M NaOH before use.

Plasmid Design, Construction, and Gene Expression

Restriction enzyme digestions, SDS-PAGE, and other standard molecular biology techniques were performed as described by Sambrook et al. (1989). Erythromycin-tailoring and resistance genes were PCR amplified from *S. erythraea* genomic DNA using the primers listed in Table S1. The NdeI/EcoRI digested fragments of the PCR products (except for genes *eryBIII*, *BV*, and *CIII*) were individually inserted into plasmid pET21c digested with the same restriction enzymes. The *eryBIII* PCR product was digested by NheI/EcoRI and inserted into pET21c using the corresponding restriction sites. Similarly, the *eryBV* and *eryCIII* PCR products were digested and inserted by NdeI/SacI and NdeI/HindIII, respectively. After transformation of the individual pET21c expression cassettes into BL21(DE3), cultures were induced overnight at 37°C, followed by sonication cell disruption. Cell lysates were clarified by centrifugation, and soluble fractions were analyzed by SDS-PAGE.

The tailoring genes that could not be expressed using pET21c (*eryBI*, *BIII*, *BVII*, and *CIV*) were transferred to plasmid pET28a by NheI/HindIII (for *eryBIII*) or NdeI/HindIII (for *eryBI*, *BVII*, and *CIV*) digestion and subsequent ligation. SDS-PAGE was performed as previously described to investigate expression from pET28a. The insertion of all erythromycin tailoring and resistance genes into individual expression plasmids was verified by Sanger sequencing at the Tufts University Core Facility.

After SDS-PAGE confirmation of gene expression, the *eryBI*, *BII*, *BIII*, *BIV*, *BV*, *BVI*, *BVII*, and *ermE* genes were combined (in order) into one operon by sequential XbaI/SpeI and SacI digestion and ligation (Figure S3). The resulting plasmid pHZT1 was a derivative of the kanamycin-resistant pET28a plasmid. Genes *eryCI*, *CII*, *CIII*, *CIV*, *CV*, *CVI*, *eryF*, *eryG*, and *eryK* were combined (in order) into one operon by sequential XbaI/SpeI and HindIII digestion and ligation. The resulting plasmid pHZT2 was a derivative of the ampicillin-resistant pET21c plasmid.

In order to construct a plasmid carrying an additional *eryK* gene, PCR and PstI/HindIII digestion preceded ligation into the streptomycin-resistant pCDFDuet-1 vector. The resulting plasmid was named pHZT4. In addition the chaperone expression plasmids pG-KJE8, pGro7, pKJE7, pG-Tf2, and pTf16 were individually co-transformed with plasmids pHZT1, pHZT2, and (when indicated) pHZT4 into *E. coli* BL21(DE3) to test the effects each chaperone or chaperone combination had on erythromycin biosynthesis.

For one-cell production of erythromycin A, plasmid pBP130 containing the genes for DEBS2 and DEBS3 (Pfeifer et al., 2001) was modified by replacing the ampicillin-resistance marker with an apramycin (apr)-resistance marker though λ-Red recombination (Datsenko and Wanner, 2000). Specifically, the apr-resistance gene was PCR amplified using plasmid pSBAC as a template (Liu et al., 2009) (Table S1). Purified PCR product and plasmid pBP130 were co-transformed into *E. coli* TOP10 harboring pKD46 (Datsenko and Wanner, 2000) through electroporation, and the resulting culture was grown in LB medium overnight at 37°C. Plasmid product was then isolated from the overnight culture and transformed into *E. coli* TOP10 for selection on an LB-agar plate containing 50 mg/l apr. Positive colonies were cultured and the resulting plasmids assessed by restriction analysis. The final plasmid was named pBPJW130.

In a similar fashion the kanamycin resistance marker on plasmid pBP144 (containing the genes required for the PCC and DEBS1; Pfeifer et al., [2001]) was changed to a tetracycline resistance marker. Here, the tetracycline-resistance gene was PCR amplified from plasmid pBR322 (Table S1). The kanamycin marker of pBP144 was replaced analogous to the procedure to modify pBP130, and the resulting plasmid was named pBPJW144.

For analog production, plasmids pBP165 (kanamycin resistant) and pBP173 (ampicillin resistant) were introduced to replace pBP144 and pBP130, respectively. Plasmid BP165 was described previously (Pfeifer et al., 2001) and contains the rifamycin-loading di-domain instead of the native DEBS1 di-domain. Plasmid BP173 derives from the constructs presented in Liu et al. (1997), in which the acyltransferase domain of module 6 is replaced with the rapamycin module 2 acyltransferase such that the extender unit accepted at this position is malonyl-CoA.

Erythromycin Biosynthesis and Analysis

For serial, two-cell erythromycin production, 6dEB was first generated using strain BAP1(pBP130/pBP144) as previously described (Zhang et al., 2010). For 6dEB analog **3**, BAP1(pBP173/pBP144) was cultured in 100 ml production medium containing 100 mg/l carbenicillin, 50 mg/l kanamycin, 100 μM isopropyl β-D-1-thiogalactopyranoside (IPTG), and 20 mM propionate at 22°C for 7 days. Analog **1** was produced similarly (Pfeifer et al., 2001) from BAP1 (pBP130/pBP165) with 20 mM of benzoate (stock solution adjusted to pH 7) included with the propionate, IPTG, and selection antibiotics. Native 6dEB and both analogs were extracted with ethyl acetate, dried, and resuspended

in methanol. The 6dEB compound was quantified using an HPLC-Evaporative Light Scattering Detector, and the 6dEB analogs were quantified by MS as previously described (with purified 6dEB as a standard) (Zhang et al., 2010).

To complete the two-cell production process, an overnight culture of *E. coli* BL21(DE3)(pHZT1/pHZT2/pHZT4/pGro7) was inoculated (5% v/v) into 1 ml production medium containing 100 mg/l carbenicillin, 50 mg/l kanamycin, 20 mg/l chloramphenicol, 50 mg/l streptomycin, 100 μM IPTG, and 2 mg/ml arabinose (pGro7 required arabinose induction) and cultured at 22°C for 24 hr. The 6dEB or 6dEB analog methanol extracts (produced as described in the preceding paragraph) were then added to final polyketide concentrations of 50 mg/l, and erythromycin production commenced by culturing for an additional 3 days at 22°C.

For one-cell erythromycin A production, an overnight culture of *E. coli* BAP1(pBPJW130/pBPJW144/pHZT1/pHZT2/pHZT4/pGro7) was inoculated (5% v/v) into 1 ml production medium containing 100 mg/l carbenicillin, 50 mg/l kanamycin, 20 mg/l chloramphenicol, 50 mg/l streptomycin, 50 mg/l apr, 5 mg/l tetracycline, 100 μM IPTG, 2 mg/ml arabinose, and 20 mM propionate and grown at 22°C for 7 days.

One milliliter of the *E. coli* cultures containing erythromycin A or the erythromycin analogs was extracted with 0.5 ml ethyl acetate. After centrifugation at 12,000 rpm for 1 min, the extract was transferred to a 1.5 ml micro-centrifuge tube, air dried, and resuspended in 100 μl of methanol. Samples were then analyzed using an LTQ XL Linear Ion Trap Mass Spectrometer (Thermo Electron Corporation) coupled with a Finnigan Surveyor LC system (Thermo Electron Corporation) with an Agilent ZORBAX Eclipse XDB-C18 HPLC column (Santa Clara, CA, USA). All MS analyses used electrospray ionization and were conducted in positive ion mode. A linear gradient of 100% water to 100% acetonitrile over 15 min was used at a flow rate of 0.6 ml/min. Where indicated, MS/MS analysis was performed using a collision energy of 25 V.

To quantify erythromycin A and analog production, an LC-MS calibration curve was prepared using commercially available erythromycin A as an external standard and roxithromycin as an internal standard. Known amounts of erythromycin A were added to *E. coli* BL21(DE3) or BAP1 cultures grown under the same conditions described above. After ethyl acetate extraction, the samples were dissolved in 100 μl of methanol containing 2.5 mg/l roxithromycin and subjected to LC-MS analysis to prepare the calibration curve. A portion of the extracts resulting from the erythromycin producing *E. coli* cultures was similarly mixed with roxithromycin and analyzed against the calibration curve. The ratio of the erythromycin A and roxithromycin standard peak areas was correlated with erythromycin A concentrations to quantify experimental erythromycin production with a suitable calibration curve made before every experimental analysis. All reported titers represent at least three independent experiments. Negative controls included (1) experiments reliant on strain BAP1 without the required production plasmids, (2) uninduced cultures, and (3) the replacement of pHZT2 with an empty pET21c expression vector.

Bacillus subtilis Growth Inhibition Bioassay

To confirm the antibiotic activity of *E. coli*-derived erythromycin A, a *B. subtilis* zone-of-inhibition bioassay was performed. Methanol solutions of erythromycin extracts were adjusted to specific concentrations based upon the results from quantification. This solution was then added to a filter paper disk and placed upon an LB agar plate prepared by mixing 20 μl of an overnight *B. subtilis* culture with 20 ml liquified LB agar (maintained at 45°C). After overnight incubation at 37°C, *B. subtilis* inhibition zones were assessed for positive control, negative control, and experimental filter disk samples. The assay was repeated a minimum of three times from independent culture experiments to ensure reproducibility.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article online at doi:10.1016/j.chembiol.2010.09.013.

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