Multiplexed Integrating Plasmids for Engineering of the Erythromycin Gene Cluster for Expression in Streptomyces spp. and Combinatorial Biosynthesis

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Bacteria in the genus Streptomyces and its close relatives are prolific producers of secondary metabolites with antibiotic activity. Genome sequencing of these bacteria has revealed a rich source of potentially new antibiotic pathways, whose products have never been observed. Moreover, these new pathways can provide novel genes that could be used in combinatorial biosynthesis approaches to generate unnatural analogues of existing antibiotics. We explore here the use of multiple orthologous integrating plasmid systems, based on the int/attP loci from phages TG1, SV1, and φBT1, to express the polyketide synthase (PKS) for erythromycin in a heterologous Streptomyces host. Streptomyces strains containing the three polyketide synthase genes eryAI, eryAII, and eryAIII expressed from three different integrated plasmids produced the aglycone intermediate, 6-deoxyerythronolide B (6-dEB). A further pair of integrating plasmids, both derived from the φC31 int/attP locus, were constructed carrying a gene cassette for glycosylation of the aglycone intermediates, with or without the tailoring gene, eryF, required for the synthesis of erythronolide B (EB). Liquid chromatography-mass spectrometry of the metabolites indicated the production of angolosaminyl-6-dEB and angolosaminyl-EB. The advantages of using multiplexed integrating plasmids for engineering expression and for combinatorial biosynthesis were demonstrated.
Erythromycin A is a bacteriostatic macrolide antibiotic produced from *Saccharopolyspora erythraea* (formerly *Streptomyces erythreus*) (13). The biosynthesis of erythromycin can be divided into two stages (14). First, the modular polyketide synthase (PKS) complex, 6-deoxyerythronolide B (6-dEB) synthase, catalyzes the sequential condensation of proprionyl-coenzyme A (CoA) and six methylmalonyl-CoA precursors to generate 6-dEB, the first isolatable intermediate in the pathway (Fig. 1). The second stage is the conversion of 6-dEB to erythromycin A, starting with the conversion of 6-dEB to erythronolide B (EB) by EryF hydroxylase (15). Two deoxysugars are then transferred to the aglycone ring to generate the first bioactive intermediate, erythromycin D (15); EryBV glycosyltransferase transfers L-mycarose to yield 3-O-mycarosylerythronolide B and then EryCIII, activated by EryCII, transfers D-deoxydesosamine to the C-5 hydroxyl (14, 16). The genes required for the biosynthesis of the activated sugars, TDP-deoxymycarose and TDP-deoxydesosamine, are all encoded within the erythromycin gene cluster (16). The final two tailoring steps, hydroxylation of C-12 by EryK hydroxylase (17), and methylation the 3'-OH of mycarose by EryG methyltransferase lead to the final product erythromycin A (18) (Fig. 1).

The biosynthetic pathway of erythromycin A offers multiple opportunities for combinatorial biosynthesis and the production of unnatural analogues. Genetic manipulation of such a large cluster comprising 55 kbp and 22 genes represents a daunting challenge (Fig. 1) (19). Engineering the erythromycin gene cluster in the native strain presents further difficulties due to the fastidious nature of *S. erythraea* and the low frequency of transformation by large plasmids (20). Despite these, previous researchers have been immensely successful at rational alteration of the pathway to produce new analogues of the intermediate 6-dEB (21–23). Several of these studies have relied on expression in a heterologous host, *S. coelicolor*, *S. lividans*, or *E. coli* (24). Using a freely replicating SCP2*-derived plasmid encoding all three of the PKS enzymes (DEBS1, DEBS2, DEBS3, encoded by *eryAI*, *eryAII*, and *eryAIII*), Kao et al. was able to express 6-dEB and 8,8a-deoxyoleandolide in *S. coelicolor* (21). Further work by McDaniel et al., using the same genetic construct as used by Kao et al., demonstrated that the aglycone ring could be modified by substitution of enzyme domains from the homologous rapamycin pathway to produce 61 6-dEB analogues (25). At the same time, Xue et al. placed the three *eryA* genes on different plasmids using SCP2* for
ereAI and ereAIII and the φC31 int/attP site for ereAIII (26). Since two of the plasmids are derived using the same replicon, SCP2*, selection had to be constantly applied to ensure against plasmid loss (26). Despite this and other potential problems that were predicted to arise through the use of vectors derived from the same incompatibility group Xue et al. demonstrated the principle of in trans expression of the PKS genes in the heterologous host. Moreover, placing the three ereA genes on different plasmids greatly facilitated combinatorial engineering of the ereAI, ereAI, and ereAIII genes.

Here, we adapt and improve the strategy taken by Xue et al., and we show that the expression of the ereAI, ereAI, and ereAIII genes can be achieved from three orthogonal integrating vectors in S. coelicolor. Using a fourth plasmid, we demonstrate how the tailoring steps downstream of the synthesis of 6d-EB and EB might be easily modified.

MATERIALS AND METHODS

Bacterial strains and culture conditions. _E. coli_ strain DH5α, _E. coli_ strain ET12567(pUZ8002), and _E. coli_ strain BW25113(pIJ790) were grown as described elsewhere (27, 28). _E. coli_ strain DH5α was used for plasmid propagation and subcloning while _E. coli_ strain BW25113(pIJ790) was used for recombineering using the REDIRECT method (27). The DNA methylation deficient strain of _E. coli_, ET12567(pUZ28002) was used as the donor plasmid for conjugation for _Streptomyces_ species as described previously (29).

_Streptomyces_ strains were maintained on Soya Mannitol (SM) agar at 30°C (30). _Streptomyces_ strains used as heterologous expression hosts were _Streptomyces coelicolor_ IAM1929, a _aphIV_ derivative of the wild-type strain _M145_ (31), _Streptomyces coelicolor_ M512 (ΔredD ΔactII-oriF SCP1-SCP2*) (32), _Streptomyces coelicolor_ M1152 (ΔactI ΔredD Δerp Δdaa rpO(B)1987), _Streptomyces coelicolor_ M1154 (ΔactI ΔredD Δerp Δdaa rpO(B)1987), _S. lividans_ TK24 (str-6 SLP2 ΔLP3*) (30), _Microccocus luteus_ (Fleming strain) was grown in tryptic soy broth (TSB) at 30°C and was used as a challenge organism for the detection of antibiotic activity.

DNA manipulations. Chemically competent _E. coli_ cells were prepared, stored, and used in the transformation procedure as described previously (28). Plasmid DNA extraction from _E. coli_ was performed using QIAprep spin miniprep kit according to the protocol supplied by the manufacturer (Qiagen). Restriction enzymes used during the study were obtained from New England BioLabs (NEB), and the digestion procedure was carried out according to the manufacturer’s instructions. Phusion high-fidelity DNA polymerase (NEB) was used for PCR amplification unless otherwise stated. The primers used in the present study are listed in Table 1. Overlap extension PCR was used to attach two or more DNA fragments when required, as described previously (34). An In-Fusion HD cloning kit (Clontech) was used generally for cloning DNA fragments according to the protocol supplied by the manufacturer.

Plasmid constructions. (i) Construction of pRF10. The _int/attP_ region of the phage _Tg1_ was amplified by PCR using _Tg1_ genomic DNA as a template and the primer _M5403_. The amplified product fragment was then digested using _SpeI_ and _HpaI_ and ligated using NEB quick ligation kit to the _SpeI_/*HpaI_ 1.994-bp fragment from _pMS82_ to generate _pRF10_. The ligation mixture was introduced into _E. coli_ by transformation and used in a conjugation assay to verify efficient integration of _pRF10_ into the _S. coelicolor_ genome, followed by rescue the large EcoRI fragment containing all of the _eryA_ genes (35). Upstream of _ereAI_ in _pIB023_ is the actII promoter and the _actII-orf4_ gene encoding the activator of _actLP_ (35). The recombineering procedure of Gust et al. (27) was used to replace all of the DNA from the 3’ end of _ereAI_ to the 3’ end of _ereAIII_ in _pLB023_ with an overlap extension PCR product encoding _aac(3)Iv-orfT-TG1-int/attP_ (34) generated from two PCR products. The _aac(3)Iv-orfT_ disruption cassette was amplified by PCR using _pIJ773_ as the template and primers _PBF20A-for_ and _PBF20B-rev_. The _TG1-int/attP_ locus was amplified using plasmid _pRF10_ with the primers _PBF20B-for_ and _PBF20B-rev_ and then used to create a single amplified product of 3,529 bp.

Primers _PBF20A-for_ and _PBF20B-rev_ contained 39 nucleotides (nt) of sequence that were identical to the endpoints of the DNA to be retained in _pLB023_ and were necessary to mediate recombineering between the _aac(3)Iv-orfT-TG1-int/attP_ PCR overlap extension product and _pLB023_ to remove the _ereAI_ and _ereAIII_ genes. Recombineering was performed by electroporation of the 3,529-bp PCR fragment into _E. coli_ strain _BW25113_(_pIJ790, pIB023_) after induction of the _gam_, _redA_, and _redB_ genes by arabinose (27). Selection for apramycin resistance yielded the desired plasmid, _pBF20_ (Fig. 2), which was confirmed by restriction analysis and nucleotide sequencing.

(ii) Construction of the expression plasmid _pBF22_. _pBF22_ is an SV1 _int/attP*-based integrating plasmid (11) containing the _ereAI_ gene under the control of the actII promoter and encodes the kanamycin-resistant gene (_aphII_) for selection (Fig. 2). To generate _pBF22_, _pIB023_ was digested with _BglII_ and _PacI_, and the 13,582-bp DNA fragment was purified. This DNA fragment contains a 5’ truncated _ereAI_ gene downstream of the _actII_ promoter, the thioestrepton resistance gene (_str_), the ampicillin resistance gene (_bla_), and the _E. coli_ _colE1_ plasmid origin of replication. The 390-bp region encoding the 3’ end of the _ereAI_ gene was amplified by PCR using _pIB023_ as a template and the primers _PBF22A-for_ and _PBF22B-rev_ and digested with _BglII_ and _PacI_. The 13,582-bp fragment and the 390-bp fragments were ligated together to form plasmid _pBF22A_. The _SV1-int/attP_ locus was amplified by PCR using _pBF3_ and the primers _PBF22F-for_ and _PBF22B-rev_ and inserted by _In-Fusion_ cloning into _pBF22A_ with _XbaI_ to give plasmid _pBF22_. The _aphII_ and _oriT_ fragments were amplified separately by using the primer pairs _PBF22C-for/PBF22C-rev_ and _PBF22D-for/PBF22D-rev_, respectively, and the templates _pNRT4_ (10) and _pIJ778_ (27), respectively. Overlap extension PCR was then used with the primers _PBF22D-for_ and _PBF22C-rev_ to fuse the _aphII_ and _oriT_ PCR fragments to generate a 1,259-bp fragment, which was inserted by _In-Fusion_ cloning into _pBF22B_ linearized with _SfiI_. The final plasmid, _pBF22_, was confirmed by restriction analysis and nucleotide sequencing.

(iii) Construction of the expression plasmid _pBF24_. _pBF24_ is _φBT1 _int/attP*-based integrating plasmid (10) harboring the _ereAI_ gene downstream of the actII promoter and containing _ermE_ for selection (Fig. 2).

_pBF24_ was created in multiple steps. A 4,079-bp fragment encoding the 5’ part of _ereAI_ was amplified using _pIB023_ as a template and the primers _PBF24A-for_ and _PBF24A-rev_ introducing a _Hpa_ restriction site at the 3’ end (_pBF24A-rev_). A 4,475-bp fragment encoding the plasmid vector sequence (containing _bla_, _str_, the actII promoter, _actII-orf4_, and the _colE1_ origin) was also amplified from _pIB023_ using the primers _PBF24B-for_ and _PBF24B-rev_. The 4,079- and 4,475-bp fragments were then ligated together by infusion to generate _pBF24A_. The remaining 3’ part of the _ereAI_ gene was amplified by using the primers _PBF24C-for_ and _PBF24C-rev_ and ligated with _pBF24A_ with _HpaI_ to form plasmid _pBF24B_. _pBF24C_ was constructed by amplification of the _ermE_ gene from _S. erythraea_ _BIOT-4480_ using the primers _PBF24D-for_ and _PBF24D-rev_ and inserting it by _In-Fusion_ cloning into _pBF24B_ with _XbaI_. The _oriT_ (amplified using the template _pIJ773_ and the primers _PBF24E-for_ and _PBF24E-rev_ and the _φBT1 _int/attP_ (amplified using _pMS82_ as the template and _pBF24F-for_ and _PBF24F-rev_)) loci were joined to generate a 2,312-bp fragment by overlap extension PCR, which was inserted.
by In-Fusion cloning into pBF24C cut with SbfI. The final plasmid, pBF24, was confirmed by restriction analysis and nucleotide sequencing.

(v) Construction of the expression plasmids pBF21, pBF23, and pBF25. Alternative versions of pBF20, pBF22, and pBF24 (Fig. 2), called, respectively, pBF21, pBF23, and pBF25 were made in which the actII-orf4/actI\(p\) promoter was replaced with the native promoter \(eryAI\)\(p\) (see Fig. S2 in the supplemental material).

To construct pBF21, pBF20 was digested with ClaI to yield three DNA fragments of 14,860, 3,521, and 156 bp. An overlap extension PCR product was generated from three PCR products: a 2,381-bp fragment containing the 5\(’\) end of the \(tsr\) gene, the ColE1 origin, and the \(bla\) gene; a 240-bp \(eryAI\)\(p\) (amplified from the \(S. erythraea\) BIOT-0666 genomic DNA using the primers PBF21A-for and PBF21A-rev); and a 154-bp fragment containing the 5\(’\) end of the \(eryAI\)\(g\) gene that was lost after cutting with ClaI. The resulting fragment (2,715 bp, obtained using the primers PBF21C-for and PBF21B-rev) was inserted by In-Fusion cloning into the purified 14,860-bp ClaI fragment to generate pBF21.

For the pBF23, and pBF25 constructions, \(eryAI\)\(p\) was amplified from the \(S. erythraea\) BIOT-0666 genomic DNA by primers PBF23-for/PBF23-rev and PBF25-for/PBF25-rev, respectively. The \(eryAI\)\(p\) fragments were...
then inserted into pBF22 and pBF24, cut with SpeI/PacI and PacI/SspI, respectively. The new plasmids were verified by restriction analysis and sequencing.

(vi) Construction of the expression plasmid pBF27N. pBF27N is an integrating plasmid encoding the C31 int/attP, derived from a plasmid containing the full angolosamine biosynthesis gene cassette under the control of the actlp promoter (36), a hygromycin-resistant gene (hyg), and oriT (Fig. 2).

The construction was done as follows: pIB023 was cut with PacI and XbaI, the 4428-bp plasmid backbone fragment (bla, tsr, and the actlp promoter) was purified, and the ends were then filled in with DNA polymerase I, large (Klenow) fragment to generate blunt ends for ligation. This blunt ended fragment was then self-ligated using Quick Ligase Enzyme (NEB) to produce pBF27A.

The hyg gene encoding hygromycin B phosphotransferase was amplified from plasmid pSMT3-M using the primers PBF27NA-for and PBF27NA-rev and inserted by In-Fusion cloning into pBF27A cut with SspI to form plasmid pBF27B. C31 int/attP locus and oriT were fused by overlap extension PCR; two amplified fragments were prepared using pSET152 as a template and primers PBF27NB-for/PBF27NB-rev and pIB773 as a template and the primers PBF27NC-for/PBF27NC-rev, and the final fused product was obtained using the primers PBF27NC-for/ PBF27NB-rev. The fragment containing C31 int/attP and oriT (2,410 bp) was inserted into pBF27B cut with SpeI by In-Fusion cloning to produce plasmid pBF27C. Finally, the fragment encoding hyg C31 int/attP and oriT was amplified by PCR using the primers PBF27ND-for and PBF27ND-rev and the template pBF27C and inserted using In-Fusion cloning into the SpeI-cut plasmid constructed by Schell et al. encoding the angolosamine biosynthesis cassette (36), thus generating plasmid pBF27N (Fig. 2).

pBF27N2 (see Fig. S2 in the supplemental material) encodes the eryF gene in addition to the angolosamine biosynthesis cassette. eryF gene was amplified from S. erythraea BIOT-0666 genomic DNA using the primer pair eryF-for/eryF-rev and inserted into pBF27N and pBF28N cut with SpeI by In-Fusion cloning to form plasmid pBF27N2.

Production and analysis of 6-dEB. Five Streptomyces strains were used for 6-dEB production: S. coelicolor J1929, S. coelicolor M512, S. lividans TK24, S. coelicolor M1152, and S. coelicolor M1154. Each strain received either pBF20, pBF22, and pBF24 expressing the eryA genes from the actII-orf4/actIp activator/promoter or pBF21, pBF23, and pBF25 in which actII-orf4/actIp was exchanged for the native eryA promoter. For each strain, the three plasmids were introduced sequentially by conjuga-

FIG 2 Plasmid constructs for the expression of 6dEB and the angolomycin glycosylation gene cassette.
tion from *E. coli*. After introduction of the eryA genes into the host strains, 10⁶ spores from three independent lines for each strain were cultured with antibiotics in 25 ml of EVL medium (corn steep solids, 15 g/liter; sucrose, 30 g/liter; ammonium sulfate, 4 g/liter; CaCO₃, 6 g/liter) as seed culture medium for 3 days at 30°C, and then 1 ml of inoculum was transferred to 25 ml of fermentation medium (soy bean flour, 36 g/liter; corn starch, 36 g/liter; ammonium sulfate, 2.4 g/liter; CaCO₃, 7.2 g/liter; soybean oil, 5 g/liter) fed with 1.2 ml of 40% glucose and 0.2 ml of propan-1-ol at 30°C for 6 days without added antibiotics. Triplicate samples (0.75 ml) were withdrawn from each flask and added to ethyl acetate–0.1% NH₄OH (0.75 ml), followed by shaking (15 min). After centrifugation at 13,000 rpm for 20 min, the organic solvent fraction was removed, dried, and stored at −20°C.

Dried extract was resuspended in methanol (500 μl), and an aliquot (5 μl) was injected for analysis by high-pressure liquid chromatography (HPLC; Dionex Ultimate 3000) on a reverse-phase halo C18 column (2.7 μm) was injected for analysis by high-pressure liquid chromatography (Bruker). The peak areas were integrated from the EICs using version 3.0 (Bruker). The peak areas were integrated from the EICs using version 3.0 of the Find algorithm in DataAnalysis with a sensitivity of 50% and absolute intensity threshold of 1,000 counts. These peak area values were entered directly into an Excel spreadsheet to calculate peak area ratios between 6-dEB and EB.

In the sample extractions, EB was added as an internal standard. EB was prepared (10 mM solution) and then diluted to 15 μM in solvent (dilution of 45 μl in 30 ml of solvent). EB-spiked solvent (0.75 ml containing 11.25 nmol) was added to 0.75 ml of culture, and the total was extracted, dried, and resuspended in 500 μl of methanol. Then, 5 μl was used for analysis. The peak area ratios of the internal standard to 6-dEB for each sample was determined. The concentration of 6-dEB in the samples was calculated using the calibration curve and the known amount of internal standard present in the sample (113 pmol of EB).

**Production and analysis of erythromycin analogues.** *S. coelicolor* M512, *S. lividans* TK24, and *S. coelicolor* M1152, each containing pBF20, pBF22, and pBF24, were used as recipients for either pBF27N or pBF27N2. The seed cultures, fermentation medium, extraction method, and the liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) were performed as described for the 6-dEB extractions with the following modifications. Neither seed nor fermentation media contained antibiotics, and the cultures were fermented for 8 days. The Luna C₁₈ column (3 μm, 2 by 150 mm) was used for HPLC analysis, instead of halo C₁₈ column (2.7 μm, 2 by 100 mm), and roxithromycin (2 μM; Sigma-Aldrich) was added to the organic solvent as an internal standard.

**Bioassay of erythromycin analogues.** The antibacterial activity of the erythromycin analogues was evaluated by the agar diffusion method using *Micrococcus luteus* as the indicator organism (37). Briefly, TSB-agar medium was initially poured into petri dishes and left to solidify. A second portion of TSB-agar containing 50 μl of a *Micrococcus luteus* overnight culture (grown 18 h at 30°C in Luria-Bertani medium) was added. Aseptically, holes were punched, and then 50 μl of the sample extract was loaded. After 48 h of incubation at 30°C, the growth inhibition zones of *Micrococcus luteus* were measured and compared to those of the appropriate control.

**RESULTS**

**Introduction of integrating plasmids and validation of engineered *Streptomyces* strains.** Several *Streptomyces* strains with different genotypes were used as heterologous expression hosts. *S. coelicolor* J1929 (ΔpgrY [31]) was used instead of the wild-type strain M145. J1929 is our laboratory standard because it is sensitive to dC31 and dBT1. *S. coelicolor* M512 (AredΔ actII-orf4 SCP1− SCP2−) [32], *S. coelicolor* M1152 [ΔactD Δred Δepk Δcla rpOB(C1298T)], and *S. coelicolor* M1154 [ΔactD Δred Δepk Δcla rpOB(C1298T) rpsL(A262G)] (33–40). *Streptomyces lividans* TK24 (str-6 SLPS2− SLPS3+) has also been shown by others to be a useful expression host (41).

The efficiency of conjugation and site-specific integration of the plasmids encoding the erythromycin polyketide synthases or the sugar biosynthesis genes from the angolamycin pathway was routinely between 4 × 10⁴ and 9.5 × 10⁴ per 10⁶ spores regardless of the host strain (see Table S1 in the supplemental material). The days taken to sporulate by the plasmid containing exconjugants were the same as the parental, plasmid-free controls, and there were no observable differences in colony size or pigmentation.

Genomic DNA from the recipient strains was extracted to assay whether the plasmids had successfully integrated into the host chromosomes. A 1,241-bp DNA fragment from eryA gene was amplified by PCR, thus confirming the integration of pBF20 and pBF21. The integration of pBF22 and pBF23 was confirmed by amplification of a 1,969-bp DNA fragment from the SV1 integratt region. A 1,862-bp DNA fragment was amplified from eryAll gene to confirm the integration of pBF24 and pBF25. Finally, a 2,97-bp DNA fragment was amplified from the dC31 int gene to confirm the integration of pBF27N and pBF27N2.

**Production of the erythromycin intermediate 6-dEB.** The expected ion (m/z = 409.3) indicative of 6-dEB was present in all strains expressing the eryA genes from the actl/p promoter (Fig. 3).

Maximum 6-dEB production was from *S. lividans* TK24::pBF20::pBF22::pBF24 and *S. coelicolor* J1929::pBF20::pBF22::pBF24 with yields of ~12 mg/liter. In these fermentations *S. coelicolor* J1929::pBF20::pBF22::pBF24 and *S. lividans* TK24::pBF20::pBF22::pBF24 achieved the highest biomasses (0.09 and 0.11 g [dry weight] per 5 ml of culture, respectively) compared to the remaining strains (0.05, 0.055, and 0.06 g [dry weight] per 5 ml of culture for M1154::pBF20::pBF22::pBF24, M1152::pBF20::pBF22::pBF24, and M512::pBF20::pBF22::pBF24, respectively); however, overall, there does not appear to be a simple relationship between the yield and the biomass achieved.

The production of 6-dEB was also detected from *Streptomyces* strains containing the plasmids pBF21, pBF23, and pBF25 expressing the eryA genes from the native eryAl promoter, but the
yields were consistently lower than from the actlp promoter (Fig. 4); the highest yielding strain (−6 mg/liter) was S. lividans TK24::pBF20::pBF22::pBF24 fermentation. Kao et al. (21) also expressed 6-dEB in S. coelicolor, and these authors also detected a side product, 8,8a-deoxyoleandolide, which is thought to be a product of the DEBS PKS using acetyl-CoA as a starter in place of proprionyl-CoA. We also detected 8,8a-deoxyoleandolide in the fermentation extracts, and the yields were ca. 20% of the yield of 6-dEB. A molecular ion m/z 409.3 indicative of 6-dEB production was not detected in the medium-only control.

Production of erythromycin analogues in Streptomyces coelicolor. As our constructs were capable of producing 6-dEB in S. coelicolor, we tested whether we could generate glycosylated derivatives by addition of a fourth plasmid. Schell et al. showed that S. erythraea containing the gene cassette encoding angAI, angAII, angMIII, spnO, ang-ORF14, angB, angMI, and tylMII, expressed from the actlp promoter, could convert exogenously added tyloactone to a new compound, 5-O-β-D-angolosaminyltylactone (36). We therefore constructed an integrating plasmid, pBF27N, based on the φC31 int/attP locus and encoding the angolomycin biosynthesis gene cassette (Fig. 3), that could be introduced into the Streptomyces strains producing 6-dEB. In addition, pBF27N was modified to encode EryF, the hydroxylase required for the production of EB from 6-dEB, thus generating pBF27N2. The addition of eryF would test whether EB could be produced and provide
a second aglycone as a substrate for the heterologous glycosyl transferases encoded in the angolomycin gene cassette, \( \text{tylMII/angMIII} \).

LC-ESI-MS was used to detect the expected metabolites in extracts from the fermented cultures (Fig. 4 and 5). \( \text{S. coelicolor M1152::pBF20::pBF22::pBF24::pBF27N} \) was expected to produce angolosaminyl-6-dEB (\( m/z \) 544.5), and the corresponding ion was detected but clearly eluting at two retention times (6.6 and 7.3 min).

![FIG 4 Production of angolosaminyl-6-dEB. (A) HPLC base peak chromatogram (BPC; see legend for Fig. 3) from extracts of \( \text{S. coelicolor M1152::pBF20::pBF22::pBF24::pBF27N} \) and extracted ion chromatograms (EICs) for the putative angolosaminyl-6-dEB isomers (\( m/z \) 544.5, peaks 1 and 2; retention times, 6.6 and 7.3 min, respectively) and a putative angolosaminyl-EB (\( m/z \) 560.4, peak 3; retention time, 5.2 min). (B) MS2 analysis of putative angolosaminyl-EB \( m/z \) 560.4, (retention time 5.2 min, peak 3; top line) and putative angolosaminyl-6-dEB \( m/z \) 544.4 isomers (retention times, 6.6 and 7.3 min, peaks 1 and 2, middle and bottom, respectively). All three MS2 analyses show the presence of \( m/z \) 158.1, which is consistent with all three compounds containing angolosamine.](http://aem.asm.org/content/81/24/8409)

![FIG 5 Production of angolosaminyl-6-dEB and angolosaminyl-EB. (A) HPLC base peak chromatogram (BPC; see legend for Fig. 3) in extracts of \( \text{S. coelicolor M1152::pBF20::pBF22::pBF24::pBF27N} \) and extracted ion chromatograms (EICs) for the putative angolosaminyl-6-dEB isomers (\( m/z \) 544.5, peaks 1 and 2) and \( m/z \) 560.4, eluting at three retention times (peaks 3, 4, and 5 at 5.2, 7.0, and 7.8 min, respectively). (B) MS2 analysis of the putative angolosaminyl-EB \( m/z \) 560.4 (peak 3, retention time, 5.2 min) indicated the presence of angolosamine (\( m/z \) 158.1, top line; see also Fig. 4). MS2 analysis of \( m/z \) 560.4, retention time 7.0 showed the unexpected presence of \( m/z \) 174, which suggests that the parent ion could be mycaminosyl-6-dEB (third line down). MS2 analysis of \( m/z \) 544.4 (peaks 1 and 2, retention times, 6.7 and 7.3 min) indicated that both compounds contain angolosamine (\( m/z \) 158.1) and are predicted to be angolosaminyl-6-dEB (see also Fig. 4).](http://aem.asm.org/content/81/24/8409)
This ion was observed at three retention times: 5.2 min, 7.01 min, and 7.01 min, respectively (Fig. 5). MS2 analysis of both peaks showed that both contained m/z 544.5, consistent with both containing angolosamine. We propose that the two retention times for m/z 544.5 correspond to 5-O-angolosaminyl-6-DEB and 3-O-angolosaminyl-6-DEB. No ion corresponding to that expected of bis-O-angolosaminyl-6-DEB, suggesting that the single glycosylated product cannot act efficiently as a substrate for attachment of a second angolosaminyl residue. Unexpectedly, we observed a very small amount of an ion m/z = 560, a retention time of 5.2 min, which could be angolosaminyl-EB (see below), and this was supported by the MS2 data, which indicated that this ion did indeed contain angolosamine. The LC-ESI-MS spectra also indicated the presence of unglycosylated 6-DEB and EB, with peaks at m/z 409 and m/z 425 (and retention times corresponding to standards for these compounds; Fig. 4). A search for the ion corresponding to angolosaminyl-6-DEB (m/z 544.5) in extracts of S. coelicolor M1152::pBF20::pBF22::pBF24::pBF27N and pBF20::pBF22::pBF24 without the angolomycin gene cassette showed no peak higher than the baseline noise, indicating that the glycosylation of 6-DEB was dependent on the presence of pBF27N. 

S. coelicolor M1152::pBF20::pBF22::pBF24::pBF27N was expected to produce angolosaminyl-EB, with a predicted m/z of 560. This ion was observed at three retention times: 5.2 min, 7.01 min, and a very small peak at 7.8 min (Fig. 5). MS2 indicated the presence of m/z 158.1 from the peak at 5.2 and m/z 174.1 from the peak at 7.01 min, suggesting the presence of angolosaminyl-EB and, possibly, mycaminosyl-6-DEB, respectively (Fig. 5). Mycaminose only differs from angolosamine by the presence of an additional hydroxyl group, explaining the mass differences observed in the MS analysis (Fig. 1 and 5). Intermediates in the production of angolosaminyl-EB were also observed as before, i.e., angolosaminyl-6-DEB isomers were both detected (m/z 544.5, 6.6 and 7.3 min, Fig. 5), and both were shown by MS2 to contain angolosamine. A search for m/z 560 in extracts of S. coelicolor M1152::pBF20::pBF22::pBF24 without the angolomycin gene cassette did show a peak higher than the baseline noise but still 100-fold lower intensity than those attributed to angolosaminyl-EB and the putative mycaminosyl-6-DEB, indicating that these metabolites were dependent on the presence of pBF27N.

Without purified angolosaminyl-6-DEB or angolosaminyl-EB standards, the yields of the analogues could not be accurately determined, but we used relative signal intensities and the internal standard, roxithromycin, as a guide to estimate the relative amounts of compounds obtained from the fermentations. The yield of 6-DEB in the M1152::pBF20::pBF22::pBF24::pBF27N cultures (six replicates) was ~3 mg/liter, and the angolosaminyl-6-DEB isomers amounted to approximately one-tenth to one-fifth of the yield of 6-DEB. The extracts from M1152::pBF20::pBF22::pBF24 (three replicates) contained ~8 and 3 mg/liter of 6-DEB and EB, respectively. The yields of angolosaminyl 6-DEB or angolosaminyl-EB were estimated to be ~3-fold less than those observed for 6-DEB and EB. The signal for the putative mycaminosyl-6-DEB was less than one-tenth of that for 6-DEB.

Only traces of m/z 544, corresponding to angolosaminyl-6-DEB, were detected in S. coelicolor M512::pBF20::pBF22::pBF24::pBF27N (data not shown). Both M512 and S. lividans containing pBF20::pBF22::pBF24::pBF27N produced 6-DEB, and S. lividans::pBF20::pBF22::pBF27N produced both 6-DEB and EB (data not shown).

Schell et al. also generated a gene cassette (containing tylAI, tylAII, tylMI, tylMB, tylNa, tylMI, and tylMI) for the synthesis and transfer of TDP-mycaminose to aglycone rings in biotransformation experiments (36). The plasmid containing this gene cassette was modified with the φC31 intaatt site and hygromycin marker and introduced into strain expressing 6-DEB, but no glycosylated erythromycin analogues were observed in the fermentation extracts.

**Plasmid stability in S. coelicolor M1152 producing angolosaminyl-6-DEB angolosaminyl-EB.** To test whether strains containing the four integrating plasmids; pBF20, pBF22, pBF24, and pBF27N were stable in S. coelicolor M1152, spores were grown on Soya Mannitol (SM) agar without antibiotics at 30°C. The spores were harvested, and titers were determined on nonselective SM agar and on selective SM agar containing each of the markers present on the four plasmids. Since there was no significant difference in the spore counts between all five plates for each strain, we conclude that the plasmid integrations are stable (see Table S1 in the supplemental material). As a further test, S. coelicolor M1152::pBF20::pBF22::pBF24 was subjected to two rounds of sporulation without selection, and there was no loss of plating efficiency on any of the selective plates.

**Antibacterial activity in extracts containing glycosylated 6-DEB and EB.** It is known that unglycosylated macrodides from the erythromycin pathway lack antibiotic activity (42). We therefore sought to determine whether the glycosylation of 6-DEB or EB with angolosamine could confer antibiotic activity. Extracts from the fermented cultures of S. coelicolor M1152::pBF20::pBF22::pBF24::pBF27N were dissolved in dimethyl sulfoxide and assayed for the antibacterial activity using a disc diffusion assay. The extract from M1152::pBF20::pBF22::pBF24::pBF27N (Fig. 6A) reliably produced a significantly bigger zone of inhibition than those from both M1152::pBF20::pBF22::pBF24::pBF27N (Fig. 6B) and the plasmid-free control extract (Fig. 6C). Since both plasmid-containing strains produced mixtures of compounds, including an-

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**Fig. 6** Antibiotic activity in the extracts from S. coelicolor M1152::pBF20::pBF22::pBF24::pBF27N. Inhibition zones from extracts of S. coelicolor M1152::pBF20::pBF22::pBF24::pBF27N (A), M1152::pBF20::pBF22::pBF24::pBF27N (B), and for plasmid-free M1152 (C). This is a representative plate from three independent experiments.
gulosaminyl-EB and angolosaminyl-6-deB, it was not possible to ascertain the identity of antibiotic itself. However, the antibiotic activity might just be a consequence of higher yield of the glycosylated intermediates produced by M1152: pBF20::pBF22::pBF24::pBF27N2 (see above).

DISCUSSION

In this study we set out to demonstrate the utility of using multiplexed integrating vectors to engineer antibiotic pathways for expression and combinatorial biosynthesis in a heterologous host. The erythromycin pathway was chosen as an example since it is large, it is well understood, and it still has huge potential for the rational design and expression of analogues. The strategy here was to use four orthogonal integrating vectors based on the Streptomyces phase integrases TG1, BT1, SV1 and δC31, each with its cognate attP site. These four integrases use different attB sites in S. coelicolor or S. lividans, resulting in the stable integration at different genetic loci (8). The genes encoding eryAI, eryAII, and eryAIII genes were inserted into TG1 int/attP-, SV1 int/attP-, and BT1 int/attP-containing plasmids, respectively, while tailoring genes were inserted into the δC31 int/attP-containing plasmid. This arrangement provides a useful platform for engineering the PKS enzymes and the tailoring enzymes and facilitates combining the engineered constructs once generated.

The advantage of using integration vectors derived using phase integrases is their stability, postintegration, due to the directional properties of the integrases (8, 9). Integrases are able to mediate the integration reaction to generate the prophage but require a second phase-encoded protein, the recombination directional factor, or RDF to activate excision. We verified here that the integrated plasmids used in our constructs were stable in the absence of antibiotic selection, and we showed also that fermentations for metabolite production could be performed in the absence of selection (Fig. 3 to 5). The stability of the integrated plasmids improves upon previous work that has utilized replicating plasmids that have required selection for maintenance (21, 26). A number of novel integration systems are becoming available (G. Taylor, P. Fogg, and M. C. M. Smith, unpublished data) that can add to or replace the δC31-, TG1-, δBT1-, and SV1-derived systems used here. The number of plasmids encoding orthogonal integrases is unlikely to be restrictive in this approach; the upper limit is more likely to be limited by the number of markers available for the selection of exconjugants.

Expression of 6-deB was robust in the heterologous hosts, with yields varying from ~1 mg/liter to >10 mg/liter, without any attempt at optimization. The actI/actII-orf4 promoter/activator once again proved that it is reliable for expression of non-native antibiotic pathways in S. coelicolor and S. lividans. Building on this expression, we generated strains that had the potential to express novel erythromycin analogues. Schell et al. previously constructed gene cassettes for the synthesis of the deoxy sugars, D-angolosamine and D-mycaminose (36). S. erythraea lacking the ability to synthesize the aglycone and deoxy sugars but expressing the angolosamine cassette is able to transform exogenously added EB to 3-O-β-D-angolosaminyl-EB (36). We used these cassettes to test whether we could generate angolosaminyl or mycaminosyl derivatives of 6-deB and EB in the heterologous host Streptomyces, into which the genes for aglycone biosynthesis had also been introduced. Two isomers of the expected mass for angolosaminyl-6-deB were detected, possibly showing that 6-deB can by glycosylated on either the three- or five-position hydroxyls in the aglycone ring (Fig. 4 and 5). We could also detect a mass consistent with angolosaminyl-EB, and it seems highly likely that this corresponds to the 3-O-β-D-angolosaminyl-EB identified by Schell et al. (36).

Some unexpected EB and 6-deB derivatives were detected. Plasmids pBF27N and pBF27N2 both contain the angolosamine cassette, but only pBF27N2 encodes EryF, the enzyme that introduces the hydroxyl group at C-6 in 6-deB to generate EB. Despite this, S. coelicolor M1152::pBF20::pBF22::pBF24::pBF27N appears to be synthesizing angolosaminyl-EB in addition to angolosaminyl-6-deB, the expected product (Fig. 4). The same two products were also observed from S. coelicolor M1152::pBF20::pBF22::pBF24::pBF27N2, which contains eryF, but this strain is producing a different side product whose MS spectra are consistent with mycaminosyl-6-deB (Fig. 5). We cannot at this stage attribute specific host- or plasmid-encoded enzyme activities to these side products.

This angolosamine cassette encodes the tylMII glycosyltransferase whose natural activity is to transfer TDP-D-mycaminose to the tylactone ring and is activated by the product of tylMIII (36, 43, 44). tylMII has been shown previously to have a broad range of substrate specificities, both for the activated sugar and for the aglycone (36, 45). We can therefore add the ability to transfer angolosamine to 6-deB to the range of TylMII activities. The glycosylation of 6-deB and EB, however, was not efficient, since only a fraction of 6-deB and EB were converted. In tylosin biosynthesis, the TylMII glycosyl transferase is normally activated by TylMIII but in pBF27N2, AngMII, the TylMIII homologue and the activator of AngMII, is used (36, 43). It is not known how compatible this pair of proteins (TylMII/AngMII) are in mediating the transfer of angolosamine to aglycones. In short, there are viable avenues to pursue to optimize the in vivo glycosylation of the aglycone using novel sugar biosynthesis pathways and matching them with different glycosyltransferases.

Extracts producing a mixture of glycosylated analogues, including angolosaminyl-6-EB, angolosaminyl-EB, and possibly mycaminosyl-6dEB, had antibiotic activity. It is not clear at this stage which of these compounds might have antibiotic activity, and we cannot rule out activation of a cryptic pathway in S. coelicolor (Fig. 6).

Enabling a “plug-and-play” synthetic biology approach to cloning, expression, and modifying antibiotic pathways is a desirable aim, but it is a challenge when the pathways involve many enzymes, including multidomain assembly line proteins such as the PKSs. However, using multiplexed integration vectors to divide and distribute different functional parts of the gene clusters is a useful first step, facilitating combinatorial biosynthesis and the optimization of genetic constructs.

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