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ORIGINAL PAPER

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Rapid engineering of polyketide overproduction by gene transfer to industrially optimized strains

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Abstract Development of natural products for therapeutic use is often hindered by limited availability of material from producing organisms. The speed at which current technologies enable the cloning, sequencing, and manipulation of secondary metabolite genes for production of novel compounds has made it impractical to optimize each new organism by conventional strain improvement procedures. We have exploited the overproduction properties of two industrial organisms—*Saccharopolyspora erythraea* and *Streptomyces fradiae*, previously improved for erythromycin and tylosin production, respectively—to enhance titers of polyketides produced by genetically modified polyketide synthases (PKSs). An efficient method for delivering large PKS expression vectors into *S. erythraea* was achieved by insertion of a chromosomal attachment site (*attB*) for ϕC31-based integrating vectors. For both strains, it was discovered that only the native PKS-associated promoter was capable of sustaining high polyketide titers in that strain. Expression of PKS genes cloned from wild-type organisms in the overproduction strains resulted in high polyketide titers whereas expression of the PKS gene from the *S. erythraea* overproducer in heterologous hosts resulted in only normal titers. This demonstrated that the overproduction characteristics are primarily due to mutations in non-PKS genes and should therefore operate on other PKSs. Expression of genetically engineered erythromycin PKS genes resulted in production of erythromycin analogs in greatly superior quantity than obtained from previously used hosts. Further development of these hosts could bypass tedious mutagenesis and screening approaches to strain improvement and expedite development of compounds from this valuable class of natural products.

Keywords Polyketide · 6-Deoxyerythronolide B · Erythromycin · Tylosin · Antibiotic

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Introduction

Natural products known as secondary metabolites contribute major products for pharmaceutical, agricultural and veterinary uses. The compounds include polyketides, β-lactams, non-ribosomal peptides and others, and are generally obtained by fermentation of microorganisms. The polyketides are an extremely diverse class of metabolites that include important therapeutic agents for antibacterial (erythromycin, tylosin, rifamycin), immunosuppressive (FK506, rapamycin), cholesterol-lowering (lovastatin), and other uses [19]. The cloning of biosynthetic gene clusters in the past decade and subsequent elucidation of how the corresponding enzymes create their product structures have led to the emergence of combinatorial biosynthesis technologies aimed at generating novel compounds through genetic manipulation of these systems. This technology has been highlighted by, though not limited to, the engineering of modular polyketide synthases (PKSs), multifunctional enzymes with active sites arranged in modules (Fig. 1), whose catalytic domains or entire modules have been mutated, substituted, deleted, and added to change the specificity or sequence of reaction steps in the polyketide chain assembly process [12].

Although the erythromycin PKS, or 6-deoxyerythronolide B synthase (DEBS) [6, 7], has served as the primary model system for developing ways to manipulate PKSs, many others, including those that produce picromycin, tylosin, oleandomycin, rapamycin and FK520, are now routinely engineered to synthesize new molecules [13, 23, 27, 33]. The increasing number of novel polyketide structures that are generated under-

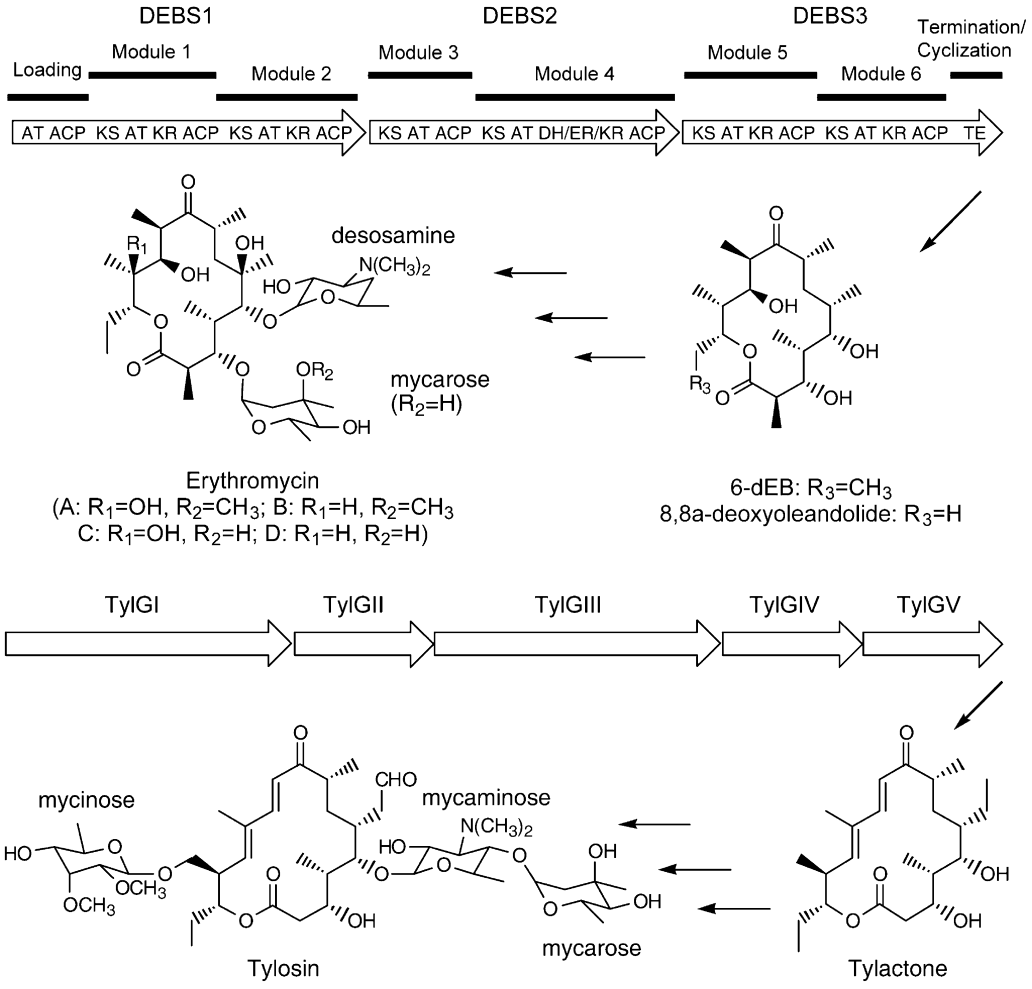

Fig. 1 Erythromycin and tylosin biosynthesis. The erythromycin polyketide synthase (PKS) 6-deoxyerythronolide B synthase (DEBS) consists of six modules, a loading domain and a thioesterase which assemble 6-dEB from a propionyl-CoA and six methylmalonyl-CoA precursors. Each of the subunits, which contain two modules, is over 350 kDa. Likewise, tylactone, the product of the tylosin PKS, is synthesized from two malonyl-CoA, four methylmalonyl-CoA, and one ethylmalonyl-CoA precursor. Tylactone and 6-dEB are subsequently converted to tylosin and erythromycin by post-PKS glycosylation and oxidation reactions. *ACP* Acyl carrier protein, *AT* acyl transferase, *KS* ketosynthase, *DH* dehydratase, *ER* enoylreductase, *KR* ketoreductase, *TE* thioesterase

scores the remarkable structural plasticity and substrate tolerance of modular PKSs. Yet, a problem that is faced in the application of genetic engineering approaches to polyketide production is that strains containing engineered or hybrid PKSs generally produce much less polyketide product than the parent organism, presumably due to lower productivity of the engineered enzyme [18]. The lower titers can hinder development of such compounds as drug candidates. In order to obtain practical amounts of materials, the producing strains must be "improved" for production.

The conventional route to secondary-metabolite-overproducing strains—repeated cycles of random mutagenesis and screening for higher producers —has been used to achieve multi-gram/liter titers for many currently important polyketides (e.g. erythromycin, FK506, lovastatin, tylosin, etc.) [31], but is tedious and has taken many years to achieve in each case. Although this approach could be applied to strains carrying engineered PKSs, it is impractical with the numbers of engineered strains generated by combinatorial biosynthesis. Recombinant DNA methods have been used to engineer antibiotic-overproducing microorganisms [2] and recently a whole 'genome shuffling' approach to strain improvement was reported with a tylosin-producing organism [34]. A drawback to these methods, however, is the requirement of genetic protocols that are specific to each strain, which can be particularly challenging for poorly characterized or genetically intractable microorganisms (e.g., marine microorganisms).

Clearly, it would be advantageous to have a host microorganism that possesses the intrinsic capacity to overproduce *any* secondary metabolite for a particular class. The genes encoding a particular secondary metabolite could then be introduced to directly establish an overproducing organism. Not only would such strains benefit combinatorial biosynthesis, but also the development of newly discovered natural products from

organisms that are difficult or impossible to culture. Cloning, sequencing and heterologous expression of secondary metabolite gene clusters is now routine, and current high-throughput gene sequencing technology makes this approach potentially more cost effective than the development of a poorly producing strain.

Here we examine whether overproduction characteristics of industrial strains that have been optimized to produce a specific polyketide are encoded in the corresponding biosynthetic enzymes or in the 'background' genotype of the strain and, therefore, whether they could serve as general hosts for overproduction of polyketides from genetically engineered PKSs. PKS gene delivery and expression systems were developed for overproducing strains of *Saccharopolyspora erythraea* and *Streptomyces fradiae* previously optimized by conventional strain improvement; these strains yield over 1 g erythromycin/l and 2 g tylosin/l, respectively, in shake flasks. Functional expression of PKS genes in these strains, including genetically engineered PKSs and PKSs cloned from 'wild-type' organisms, indicates the utility of this approach to increasing titers of polyketides derived from combinatorial biosynthesis and potentially other types of natural products.

Materials and methods

Strains, culture conditions, and genetic manipulation

Saccharopolyspora erythraea K41-135 was obtained from Solidago AG (Bern, Switzerland). *S. erythraea* strains were maintained on M1 agar medium [9] and engineered by conjugation using *Escherichia coli* ET12567/pUB307 [8] containing the donor plasmid. Conjugation was carried out according to the reported procedure [14] using R5 agar with 50 mg nalidixic acid/ml. *Streptomyces fradiae* KOS155-3C was obtained from the State Scientific Center of Russian Federation and engineered by conjugation using *E. coli* DH5α/pUB307 containing the donor plasmid. AS1 agar [1] was used for propagation and conjugation experiments with *S. fradiae* strains. Transconjugants were selected with 3 ml of soft agar containing 1 mg of apramycin and 1 mg nalidixic acid. *Streptomyces coelicolor* CH999 [17] and *Streptomyces lividans* K4-114 [35] were transformed by standard protoplast procedures [14]. DNA cloning and manipulation were done in *E. coli* DH5α unless specified otherwise.

Construction of *S. erythraea* and *S. fradiae* 'clean-host' derivatives

PKS genes in *S. erythraea* K41-135 and *S. fradiae* KOS155-3C were deleted as follows. Two fragments flanking the *eryA* genes were PCR-amplified from *S. erythraea* K41-135 gDNA using the following primers: *eryAI* left flank, for 5′-TTTGCATGCGGCCACGCGCACGTCGTGACC, rev 5′-TTAAGCTTCATATGTCCCCCTACTCGACGACCAC; *eryAIII* right flank, for 5′-TTTGGATCCGGCGGAGGGAATACATGACCACGAC, rev 5′-TTTGAATTCCCGCTCGCTGAAGTCCAGGCT. The two fragments were cloned into pSET152 [4] using the underlined restriction sites to make pKOS97-49a. The ϕC31 *attB* sequence was inserted between the *Hin*dIII and *Bam*HI sites of pKOS97-49a using two annealed oligonucleotides (for 5′-AGCTTCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTAACTAGTG, rev 5′-GATCCACTAGTTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGA), to generate pKOS024-87. pKOS0134-04 was made by inserting a ~300 bp *Nhe*I–*Bam*HI fragment containing the *ermE*p* (*ermE*p1 △TGG) promoter [3] between the resulting *Spe*I and *Bam*HI sites of pKOS024-87. Two fragments flanking the *tylG* genes were PCR-amplified from *S. fradiae* KOS155-3C gDNA using the following primers: *tylGI* left flank, for 5′-TTTGCATGCGATGTTGACGATCTCCTCGTC, rev 5′-GGAAAGCTTCATATGTTCTCTCCGGAATGTG; *tylGVI* right flank, forward 5′-TTAAGCTTTCTAGAGAGGAGAGGCCGTGAAC, rev 5′-AAAGAATTCGAACTCGAGCACGGACTCGTTG. The two fragments were cloned into pSET152 using the underlined restriction sites to make pKOS159-5. pKOS134-04 and pKOS159-5 were used as suicide vectors for delivery and integration by stepwise double crossing-over to generate *S. erythraea* K24-1 and *S. fradiae* 159-5, respectively, the final clean-host derivatives of the overproducing strains. Double crossovers were verified by lack of polyketide production and Southern blot hybridization or PCR analysis.

Construction of PKS expression vectors

The *eryA* genes from *S. erythraea* K41-135 were isolated from a Supercos (Stratagene, La Jolla, California) library of gDNA using probes generated from the *eryA* genes of pCK7 [11]. Two overlapping cosmids encoding the entire *eryA* region were used to move the three *eryA* genes from the unique *Cla*I site at the 5′-end of *eryAI* to the unique *Eco*RI site at the 3′-end of *eryAIII* into the corresponding sites of pKAO127 [35], generating pKOS108-04. The 5′-region of *eryAI* genes from *S. erythraea* K41-135 and pCK7 were sequenced from the start codon to the *Cla*I site to ensure there were no differences.

pKOS159-8 and pKOS159-10 are derivatives of pSET152 containing the *eryA* genes under the control of the *ermE*p* promoter and the *actI*p/*actII*–ORF4 promoter–activator pair [17], respectively. A 35-kb *Nsi*I fragment from pKAO127 carrying the *eryA* genes and the *actI*p/*actII*–ORF4 region was cloned into pKOS97-64c (a pSET152 derivative containing the *ermE*p* promoter and a λ *cos* site) to make pKOS159-10. The *fd* transcriptional terminator from the pKAO127 fragment prevents expression of any genes from the *ermE*p* promoter in this plasmid. The fragment containing the *fd* terminator and *actI*p/*actII*–ORF4 segment in pKOS159-10 was removed by digestion with *Pac*I and self-ligation to generate pKOS159-8. For expression of *eryA* genes under their natural promoter, pKOS159-31 was constructed by cloning the *Nde*I–*Xba*I fragment carrying the *eryA* genes (and λ cos site) from pKOS159-10 and the *Xba*I–*Nde*I-digested PCR-amplified *eryAI* left-flank fragment from above into pSET152 digested with *Xba*I. pKOS159-33, which contains the *eryA* genes from *S. erythraea* K41-135, was constructed in an analogous way using the *eryA* fragment from pKOS108-04. Likewise, all engineered DEBS expression plasmids were made using pKOS159-33 as a scaffold and appropriate restriction enzymes to move the genetically modified *eryA* fragment from existing plasmids [18].

pKOS168-190 (obtained from H. Suzuki, Kosan Biosciences) contains the tylosin PKS (TylPKS) genes and a 1-kb fragment upstream of the *tylGI* gene, encoding the putative *tylGI*p promoter, cloned in pSET152. The source of the *tylG* genes in this plasmid is *S. fradiae* ATCC19609, a wild-type producer. In order to express the TylPKS with the *ermE*p* promoter, pKOS244-5 was constructed by cloning the *Eco*RI–*Nsi*I fragment carrying the *tylG* fragment from pKOS168-190 and the *Pac*I–*Eco*RI-digested fragment from pKOS241-34 into pKOS159-8 digested with *Pac*I–*Nsi*I.

Fermentation and analysis of polyketide products

Strains producing 6-deoxyerythronolide B (6-dEB), erythronolide B and 3α-mycarosyl-erythronolide B were analyzed by LC-MS as previously described [24]. For *S. erythraea,* recombinant strains were grown in 5 ml TSB [14] (+ 30 μg apramycin/ml) at 30 °C for 48 h and used to inoculate 50 ml F1 media [9] (+ 30 μg apramycin/

ml) in a 250-ml baffled Erlenmeyer flask. Fermentations were fed 0.15 ml sterile *n*-propanol and 0.3 ml sterile soy oil daily and grown at 34 °C for 9 days. Five-liter bioreactor fermentations were carried out essentially as described [9] except F1 was used as the production medium. The concentrations of erythromycin analogues in fermentation broths were determined by on-line extraction/MS analysis using a system [24] equipped with a Turbo Ionspray source. Culture broths were clarified by centrifugation, and an internal standard of roxithromycin (Sigma, St. Louis, Mo.) was added to a final concentration of 5 mg/l, followed by four volumes of 2-propanol. Following centrifugation, 100 µl of supernatant was loaded onto a Metaguard (Ansys, Lake Forest, Calif) column (4.6 mm Inertsil ODS-3 5 µm), and washed with 50% 5 mM ammonium acetate in MeOH for 2 min. The eluate was changed to 100% 5 mM ammonium acetate in MeOH, and diverted into the mass spectrometer. The positive ion mass spectrum from 655 to 895 amu was acquired in multichannel analysis mode for 2.5 min. Erythromycin concentrations were estimated by comparing the peak intensities to an erythromycin standard calibration curve. Assignments of erythromycin analogs were based on the presence of new $m/z+$ signals corresponding to the predicted molecular weights. Additional characterization was done by LC-MS analysis, using conditions described previously [24], to distinguish between erythromycin congeners and analogs with the same molecular weights. A purified sample of 11-deoxyerythromycin B characterized by NMR spectroscopy was obtained from Dr. Gary Liu (Kosan Biosciences) and used to confirm the identity of this compound by LC-MS. Production of 10-desmethyl erythromycin compounds using a similar genetic modification in DEBS was reported previously [26] and we are confident in structural assignment of these analogs based on this precedent and mass spectra data.

S. fradiae strains were analyzed in 50 ml RM medium (g/l, 15 wheat flour, 10 corn gluten enzymatic hydrolysate, 25 beet molasses, 2.5 brewer's yeast, 1 $(NH_4)_2HPO_4$, 1 NaCl, 2 $CaCO_3$, 34 soybean oil). Seed cultures were grown in 5 ml TSB medium (+ 30 µg apramycin/ml) at 30 °C for 48 h and used to inoculate 50 ml of RM medium (+30 µg apramycin/ml) in a 250-ml baffled Erlenmeyer flask. After 7-days of growth at 30 °C, the culture broth was analyzed for tylosin production by HPLC (Metachem Metasil Basic column, 4.6×150 mm, 5 µm particle) using a linear gradient from 15 to 100% organic phase (56% methanol, 5 mM ammonium acetate) at 1 ml/min over 7 min. Tylosin was monitored by MS and UV absorption at 282 nm. Tylosin standard (Sigma, T-6134) was used to generate a calibration curve for titer determination.

Results

Comparison of DEBS from wild-type and optimized *S. erythraea* in heterologous hosts

Shake-flask cultivation of *S. erythraea* K41-135 (the erythromycin-overproducing strain) yields 1–2 g erythromycin/l in starch-based production medium F1. Under identical conditions, *S. erythraea* NRRL2338, a wild-type soil isolate, yielded ~200 mg erythromycin/l. This five-to ten-fold difference was also observed in other media commonly used for fermentation of actinomycetes. We examined whether mutations that may have been introduced in the genes encoding DEBS during strain improvement of K41-135 have led to improved catalytic properties of the PKS by expressing DEBS genes cloned from the overproducer and from a wild-type *S. erythraea* strain in heterologous hosts.

The three genes, *eryAI, eryAII, and eryAIII* (~10 kb each), which encode the three DEBS subunits, were cloned from a cosmid library of the K41-135 strain and placed in the same expression vector, pKAO127 [11, 35], used previously for heterologous expression of DEBS derived from a wild-type *S. erythraea* strain[1]. The two expression plasmids, pKOS108-04, carrying DEBS from K41-135, and pKAO127, carrying 'wild-type' DEBS, are identical except for the source of the *eryAI–III* genes. In each case, expression of the genes is controlled by the *actI*p promoter and *actII*–ORF4 regulatory protein on the expression plasmid [17]. Both plasmids were used to transform *Streptomyces lividans* K4-114 [35] and *Streptomyces coelicolor* CH999 [17], two hosts routinely used for heterologous expression of PKS genes. Multiple transformants (2–4 each) were grown in parallel and the production profiles of the polyketides 6-dEB and 8,8a-deoxyoleandolide were assessed. In both *S. lividans* and *S. coelicolor*, the DEBS genes from the two different *S. erythraea* strains yielded experimentally equivalent amounts of the polyketides under the fermentation conditions used (Table 1). These results, corroborated by additional experiments reported below, imply that if any amino acid differences exist between the two enzyme complexes, they are not the principal cause of erythromycin overproduction in strain K41-135.

Host-vector systems for PKS expression

For expressing large numbers of PKSs, the desirable features for a host-vector system are: (1) efficient methods for introducing DNA vectors carrying large PKS gene inserts (i.e. > 30 kb), (2) a background free of other competing polyketide pathways (i.e., a 'clean host'), and (3) a vector-promoter system that retains the intrinsic production capabilities of the host when used for PKS expression. For *S. erythraea*, DNA is typically introduced by protoplast transformation followed by homologous recombination into the chromosome. This method of gene delivery is relatively inefficient, particularly if double crossing-over between the introduced

Table 1 Titers of 6-deoxyerythronolide B (6-dEB) from wild-type DEBS (IMS31) and 'overproducer' DEBS (K41-135) genes expressed in *Streptomyces coelicolor* and *Streptomyces lividans*. In these hosts, 8,8a-deoxyoleandolide (8-dOle) is produced as a minor product resulting from a relaxed starter unit specificity of DEBS. *PKS* Polyketide synthase

Strain/PKS	6-dEB (mg/l)	8-dOle (mg/l)
S. coelicolor CH999		
Wild-type DEBS	40	12
K41-135 DEBS	47	13
S. lividans K4-114		
Wild-type DEBS	33	7
K41-135 DEBS	28	7

[1]The *eryA* genes in pKAO127, which is derived from pCK7 [11], originate from *S. erythraea* IMS31 [29], a wild-type erythromycin producer (L. Katz, personal communication).

and genomic DNA is required. Furthermore, it was found that the transformation efficiency of strain K41-135 was more than ten-fold lower than the NRRL2338 strain, and it was not possible to introduce DNA fragments of the desired size. Electroporation, reported for some *S. erythraea* strains [30], also failed with K41-135. Many actinomycetes permit the introduction of DNA through conjugal mating with *E. coli* mediated by the RP4 system [16]. At least one example has been reported in which conjugal transfer from *E. coli* was used to deliver DNA into *S. erythraea* with low efficiency [5]. We found that either small (< 8kb) suicide vectors or ϕC31-based vectors (i.e. pSET152 [4]) could be introduced into *S. erythraea* K41-135 at a low frequency using conjugal transfer but that transfer of larger plasmids was not possible.

Integration of ϕC31 vectors occurs by a site-specific recombination between a chromosomal (*attB*) and vector (*attP*) attachment site, analogous to phage λ in *E. coli* [28]. Screening of *S. erythraea* exconjugants obtained with pSET152 revealed that *S. erythraea* does not contain the consensus *attB* site for ϕC31 found in other *Streptomyces* hosts [15, 21], where pSET152 integrates efficiently. Sequence analysis indicated at least seven distinct locations where pSET152 integrated in *S. erythraea* K41-135 with no homology to each other or the consensus *Streptomyces attB* site (data not shown). Therefore, a 'clean-host' version of the overproducer, *S. erythraea* K24-1, was constructed in which the *eryAI–III* genes are replaced by the ϕC31 *attB* sequence from *S. lividans* (Fig. 2A). In addition, an *ermE*p* promoter [3] was engineered downstream of the *attB* site to drive expression of four erythromycin biosynthetic genes, *eryCII*, *eryCIII*, *eryBII* and *eryG*, located downstream of *eryA* in the operon (Fig. 2A). In *S. erythraea* K24-1, integration of pSET152 was found to occur specifically at the engineered *attB* locus and with at least 100-fold higher frequency than the parent strain, K41-135. This large increase in conjugation–integration efficiency allowed pSET152-derived vectors containing the entire set of DEBS genes (~30 kb) to be integrated into the K24-1 strain with fidelity.

DNA can be introduced into *S. fradiae* efficiently by conjugal transfer from *E. coli* [4] and was also possible with the overproducing strain, K155–3C, used here. A clean-host version of this strain, K159–1, was constructed analogous to *S. erythraea* by deleting the *tylGI–V* genes as described in the Materials and methods (Fig. 2B). Vectors based on the ϕC31 phage integrate site-specifically in *S. fradiae* [4] (also verified for K155-3C) and so introduction of an *attB* site was not necessary. For K159-1, the *tylGI–V* deletion was designed so that the natural promoter upstream of *tylGI* regulated expression of the deoxysugar- and polyketide-modifying genes downstream of the *tylG* locus.

Expression of native PKSs under control of different promoter systems

Different promoters were screened in each of the above hosts using DEBS or the TylPKS as reporters in their native host. Two heterologous promoters were examined, the widely used constitutive *ermE*p* [3] and the *actI*p/*actII*–ORF4 promoter-activator pair [17] used for PKS expression in *S. coelicolor*. The *eryAI*p and *tylGI*p promoter-containing regions upstream of the two PKS gene clusters were also cloned and used for expression. The sequences of these regions from the overproducing strains were examined for promoter mutations that may

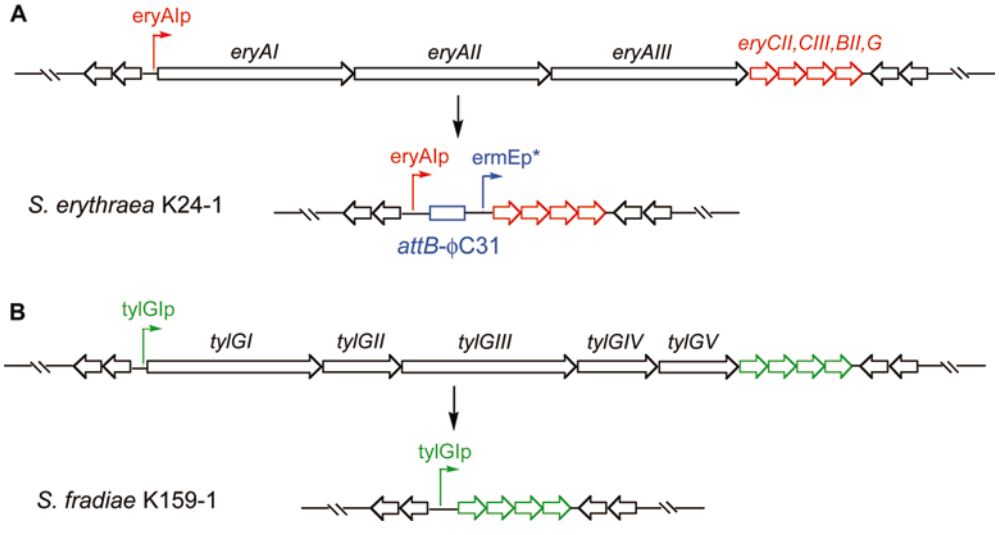

Fig. 2A, B Construction of clean hosts for integration of ϕC31-based PKS gene expression plasmids. The *eryA* genes encoding DEBS (**A**) and the *tylG* genes encoding TylPKS (**B**) were deleted from the chromosomes of *Saccharopolyspora erythraea* and *Streptomyces fradiae* overproducing strains, respectively. A promoter (*ermE*p*) was engineered upstream of the sugar biosynthesis genes in K24-1 since their transcription would be interrupted by integration of the expression vector in the *attB* site. The naturally occurring φC31 *attB* site in *S. fradiae* lies outside of the *tyl* cluster. See text for details of construction

have occurred during strain improvement, however, no differences were found compared to the corresponding wild-type strain.

A set of plasmids was constructed using the *ermE*p*, *actI*p/*actII*–ORF4, and *eryAI*p promoters with the wild-type DEBS-encoding genes from pKAO127 (see above) in a pSET152-derived vector. Likewise, a set of plasmids was constructed using *ermE*p*, and *tylGI*p with the TylPKS-encoding genes from the wild-type (ATCC19609) producing strain of *S. fradiae*. The plasmids were introduced into either *S. erythraea* K24-1 or *S. fradiae* K159-1 as appropriate and transconjugants were screened for production. The titers of polyketides in shake flasks (Table 2) revealed that only use of the native PKS promoter, *eryAI*p for *S. erythraea* and *tylGI*p for *S. fradiae*, led to high polyketide production whereas yields from the heterologous promoters, *ermE*p* (and *actI*p/*actII*–ORF4 in *S. erythraea*), were more than 25 times lower.

S. fradiae ATCC19609 produces ~five- to ten-fold less tylosin than the overproducer strain *S. fradiae* K155-3C. In *S. fradiae* K159-1 expressing the wild-type TylPKS genes from ATCC19609, tylosin production (1.3 g/l) was comparable to that of its parental strain, K155-3C (~2 g/l). The *S. erythraea* K24-1 strain expressing the DEBS genes under control of *eryAI*p produced a mixture of erythromycins (3:1 A:B ratio) and two precursors, erythronolide B and 3α-mycarosylerythronolide B, in a ~2:1:1 ratio with combined titers equal to the erythromycin titer of the parent strain K41-135. The presence of erythronolide B and 3α-mycarosylerythronolide B can be explained by suboptimal expression of the deoxysugar biosynthetic and glycosyl transfer genes (*eryCII*, *eryCIII*, and *eryBII*) that are normally under control of *eryAI*p in the unmodified *ery* gene cluster [22]. The engineered K24-1 host utilizes the *ermE*p* promoter for expression of these genes, which appears to be relatively inefficient based on expression of the DEBS genes in this strain (Table 2).

Restoration of high production levels in the overproducing hosts using the DEBS and TylPKS genes from wild-type organisms further demonstrates that the cause of overproduction is not due to mutations in the PKS genes in either strain. The genes encoding DEBS cloned from the overproducer, K41-135, (see above) were also introduced back into the *S. erythraea* K24-1 strain under control of the *eryAI*p promoter and no apparent difference in overall yield and distribution of erythromycin and its precursors was observed compared to the wild-type DEBS genes (Table 2). This confirmed our prior observation in *S. lividans* and *S. coelicolor* that the two DEBS enzymes have similar activity.

Table 2 Maximum erythromycin (*Saccharopolyspora erythraea*) and tylosin (*Streptomyces fradiae*) titers resulting from PKS expression under control of different promoter systems

Strain/PKS	Promoter	Polyketide titer (mg/l)
S. erythraea K24-1		
Wild-type DEBS	*eryAI*p	1,300[a]
Wild-type DEBS	*ermE*p*	50[b]
Wild-type DEBS	*actI*p/*act*II-4	50[b]
K41-135 DEBS	*eryAI*p	1,300[a]
S. fradiae K159-1		
Wild-type TylPKS	*tylGI*p	1,300[c]
	*ermE*p*	100[c]

[a]~800 mg total erythromycins (A and B)/l, ~250 mg erythronolide B/l and ~250 mg 3α-mycarosylerythronolide B/l
[b]Total erythromycins (A and B)
[c]Tylosin

Production of erythromycin analogs using genetically modified erythromycin PKSs in the *S. erythraea* overproducer

More than 100 analogs of 6-dEB have been produced using DEBSs that have been genetically modified by domain modification, inactivation, insertion or substitution with modules from other PKSs [18, 27, 32]. Production of these compounds occurred in a non-optimized *S. lividans* host and generally suffered from significant decreases in titer compared to 6-dEB and also were biologically inactive due to lack of the deoxysugar and oxidation modifications. The engineered *S. erythraea* K24-1 strain provided an opportunity to examine titers from some of the same engineered DEBS constructs in an industrial producer as well as to produce the biologically active erythromycin analogs. Two different modifications in DEBS module 2, a β-keto modifying domain substitution (KR2 → rapDH/ER/KR1) and an acyltransferase (AT) substitution (AT2 → rapAT2) were chosen (Table 3). The specific nature of the engineered proteins and the 6-dEB analogs produced has been described previously [18]. Each was transferred from their *S. lividans* expression vector to the integrating vector described above under control of *eryAI*p and transferred into *S. erythraea* K24-1 by conjugation.

Positive transconjugants from each of the experiments produced molecules with antibacterial activity based on agar plate bioassays with *Micrococcus luteus*. Culture broths were subjected to LC-MS analysis and compounds characterized as described in Materials and methods. As expected, each of the strains produced the predicted erythromycin analogs (Fig. 3) as a mixture of

Table 3 Shake-flask production of erythromycin analogs in *S. erythraea* K24-1

Genetic modification	Erythromycin analog	Total titer of analogs (mg/l)	Ratio of A:B:C:D congeners
KR2 → rapDH/ER/KR1	11-deoxy	85	1:5:2:2
AT2 → rapAT2	10-desmethyl	100[a]	3[b]:5:1:1

[a]Trace amounts (< 5% total) of erythromycin A and B were also observed in this strain as a result of non-stringent AT specificity
[b]Combined 10-desmethyl erythromycin A and its presumed 6,9-spiroketal form (see [26])

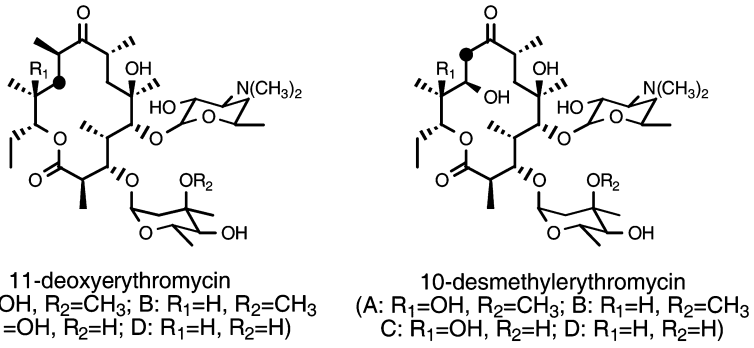

Fig. 3 Structures of erythromycin analogs produced by genetically modified DEBS in the *S. erythraea* K24–1 overproducer

the different A, B, C, and D congeners (Table 3). The different distribution of congeners compared to the parent strain may reflect different activities of the enzymes that catalyze those reactions (EryK and EryG) for the altered substrates. Also, in the K24-1 host, *eryG* is expressed by the *ermE*p* promoter at presumably reduced levels. The absence of detectable aglycone in the two strains with modified DEBS genes indicates that expression of the remaining genes by *ermE*p* (*eryCII*, *eryCIII*, and *eryBII*) was sufficient to support complete glycosylation of the polyketide precursor.

Polyketide titers of the two recombinant strains in shake-flask fermentation (Table 3) were ~5–10% of the erythromycin yield (1–2g/l) from the parent *S. erythraea* overproducer, K41-135, and are consistent with relative titer reductions observed when the same engineered DEBS genes are expressed in *S. lividans* [18]. In continuous stirred-tank fermentation, *S. erythraea* K41-135 yields over 7 g erythromycin/l, or three to seven times more product than in shake flasks. Under the same conditions in a 5-l bioreactor, the yield of 11-deoxy erythromycins produced by the strain containing DEBS (KR2 → rapDH/ER/KR1) was increased 4.5-fold to 380 mg/l.

Discussion

The genetic changes that give rise to overproduction of secondary metabolites in mutagenized strains must lie in some combination of the genes directly involved in production of the metabolite , genes which enhance the physiological environment for metabolite production (e.g. precursor and cofactor supply), and genes which regulate these processes. The broad application of such strains as 'generic' overproduction hosts requires that the first of these possibilities is not the primary determinant of overproduction and that the latter two are translatable to expression of engineered or heterologous biosynthetic enzymes. Although the two PKSs from the overproducing strains studied here may have acquired mutations during strain optimization, our experiments show that any differences between these enzymes and the corresponding PKSs from the wild-type producers do not significantly affect production levels in either optimized or non-optimized hosts. Therefore, the differences between the wild-type and overproduction strains reside in factors such as precursor metabolism and/or improved PKS gene expression.

The requirement for the use of endogenous promoters, *eryAIp* and *tylGIp* in *S. erythraea* and *S. fradiae*, respectively, to achieve high polyketide titers in the overproducers suggests that PKS gene expression levels may be a primary factor. This could be due to a higher intracellular concentration of enzyme, an extended phase of gene expression, or both. Since the cells could not be separated from the insoluble components of the production media used in the above studies it was not possible to make reliable measurements of protein content. Estimates of DEBS content in cells grown in soluble media—in which overall titers are lower but differences are still observed—do not suggest that increased enzyme concentration alone is responsible for overproduction (E. Rodriguez and R. McDaniel, unpublished observation). The identical promoter sequences in the overproducing and wild-type organisms indicate that any differences in expression are due to mutations in regulatory genes. The *actII–ORF4*/*actI*p promoter-activator pair was used previously to express the DEBS genes in a modified *S. erythraea* NRRL2338 strain, and elevated erythromycin production was reported [25]. We obtained the converse result in the *S. erythraea* overproducer, in that use of this promoter-activator resulted in more than a 20-fold decrease in erythromycin titers. Likewise, the constitutive *ermE*p* promoter, generally regarded as a strong promoter for expression in actinomycetes, produced 10- to 20-fold less polyketide in both *S. erythraea* and *S. fradiae* overproducers.

We report here a novel gene delivery and expression system for *S. erythraea* that allows rapid engineering of recombinant strains and PKS gene expression. In spite of the commercial importance of this strain and the central role of DEBS in modular PKS research, tools for gene manipulation and expression in *S. erythraea* have remained largely underdeveloped. The previous approach to gene engineering in *S. erythraea*—protoplast transformation with ensuing homologous recombination—is too inefficient for PKS engineering compared to methods available for other developed hosts [17, 20, 35]. Furthermore, industrially optimized strains of *S. erythraea* are highly refractory to protoplast transformation, resulting in the use of low-titer 'red' variants for genetic

manipulation [30]. These barriers were overcome through the creation of an artificial phage ϕC31 *attB* locus in the chromosome of *S. erythraea* coupled with the use of conjugal transfer from *E. coli* for efficient transfer of DNA vectors. Using this system it was possible to mobilize gene clusters greater than 30 kb in size into an *industrial* version of *S. erythraea*. Insertion of the ϕC31 *attB* site has been previously described for stable expression of genes in mammalian cell lines [10] and may therefore be a valuable tool with broad application in actinomycetes and other microorganisms.

The advantages of using a previously optimized industrial microorganism as a host for improved polyketide production are realized in both the overproduction properties of the strain itself and the extensive process development work needed to achieve optimal production conditions. This is apparent from the substantial quantities of 11-deoxy and 10-desmethyl erythromycin analogs obtained in the engineered *S. erythraea* overproducer. In *S. lividans* K4-114, a strain that has not undergone any strain improvement or process development, the yield of the 11-deoxy and 10-desmethyl 6-dEB analogs produced by the same DEBS constructs under current best conditions is less than 5 mg/l each. Thus, the shake-flask titers for the *S. erythraea* strains represent ~ten-fold improvements on a molar basis and the production of 380 mg 11-deoxyerythromycin/l in the optimized *S. erythraea* stirred-tank fermentation process represents more than a 50-fold improvement in titer. Further utility with the *S. erythraea* host is provided by the ability to generate the biologically active glycosylated derivatives of the structurally modified 6-dEB aglycones, and is therefore especially useful for overproduction of valuable erythromycin analogs and other compounds requiring these deoxysugars. Recent experiments with heterologous PKSs indicate that this approach can be generally adapted to a variety of PKSs and organisms. In one noteworthy example, the *S. fradiae* strain reported here has been engineered to produce over 1 g/l of a 16-membered macrolide using a heterologous PKS (E. Rodriguez, unpublished observation).

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