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A two-vector system for the production of recombinant polyketides in *Streptomyces*

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A two-vector system was developed for heterologous expression of the three genes comprising the 6-deoxyerythronolide B synthase (DEBS) polyketide gene cluster. Individual DEBS genes and pairwise combinations of two such genes were each cloned downstream of the actinorhodin (*actI*) promoter in two compatible *Streptomyces* vectors: the autonomously replicating vector, pKAO127′Kan′, and the integrating vector, pSET152. The resulting plasmids were either simultaneously or sequentially transformed into *Streptomyces lividans* K4-114. Efficient *trans*-complementation of modular polyketide synthase subunit proteins occurred when the respective genes were transcribed from the two vectors and resulted in production of the erythromycin precursor 6-deoxyerythronolide B (6-dEB). *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 46–50.

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Introduction

Polyketides comprise a group of natural products mainly produced as secondary metabolites by soil-living *Actinomycetes*. Numerous pharmaceutically important groups of compounds such as antibiotics, anti-cancer agents and immunosuppressants are polyketides. Based on their modes of synthesis two classes are distinguished: aromatic polyketides (eg actinorhodin and tetracenomycin) are synthesized by polyketide synthases (PKSs) that contain a single set of iteratively used active sites, whereas modular polyketides such as erythromycin and tylosin are synthesized by enzyme complexes consisting of large multifunctional proteins arranged as repeated modules. Each module contains the active sites used for one round of chain elongation, with each active site being used for only one reaction in the entire biosynthetic pathway. Structural diversity occurs from variations in the number and type of active sites in the PKSs. For detailed reviews on polyketides and the recent advances in manipulating modular PKSs, see Hopwood [6], Katz [9], and Carreras and Santi [3].

Katz and colleagues [4,5] reported genetically manipulating individual domains of the DEBS gene cluster in the chromosome of the native host, *Saccharopolyspora erythraea*. Deletion of a ketoreductase domain [4] and mutation of an enoyl reductase domain [5] both resulted in novel compounds. Numerous analogs of 6-deoxyerythronolide B (6-dEB) were also obtained by genetic manipulation of the 32-kb PKS gene cluster on a single plasmid and expression in heterologous host strains *Streptomyces coelicolor* CH999 and *Streptomyces lividans* K4-114 [10,14,15; McDaniel *et al*, unpublished data]. Although the plasmid system provided significant advantages, the creation of novel polyketides remained somewhat tedious. For each altered polyketide, specifically targeted genetic changes had to be introduced into individual constructs, a difficult task considering the large size of the plasmids used in the referred studies—*ca* 48 kb—and limited number of natural unique restriction sites within the cluster of three DEBS genes. We reasoned that concomitant expression of modular PKS genes from two compatible vectors would provide a simpler system for such mutagenesis studies, as well as an approach to cloning even larger PKS gene clusters. In the present work, we describe a two-vector system using an autonomously replicating vector, pKAO127′Kan′, and an integrating vector, pSET152, that expresses a functional polyketide synthase in *Streptomyces lividans*.

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Materials and methods

Bacterial strains and growth conditions

E. coli XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *ZΔM15* Tn*10* (Tet^r)]) was used as the plasmid host. All *E. coli* strains were routinely cultured in Luria broth [18] supplemented with 100 μg ml^{-1} carbenicillin and 80 μg ml^{-1} kanamycin as required. The Streptomycete host strain used, *S. lividans* K4-114, lacks most of the actinorhodin gene cluster and can be transformed with high efficiency [20]. Media used for *Streptomyces* cultivation were R5 for plates and YEME for liquid growth, both described [7]. Antibiotics for *Streptomyces* plate growth were used in concentrations of 50 μg ml^{-1} thiostrepton and 100 μg ml^{-1} apramycin, and for liquid growth 5 μg ml^{-1} thiostrepton, and 50 μg ml^{-1} apramycin.

Plasmid contructs

PCR conditions used in the generation of plasmid constructs were optimized for amplification of GC-rich *Streptomyces* DNA by the addition of 10% DMSO to each sample and raising the annealing temperature to 65°C. pKOS021-21 (Figure 1) which is similar to pKAO127′Kan′ but contains an *Avr*II site between the *ery*AI and *ery*AII genes was created by PCR amplification of a *ca* 1-kb fragment corresponding to the intervening region of the *ery*AI

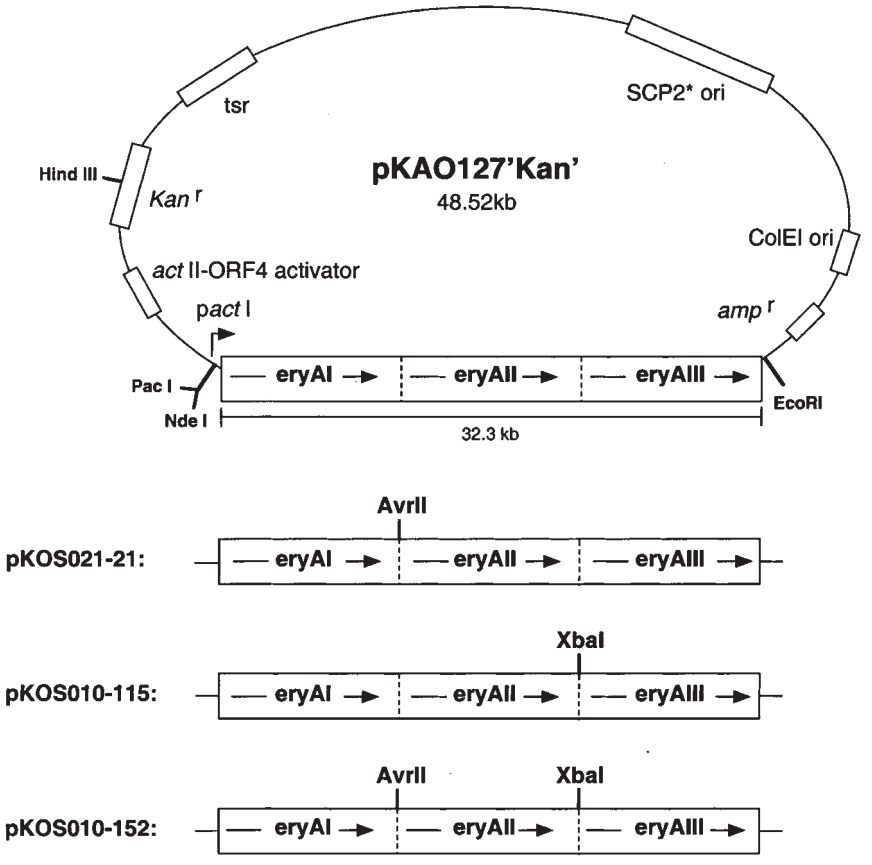

Figure 1 Schematic map of plasmid pKAO127′Kan′ and its three derivatives pKOS021-21, pKOS010-115, and pKOS010-152. The drawing is not to scale. For pKAO127′Kan′, approximate locations of genes encoding kanamycin resistance (Kan^r), thiostrepton resistance (tsr), ampicillin resistance (amp^r), the *act* II-ORF 4 activator, as well as the SCP2* and the ColEI origins of replication are shown by small bars on the circular map. The positions of the three DEBS genes (*eryAI–eryAIII*) are indicated by larger bars with vertical dotted lines indicating approximate gene boundaries. The overall size of the three DEBS genes is indicated below the plasmid map. Restriction sites (*Hind*III, *Pac*I, *Nde*I and *Eco*RI) in pKAO127′Kan′ which were used to generate various constructs described herein are shown. The position of the *act*I promoter is shown upstream of the *ery*AI gene along with an arrow denoting the start site of transcription. In the case of pKOS021-21, pKOS010-115, and pKOS010-152, only the three *ery*A genes are shown, as bars aligned directly below their counterparts on pKAO127′Kan′. The unique restriction sites, *Avr*II and *Xba*I, which were introduced at the gene junctions in the respective plasmids are indicated above the genes.

and *ery*AII genes using oligonucleotides 9 and 10 (Figure 2). The PCR fragment was digested with *Avr*II and *Kpn*I and cloned into pLitmus28 (New England Biolabs, Beverly, MA, USA). A slightly modified recombination procedure described in detail by Kao *et al* [8] was used to cross this fragment into pKAO127′Kan′. pKOS010-115 (Figure 1) was constructed in several steps. First, oligonucleotides 7 and 8 (Figure 2) were used to PCR amplify a *ca* 385-bp fragment of the DEBS gene cluster, corresponding to the 5′ end of the *ery*AIII gene. This fragment was digested with *Bgl*II and *Xba*I and cloned into pLitmus28. Using standard cloning procedures the *ery*A gene fragment was subsequently transferred in several steps into pKAO127′Kan′. pKOS010-152 (Figure 1) was constructed using the same procedures applied in the construction of pKOS010-115 and pKOS021-21.

Oligonucleotides 15 and 16 (Figure 2) were used for PCR amplification of a *ca* 550-bp fragment corresponding to the 5′ terminus of the *ery*AII gene. This fragment was digested with *Hind*III and *Pac*I and cloned into pNEB193 (New England Biolabs) and subsequently used for the construction of pKOS010-151 (Figure 3b). pKOS010-123, pKOS010-163, and pKOS021-30 (Figure 3b) were all constructed from existing pKAO127′Kan′ derivatives. The *ca* 3-kb *Hind*III/*Pac*I pKAO127′Kan′ fragment (Figure 1) originally from pRM5 [13] was transferred into pSET152; this fragment contains the phage fd transcriptional terminator and genes encoding the *act*II-ORF IV activator which is required for expression from the *act* I promoter, and ketoreductase proteins; the latter gene is not relevant for the experiments described here (see McDaniel *et al* [13] for a detailed map). Fragments carrying the individual *ery*A genes from plasmids pKOS010-151, pKOS010-163, pKOS021-30, and pKOS010-123 were then cloned into the pSET152-derived construct resulting in pKOS021-22, pKOS010-153, pKOS021-23, and pKOS021-45, respectively (Figure 3).

Production of 6dEB

The procedure used for 6-dEB purification from large R5 agar plates (*ca* 34 ml agar) was essentially as described in Kao *et al* [8]. Yields were calculated from total 6-dEB produced from one plate each that were grown for approximately 5–6 days.

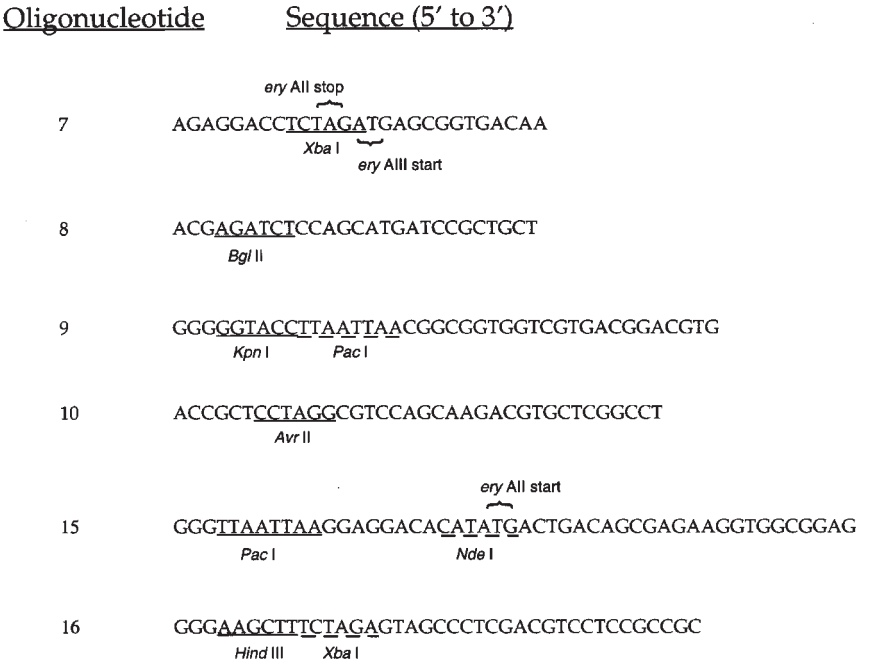

Figure 2 Oligonucleotides used for PCR in generating plasmid constructs. Oligonucleotides 7 and 8 were used to introduce an *Xba*I site between the *ery*AII and *ery*AIII genes in pK0S010-115. In oligonucleotide 7, the *Xba*I site is underlined; the TAG stop codon of the *ery*AII gene is shown by a bracket above the sequence, the ATG start codon of the *ery*AIII gene is shown by a bracket below the sequence. In oligonucleotide 8, a unique *Bgl*II site in the *ery*AIII gene is underlined. Oligonucleotides 9 and 10 were used to introduce an *Avr*II site between the *ery*AI and *ery*AII genes in pKOS021-21. In oligonucleotide 9, underlined restriction sites are *Kpn*I (single line) and *Pac*I (dashed line) which were introduced for cloning purposes. The sequence to the right of the *Pac*I site represents the 3-terminal region of the *ery*AI gene. In oligonucleotide 10, the introduced *Avr*II site is underlined. Oligonucleotides 15 and 16 were used in the construction of pKOS010-151. In oligonucleotide 15, underlined restriction sites are *Pac*I (single line) and *Nde*I (dashed line). The ATG start codon of the *ery*AII gene is indicated above the sequence by a bracket. In oligonucleotide 16, underlined restriction sites are *Hind*III (single line) and *Xba*I (dashed line).

Results

Introduction of unique restriction sites in the pKAO127′Kan′ DEBS expression vector

pKAO127′Kan′ (Figure 1) is an *E. coli/Streptomyces* shuttle vector with a copy number in *Streptomyces* of one to two (SCP2* origin of replication; [11]) that carries the three DEBS genes, *ery*AI–III, cloned downstream of the *act*I promoter (Figure 1). This replicating vector was previously shown to produce 6-deB at 10–20 mg L^{-1} in *S. lividans* and *S. coelicolor* [20]. There are no unique restriction sites within the DEBS gene cluster that permit facile cloning and subsequent manipulation of the intact individual genes. Using PCR and molecular biological as well as genetic methods [7,18], we constructed three similar plasmids, designated pKOS021-21, pKOS010-115, and pKOS010-152 (Figure 1). These carry a unique *Avr*II site between the *ery*AI and *ery*AII genes (pKOS021-21), an *Xba*I site between the *ery*AII and *ery*AIII genes (pKOS010-115), and both *Avr*II and *Xba*I sites combined (pKOS010-152). These mutations are designed to be silent in regard to the coding sequence of the DEBS gene cluster and are useful for construction of expression constructs encoding only one or two DEBS genes. Using these four plasmids we analyzed plate production levels of 6-dEB, the non-glycosylated precursor of erythromycin, in *S. lividans* K4-114. No reproducible significant difference among the strains was apparent, demonstrating that introduction of these restriction sites does not affect polyketide synthesis.

Development of a two-vector system

The *ery*A genes are chromosomal in origin and there are not many available vectors (replicating or otherwise) which can tolerate the large *ery*A gene inserts (32.3 kb for all three genes). Hence, for a second vector to be developed into an expression construct, we chose the integrating vector, pSET152, which was likely to be compatible with our first vector, the SCP2*-type replicating vector, pKAO127′Kan′. pSET152 is an integrating vector used in *S. lividans* [2]. It carries the phage ΦC31 attachment region (*attP*) and integrase gene (*int*) and the gene encoding apramycin resistance (this marker can be used in both *E. coli* and *Streptomyces*). Site-specific integration of pSET152 at the bacterial attachment site, *attB*, is mediated by the Int gene product.

Significantly, integration at *attB* has not been reported to have a negative impact on the expression of cloned genes, thus *attB* is likely to be neutral with respect to expression of PKS genes. Compatibility of pSET152 and pKAO127′-Kan′ was verified by co-transformation of the two vectors into *Streptomyces lividans* K4-114 followed by recovery of transformants containing the vectors' DNA (data not shown). Subsequently, a total of four pSET152-derived vectors were created (Figure 3a) each of which allowed for expression of one or two DEBS genes under the control of the *act*I promoter. An analogous set of expression constructs was also created in pKAO127′Kan′ (Figure 3b). Next, all pSET152-derived plasmids (pKOS021-23,

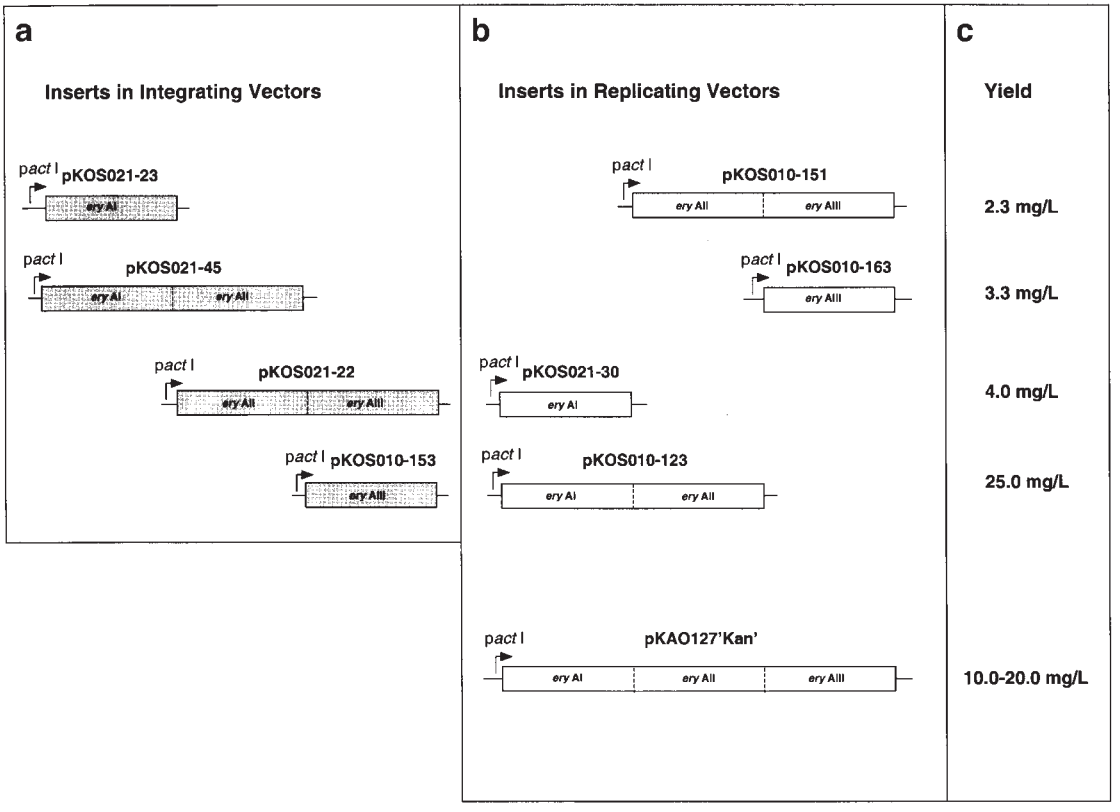

Figure 3 Combinations of the three *ery*A genes used in the two-vector system. In (a), combinations of the *ery*A genes cloned into pSET152 integrating plasmids are shown. The position of the *act*I promoter is indicated upstream of the relevant *ery*A gene(s) along with an arrow denoting the start site of transcription. The vector, pSET152 is not shown but is described in detail in the text. Similarly, in (b) combinations of *ery*A genes cloned into the replicating plasmid pKAO127′Kan′ are shown. The position of the *act*I promoter is indicated upstream of the relevant *ery*A gene(s) along with an arrow denoting the start site of transcription. For comparison to the one plasmid system, the three genes (in pKAO127′Kan′) are shown below the single and double combinations. The pKAO127′Kan′ vector is shown in detail in Figure 1. In (c) the yields are shown which were obtained when two complementing vectors and pKAO127′Kan′ (indicated to the left) were transformed into K4-114. Yields represent an average of at least two separate determinations and are calculated from total 6-dEB produced from one plate each.

pKOS021-45, pKOS021-22, and pKOS010-153) were individually transformed into *Streptomyces lividans* K4-114.

As predicted, analysis of the resulting strains revealed no 6-dEB production. Both of the two strains harboring the first gene [K4-114 (pKOS021-23) and K4-114 (pKOS021-45)] produced traces of triketidelactone indicating that: (i) these constructs are functional; and (ii) consistent with earlier observations, that the thioesterase domain encoded by DEBS module 6 is not required for cleavage of the thioester bond and subsequent cyclization to result in the lactone formation. Analogously, K4-114 strains transformed with only one each of the four pKAO127′Kan′-derivatives (pKOS010-151, pKOS010-163, pKOS021-30, and pKOS010-123; Figure 3b) gave similar results, no 6-dEB and traces of triketidelactone.

Subsequently, we transformed the complementary pKAO127′Kan′-derived plasmids into the various corresponding apramycin-resistant strains containing the pSET152-derived plasmids and analyzed the resulting double-resistant transformants for 6-deB production. At least two separate determinations were performed and confirmed that all strains carrying combinations of two complementing vectors produced 6-dEB (Figure 3c). The quantities from three of the four combinations ranged from 2.3 to 4.0 mg L^{-1} which is less than the 25 mg L^{-1} level observed with the fourth combination and the 10–20 mg L^{-1} level seen with pKAO127′Kan′ (ie, the three *ery*A genes in one expression plasmid) (Figure 3c). Thus, subunit complementation of the individual PKS proteins can occur *in trans* and there is no *cis*-requirement which necessitates expression of modular PKSs from one plasmid.

In the course of this experiment we also analyzed co-transformability of K4-114 protoplasts with two plasmids by simultaneously selecting for apramycin and thiostrepton markers. For K4-114 transformations with a single plasmid, 1 μg of plasmid DNA was used; for simultaneous transformation with two plasmids 1 μg DNA of each respective plasmid was used. Apramycin/thiostrepton double-resistant transformants were obtained with an approximate ten-fold reduced frequency compared to selection for a single drug resistance marker. We analyzed selected clones of such transformants for their ability to produce 6-dEB and found no significant difference in yields compared to clones obtained by sequential transformation procedures.

Conclusions

Successful production of polyketides in heterologous hosts would be facilitated by new methods that allow for simpler manipulations of the very large PKS gene clusters.

Although remarkable progress in the manipulation of modular PKSs has been made, the formation of the multisubunit PKS complex and the interactions of its individual protein members remains to be elucidated. Our goal was to demonstrate efficient *trans*-complementation of PKS subunit proteins from two compatible vectors, and we demonstrated a functional two-vector system by adding pSET152-derivatives to the current pKAO127′Kan′-based plasmids. Although three out of four combinations resulted in a comparatively reduced yield, the experiments clearly demonstrated that modular PKS subunits can associate with each other when transcribed from compatible vectors. The finding of certain possibly favorable pairings of expression constructs in the two-vector system indicates that other compatible *Streptomyces* vectors [12,16,17,19] could also prove useful for such experiments. We expect that this new expression technology will find broad utility in the expression of PKS genes.

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