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Approaches to stabilization of inter-domain recombination in polyketide synthase gene expression plasmids

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Abstract Regions of extremely high sequence identity are recurrent in modular polyketide synthase (PKS) genes. Such sequences are potentially detrimental to the stability of PKS expression plasmids used in the combinatorial biosynthesis of polyketide metabolites. We present two different solutions for circumventing intraplasmid recombination within the megalomicin PKS genes in *Streptomyces coelicolor*. In one example, a synthetic gene was used in which the codon usage was reengineered without affecting the primary amino acid sequence. The other approach utilized a heterologous subunit complementation strategy to replace one of the problematic regions. Both methods resulted in PKS complexes capable of 6-deoxyerythronolide B analogue biosynthesis in *S. coelicolor* CH999, permitting reproducible scale-up to at least 5-l stirred-tank fermentation and a comparison of diketide precursor incorporation efficiencies between the erythromycin and megalomicin PKSs.

Keywords 6-Deoxyerythronolide B · Erythromycin · Megalomicin

Introduction

Modular polyketide synthases (PKSs) are large, multisubunit enzyme complexes that perform the biosynthesis of polyketide secondary metabolites [10]. Examples of polyketides include the antibiotic erythromycin, the immunosuppressant FK506, and the antitumor compound epothilone. A large interest in these enzyme systems lies in the ability to manipulate the specificity or sequence of reactions catalyzed by PKSs to produce novel therapeutic compounds [9, 17]. A number of plasmid-based heterologous expression systems have been developed for the engineering and expression of PKSs, including multiple-plasmid systems for combinatorial biosynthesis [8, 18, 19].

In modular PKSs, active sites are arranged in groups (modules) which perform a single round of polyketide chain extension and modification (Fig. 1). PKS modules are typically 3.5–7.0 kb, depending on the number of active sites present in the module. Frequently, the homology between similar active site domains [e.g. ketosynthase (KS), acyltransferase (AT), ketoreductase (KR)] in a cognate PKS is greater than between domains of heterologous PKSs. Many sequenced PKS gene clusters contain at least two domains in which the DNA sequence identity is greater than 99% over significant lengths of nucleotide bases (i.e. >500 bp). For example, the KR and acyl carrier protein domains from modules 2 and 5 of the oleandomycin PKS [12, 13] each contain a 1,211-bp contiguous segment with 100% identity. In the tylosin PKS [1], three fragments (2,013–2,290 bp) from the KS and AT domains of modules 1, 4, and 6 all share a sequence identity greater than 99.5% (R. McDaniel and R. Reid, unpublished data). These repetitive sequences most likely arose from gene duplications followed by sequence divergence (gene conversion would not add modules, so multimodular PKSs must have involved duplication) during the evolution of the PKS. While these regions appear to be stable in the chromosome of the host organisms in which they are found, such duplications are potentially detrimental to the stable expression of plasmid-borne PKSs in hosts capable of homologous recombination.

In this study, we examined the effect of duplicate sequences on plasmid stability in *Streptomyces coelicolor*, a host commonly used for heterologous expression of PKSs, and compared two different approaches to creating more stable expression constructs. The

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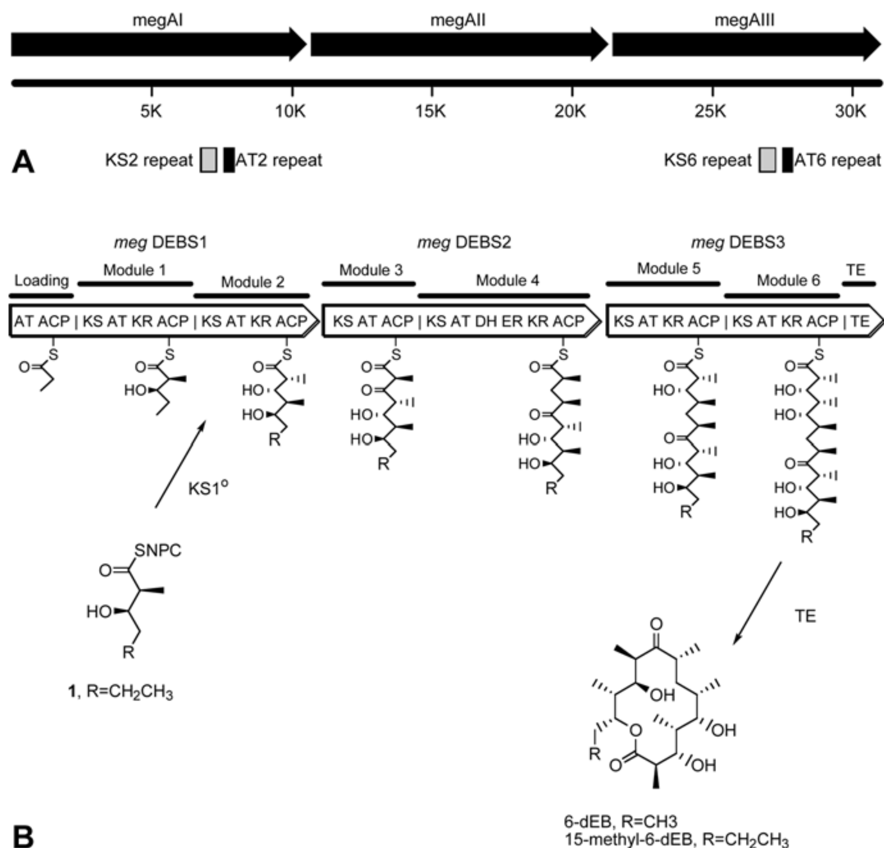

Fig. 1A, B The megalomicin polyketide synthase (PKS; *meg* DEBS) and corresponding genes. **A** The *meg* DEBS is encoded by three genes of ˜10 kb each. The regions of identical sequence between KS2/AT2 and KS6/AT6 (see text) are depicted below the genes. **B** The arrangement of modules and actives sites of *meg* DEBS are identical to the erythromycin PKS (*ery* DEBS). The natural PKS produces 6-deoxyerythronolide B (6-dEB) from propionyl-CoA and 6 methylmalonyl-CoA units. A KS1° mutation permits the incorporation of synthetic diketide intermediates to produce 6-dEB analogues. ACP Acyl carrier protein, AT acyl transferase, DH dehydratase, ER enoylreductase, KR ketoreductase, KS ketosynthase, SNPC N-propionyl-cysteamine thioester, TE thioesterase

megalomicin 6-deoxyerythronolide B (6-dEB) synthase (*meg* DEBS; Fig. 1B) contains duplicate regions comprising 615 bp in the KS domains and 426 bp in the AT domains of modules 2 and 6. The erythromycin 6-dEB synthase (*ery* DEBS) is identical in overall genetic architecture to *meg* DEBS [16], but does not possess any such redundant sequences. Recently, we reported that both *ery* and *meg* DEBS produced similar yields of 6-dEB in *Streptomyces lividans* [16]. In subsequent rounds of fermentation, titers from *meg* DEBS were consistently lower than those from *ery* DEBS. Furthermore, a significant decrease in titers was observed when *meg* DEBS was expressed in *S. coelicolor* CH999 and the titers could not be determined reproducibly (R. McDaniel, unpublished data). We therefore examined whether these observations were due to plasmid rearrangement in this host and developed two independent strategies to overcome this problem.

Materials and methods

Manipulation of DNA and organisms

Construction of plasmids was performed in *Escherichia coli* XL1-Blue (Stratagene). Standard procedures were used for growth and maintenance of *E. coli* [11] and *Streptomyces* [5]. Protoplast transformation procedures were used to introduce DNA into *Streptomyces* strains [5]. Transformants were selected using 1 mg thiostrepton and/or 2 mg apramycin overlay (2 ml) on R2YE regeneration plates.

Analysis of plasmid stability in *S. coelicolor*

Individual colonies from primary transformants were used to inoculate 5–6 ml of R5 [5] or TSB [5] media with thiostrepton (25 μg/ml) added for selection. After 3 days growth at 30 °C, 2–3% of the culture volume was used to inoculate another 5 ml culture and the remaining cells were collected for plasmid analysis. This procedure was repeated for a total of 3–4 rounds. Since the expression plasmids used in *S. coelicolor* contain elements for replication and selection in *E. coli* [8], analysis of plasmid content was performed by isolating total DNA from the harvested cells and transforming Ca^{2+} competent cells of *E. coli* XL1-Blue (Stratagene), which is deficient in homologous recombination (*recA1*). Plasmid DNAs from approximately 10–20 *E. coli* transformants were prepared and analyzed by restriction enzyme–gel electrophoresis, with comparison to the authentic starting plasmid.

Construction of *meg* DEBS (KS1°) plasmids

Plasmid pKOS108-06, encoding the wild–type *meg* DEBS, was described in [16]. Plasmid pKOS108-15 encodes the same *meg* DEBS with a point mutation (Cys729Ala) in the KS domain of module 1 (KS1°), as previously described for *ery* DEBS [4]. This was constructed as follows. A 22-kb *Eco*RI–*Bgl*II fragment containing the *megAI* and *megAII* genes was subcloned into pLitmus 28 (Stratagene). The 2.4-kb *Eco*RI–*Sph*I fragment of this plasmid containing the KS1 domain was replaced with the same fragment in

▶

Fig. 2 Alignment of the identical DNA sequences in *meg* modules 2 and 6 (megKS2/KS6, megAT2/AT6) with the reengineered segments for module 6 (megKS6*, megAT6*). All of the engineered nucleotide sequences conserve the amino acid sequence of the *meg* DEBS. Restriction sites used in assembly of the synthetic fragments are shown

which the above mutation had been introduced by PCR mutagenesis (nt 2185–2190 of *megAI* changed to GCTAGC). The resulting plasmid, pKOS024-86, contained an *Nhe*I restriction site at the location of the amino acid substitution. The 22-kb *Eco*RI–*Bgl*II fragment from pKOS024-86 was then used to replace the corresponding fragment from pKOS108-06 to create pKOS108-15. Likewise, the 22-kb *Eco*RI–*Bgl*II fragment from pKOS024-86 was used to replace the corresponding fragment from pKOS024-24 [16] to generate pKOS108-14, which contained only the *megAI* (KS1°) and *megAII* genes.

Construction of *meg* DEBS genes with synthetic codons

Three separate pieces of DNA, a *Pst*I–*Bam*HI fragment (nt 26,739–26,947), a *Bam*HI–*Bsm*I fragment (nt 26,947–27,267), and a *Sfa*NI–*Fse*I fragment (nt 27,697–27,987), covering the KS6 and AT6 regions of the *megAIII* gene were synthesized (Retrogen) and cloned into pCR-Blunt II-TOPO (Invitrogen). Each of the DNA segments altered the natural codons to those shown in Fig. 2.

```
               Pst I
megKS2/6     1 TGCAGCGGTTGTCGGTGGCGGTGCGGGAGGGGCGTCGGGTGTTGGGTGTGGTGGTGGGTTCGGCGGTGAATCAGGATGGG
megKS6*      1 TGCAGCGCCTCTCCGTCGCCGTCCGCGAGGGCCGCCGAGTCCTCGGCGTCGTCGTCGGCTCGGCCGTCAACCAAGACGGC
               *******  * ** ** ** ** ** ***** ** ** **  * ** ** ** ** ** ***** ** ** ** ** **

megKS2/6    81 GCGAGTAATGGGTTGGCGGCGCCGTCGGGGGTGGCGCAGCAGCGGGTGATTCGGCGGGCGTGGGGTCGTGCGGGTGTGTC
megKS6*     81 GCGTCAAACGGCCTCGCCGCGCCCTCCGGCGTCGCCCAGCAGCGCGTCATACGCCGCGCGTGGGGACGCGCCGGAGTATC
               ***   ** **  * ** ***** ** ** ** ******** ** ** ** ** ******** ** ** ** ** **
                                                                 BamH I
megKS2/6   161 GGGTGGGGATGTGGGTGTGGTGGAGGCGCATGGGACGGGGACGCGGTTGGGGGATCCGGTGGAGTTGGGGGCGTTGTTGG
megKS6*    161 GGGCGGCGACGTCGGAGTCGTCGAGGCCCACGGCACCGGCACCCGCCTCGGGGATCCCGTCGAGCTGGGCGCCCTCCTGG
               *** ** ** ** ** ** ** ***** ** ** ** ** ** **  * ******** ** *** **** **  *  ***

megKS2/6   241 GGACGTATGGGGTGGGTCGGGGTGGGGTGGGTCCGGTGGTGGTGGGTTCGGTGAAGGCGAATGTGGGTCATGTGCAGGCG
megKS6*    241 GCACGTACGGCGTCGGCCGCGGCGGCGTCGGCCCGGTCGTCGTCGGCAGCGTCAAGGCCAACGTCGGCCACGTCCAGGCC
               * ***** ** ** ** ** ** ** ** ** ***** ** ** **    ** ***** ** ** ** ** ** *****

megKS2/6   321 GCGGCGGGTGTGGTGGGTGTGATCAAGGTGGTGTTGGGGTTGGGTCGGGGGTTGGTGGGTCCGATGGTGTGTCGGGGTGG
megKS6*    321 GCGGCCGGCGTCGTCGGGGTCATCAAGGTCGTCCTCGGCCTCGGCCGCGGGCTGGTCGGCCCGATGGTCTGCCGCGGCGG
               ***** ** ** ** ** ** ******** **  * **  * ** ** *** **** ** ******** ** ** ** **

megKS2/6   401 GTTGTCGGGGTTGGTGGATTGGTCGTCGGGTGGGTTGGTGGTGGCGGATGGGGTGCGGGGGTGGCCGGTGGGTGTGGATG
megKS6*    401 CCTCAGCGGCCTCGTCGACTGGTCGTCCGGCGGCCTGGTCGTCGCGGACGGGGTCCGCGGCTGGCCGGTCGGCGTCGACG
                 *    **  * ** ** ******** ** **  **** ** ***** ***** ** ** ******** ** ** ** *
                                                         Bsm I
megKS2/6   481 GGGTGCGTCGGGGTGGGGTGTCGGCGTTTGGGGTGTCGGGGACGAATGCTCATGTGGTGGTGGCGGAGGCGCCGGGGTCG
megKS6*    481 GCGTCCGCCGGGGCGGCGTCTCGGCGTTCGGCGTCAGCGGGACGAATGCTCATGTGGTGGTGGCGGAGGCGCCGGGGTCG
               * ** ** ***** ** ** ******** ** **    ******************************************

megKS2/6   561 GTGGTGGGGGCGGAACGGCCGGTGGAGGGGTCGTCGCGGGGGTTGGTGGGGGTGG 615
megKS6*    561 GTGGTGGGGGCGGAACGGCCGGTGGAGGGGTCGTCGCGGGGGTTGGTGGGGGTGG 615
               *******************************************************
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megAT2/6     1 CCGGTGTGGTGTCGGGGGTGGCGTCGGGTGGTGGTGTGGTGTTTGTTTTTCCTGGTCAGGGTGGTCAGTGGGTGGGGATG
megAT6*      1 CCGGTGTGGTGTCGGGGGTGGCGTCGGGTGGTGGTGTGGTGTTTGTTTTTCCTGGTCAGGGTGGTCAGTGGGTGGGGATG
               ********************************************************************************
                                                                 SfaN I
megAT2/6    81 GCGCGGGGGTTGTTGTCGGTTCCGGTGTTTGTGGAGTCGGTGGTGGAGTGTGATGCGGTGGTGTCGTCGGTGGTGGGGTT
megAT6*     81 GCGCGGGGGTTGTTGTCGGTTCCGGTGTTTGTGGAGTCGGTCGTGGAGTGCGATGCGGTCGTGTCGAGCGTCGTCGGCTT
               ***************************************** ******** ******** ******   ** ** ** **

megAT2/6   161 TTCGGTGTTGGGGGTGTTGGAGGGTCGGTCGGGTGCGCCGTCGTTGGATCGGGTGGATGTGGTGCAGCCGGTGTTGTTCG
megAT6*    161 CAGCGTGCTGGGCGTCCTGGAGGGCCGCAGCGGCGCCCCGAGCCTGGACCGCGTCGACGTGGTCCAGCCGGTCCTGTTCG
                   *** **** **  ******* **    ** ** ***    **** ** ** ** ***** ********  ******

megAT2/6   241 TGGTGATGGTGTCGTTGGCGCGGTTGTGGCGGTGGTGTGGGGTTGTGCCTGCGGCGGTGGTGGGTCATTCGCAGGGGGAG
megAT6*    241 TGGTCATGGTCAGCCTGGCCCGCCTGTGGCGCTGGTGCGGCGTGGTCCCGGCCGCCGTGGTCGGCCACAGCCAGGGCGAG
               **** *****     **** **  ******* ***** ** ** ** ** ** ** ***** ** **    ***** ***

megAT2/6   321 ATCGCGGCGGCGGTGGTGGCGGGGGTGTTGTCGGTGGGTGATGGTGCGCGGGTGGTGGCGTTGCGGGCGCGGGCGTTGCG
megAT6*    321 ATCGCCGCCGCGGTCGTGGCCGGCGTCCTGAGCGTCGGCGACGGCGCCCGCGTCGTGGCCCTGCGCGCCCGCGCCCTGCG
               ***** ** ***** ***** ** **  **   ** ** ** ** ** ** ** *****  **** ** ** **  ****
                     Fse I
megAT2/6   401 GGCGTTGGCCGGCCACGGCGGCATGG 426
megAT6*    401 CGCCCTGGCCGGCCACGGCGGCATGG 426
                **  *********************
```

The cloned synthetic fragments were verified by DNA sequencing and used to construct pKOS097-152a as follows. First, a cassette containing the phage λ *cos* site was introduced downstream of the *megAIII* gene. A 350-bp segment at the end of the *megAIII* gene was PCR-amplified using the two oligonucleotide primers 5'-d(TTTGACGTGTACCCACCCGGTCACCAGGAG) and 5'-d(TTTGAATTCTCTAGATCATGCCCTCTCCCCGCTCAACAACCAGGC) and cloned into pCR-Blunt II (Invitrogen) to create pKOS097-87B. The 9.2-kb *Bgl*II–*Xba*I fragment containing most of *megAIII* from pKOS108-06 was subcloned into pLitmus28 (New England BioLabs) to generate pKOS097-81. The 4.3-kb *Pst*I–*Xba*I fragment from pKOS097-81was then subcloned into pLitmus28 to make pKOS097-84. The 0.35-kb *Afl*III–*Eco*RI fragment from pKOS097-87B and the 4.0-kb *Afl*III–*Pst*I fragment from pKOS097-84 were ligated together with *Pst*I–*Eco*RI digested pLitmus38 (New England BioLabs), resulting in pKOS097-90. This was followed by insertion of the 4.9-kb *Spe*I–*Pst*I fragment from pKOS097-81 into the corresponding sites of pKOS097-90 to generate pKOS097-90A. The 9.2-kb *Bgl*II–*Eco*RI fragment of pKOS097-92a was combined with the *megAI* and *megAII* genes in pHU152', a cloning vector containing the desired *cos* site. This plasmid was named pKOS097-92 and contains the *megAI–AIII* genes with a *cos* site downstream of *megAIII*, flanked by an *Xba*I site. Next, the *Pst*I–*Bsm*I fragment in pKOS097-90 was replaced with the two *Pst*I–*Bam*HI and *Bam*I–*Bsm*I synthesized DNA fragments from above. The *Pst*I–*Sfa*NI fragment from pKOS097-90 and the synthesized *Fse*I–*Sfa*NI fragment were joined together to replace the *Pst*I–*Fse*I fragment of pKOS097-90 to make pKOS097-152. Finally, pKOS097-152a was constructed by a four-fragment ligation, using the 4.9-kb *Bgl*II–*Pst*I fragment from pKOS098-81, the 2.6-kb *Pst*I–*Blp*I fragment from pKOS097-152, the 2.0-kb *Blp*I–*Xba*I fragment from pKOS097-92, and pKOS10814 digested with *Bgl*II–*Xba*I. The ligation mixture was packaged in vitro using a Gigapack-III gold kit (Stratagene) and was then used to transfect *E. coli* Xl1-Blue. One plasmid from such transfectants containing the expected banding pattern in agarose gel after *Bam*HI digestion was named pKOS97-152a.

Fermentation and analysis of polyketide titers

Fermentation studies were initiated from frozen cell-bank vials, as described previously [2]. Primary seed cultures were established by inoculating 50 ml of FKA medium [2] with a cell-bank vial and cultivating for 3 days. For shake-flask studies, replicate flasks containing 35 ml of production medium were inoculated with 1.75 ml (5% v/v) of the primary seed culture. For the production of 15-methyl-6-dEB, 40% racemic (2*S*,3*R*; 2*R*,3*S*)-2-methyl-3-hydroxy-hexanoic acid, and *N*–propionylcysteamine thioester (diketide SNPC) in dimethylsulfoxide were fed (2 g/l final) at 2 days after inoculation and maintained above 1 g/l. Flasks were incubated for 6–10 days with 1-ml samples withdrawn as necessary and stored at −20 °C until analysis. All media were supplemented with 10 ml of 50% (v/v) Antifoam B (J.T. Baker, Phillipsburg, N.J.) per liter of culture volume as post-sterile additions. Seed cultures were also supplemented with 50 mg thiostrepton/l (Calbiochem, La Jolla, Calif.).

Bioreactor studies were performed in B. Braun MD 5-l fermentors with 3 l of FKA medium without HEPES, operated at 30 °C, pH 6.5, 0.3 vvm airflow, and 600 rpm agitation. Dissolved oxygen concentration and pH were monitored using autoclavable electrodes (Mettler Toledo, Wilmington, Mass.). Under these operating conditions, dissolved oxygen was maintained above 50% by automatically increasing agitation as necessary. Foaming was controlled by automatic addition of 50% (v/v) Antifoam B solution. The pH was controlled by automatic addition of 2.5 N sodium hydroxide or sulfuric acid. Bioreactors were inoculated with 5% (v/v) secondary seed culture prepared by sub-culturing 25 ml of primary seed into 500 ml of FKA and cultivating for 2 days. Samples were withdrawn as necessary and stored at −20 °C for later analysis.

Quantification of diketide-SNPC and 15-methyl-6-dEB was performed using a Hewlett–Packard 1090 HPLC equipped with a diode array detector and an Alltech 500 evaporative light-scattering detector as described previously [6]. Measurement uncertainty was typically 10% by this procedure.

Results and discussion

Analysis of *meg* DEBS expression plasmid stability in *S. coelicolor*

Two *meg* DEBS expression plasmids were used to transform *S. coelicolor* CH999 [8]. Plasmid pKOS108-6 (Fig. 3) encodes the wild-type *meg* DEBS and pKOS108-15 (Fig. 3) encodes the same *meg* DEBS with a point mutation (KS1°) of the active site cysteine in the ketosynthase domain of module 1, as previously described for *ery* DEBS [3, 4]. Efficient transformation of *S. coelicolor* requires the use of unmethylated DNA that is typically prepared from methylation-deficient *E. coli* strains, such as ET12567 (*dam13::Tn9, dcm6*) [7]. However, in the case of pKOS108-6 and pKOS108-15, we were unable to passage the DNA through ET12567 without rearrangement, presumably due to intra-plasmid recombination (ET12567 is a RecA^{+} strain). Therefore, these plasmids were first passaged through *S. lividans* JT46 (*rec46*), a strain deficient in intra-plasmid recombination [15], in order to generate DNA suitable for introduction into *S. coelicolor*.

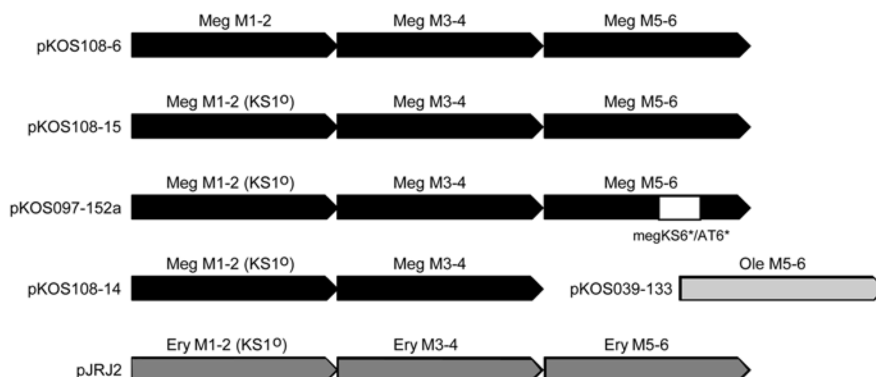

Fig. 3 Engineered PKS plasmids used in this study. Consult text for details

Results from the analysis of plasmid stability, performed as described in the Materials and methods section, are summarized in Fig. 4. By the end of the third round of propagation, an average of only 10% of the plasmids rescued from *S. coelicolor* appeared to be the same as the starting expression plasmid, pKOS108-6 or pKOS108-15. Of the remaining plasmids observed, >90% contained restriction fragments that were consistent with a large deletion of the *meg* PKS genes (Fig. 5). These data are consistent with the belief that, in *S. coelicolor*, plasmids pKOS108-6 and pKOS108-15 undergo recombination between the homologous regions of modules 2 and 6 at a significant frequency. In the case of cultures containing plasmid pKOS108-15, production of metabolite (6-dEB) as a contributing factor for plasmid instability can be ruled out, since the KS1° mutation renders the PKS inactive.

Gene synthesis to alter codon usage of the *megAIII* gene

To prevent homologous recombination in the *meg* DEBS expression plasmids, the DNA coding sequences for the KS and AT regions of module 6 that are identical to module 2 were changed with the synthesized DNA fragments (*meg*KS6*, *meg*AT6*) shown in Fig. 2. These nucleotide changes maintain the exact amino acid sequence of the natural *meg* DEBS protein, but reduce the DNA sequence identity from 100% to 70%, with the longest contiguous segment of identity being 121 bp. The final expression plasmid, pKOS97-152a (Fig. 3), is identical to pKOS108-15, except for the altered sequences shown in Fig. 2 and the substitution of ~0.4 kb of DNA downstream of *megAIII* in pKOS108-15 with a λ *cos* site (used to facilitate plasmid construction).

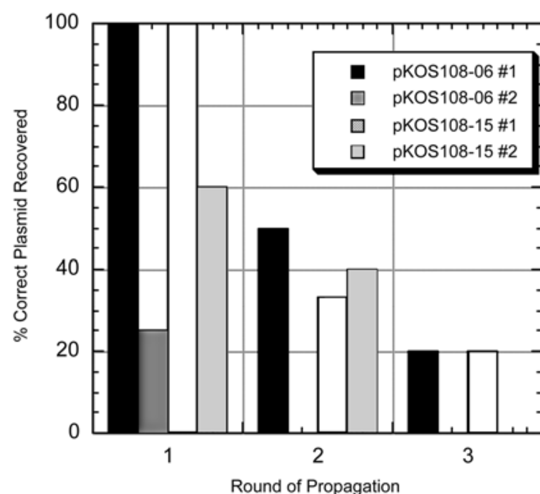

Fig. 4 Frequency of *meg* expression plasmid rearrangement in *S. coelicolor*. Two independent primary transformants each of *S. coelicolor* CH999/pKOS108-06 (*meg* DEBS) and *S. coelicolor* CH999/pKOS108-15 [*meg* DEBS (KS1°)] were analyzed as described in Materials and methods

Plasmid pKOS097-152a was introduced into *S. coelicolor* CH999 as above. However, unlike pKOS108-15, it was possible to generate unmethylated plasmid DNA using *E. coli* ET12567 without detectable rearrangement. *S. coelicolor* CH999/pKOS097-152a transformants were propagated and checked for plasmid stability in a manner similar to the above procedure for pKOS108-15. Based on restriction enzyme analysis and comparison with the authentic starting plasmid, there appeared to be no rearrangement of pKOS097-152a after four rounds of propagation in *S. coelicolor* CH999. Thus, the altered coding sequences in module 6 of *meg* DEBS was sufficient to prevent plasmid rearrangement due to intraplasmid recombination. This was further supported by reproducible measurement of polyketide titers from this strain.

Subunit complementation from heterologous PKSs

A method for hybrid PKS construction was recently described in which protein subunits from related PKS families were used to form heterologous PKS complexes with full complementation of enzymatic activities [14]. For example, the third subunit of the *ery* DEBS and oleandomycin PKSs (DEBS3 and OleAIII, respectively, each encoding modules 5 and 6 of their PKSs) were co–expressed with the first two subunits of the picromycin PKS (PikAI and PikAII, encoding modules 1–4 of the picromycin PKS) to generate hybrid 14-membered macrolactones. We therefore decided to implement this strategy as an alternative approach to codon engineering, to overcome the plasmid instability of pKOS108-15.

The OleAIII subunit was used to replace *meg* DEBS3, even though *ery* DEBS3 has greater homology to *meg* DEBS3, to perform a more rigorous test of heterologous subunit complementation. Plasmid pKOS039-133, used in prior complementation experiments with the picromycin PKS, encodes OleAIII (Fig. 3) [14]. This vector was combined with the replicating plasmid pKOS108-14, identical to pKOS108-15 except *megAIII* is deleted, in *S. coelicolor* CH999. Since pKOS039-133 is a chromosomal integrating vector, analysis of plasmid stability was performed for pKOS108-14. After four rounds of propagation, there was no evidence of plasmid rearrangement in pKOS108-14, when analyzed by the above method, and the strain produced polyketide with reproducible titers.

Analysis of 6-dEB production from engineered PKSs

Several analogues of 6-dEB have been generated by precursor directed biosynthesis in which chemically synthesized *N*-acyl cysteamine thioester diketides are fed to *S. coelicolor* CH999 expressing *ery* DEBS (KS1°) [3] (reviewed in [9]). Initially, we wished to examine the relative efficiencies of diketide processing between *meg*

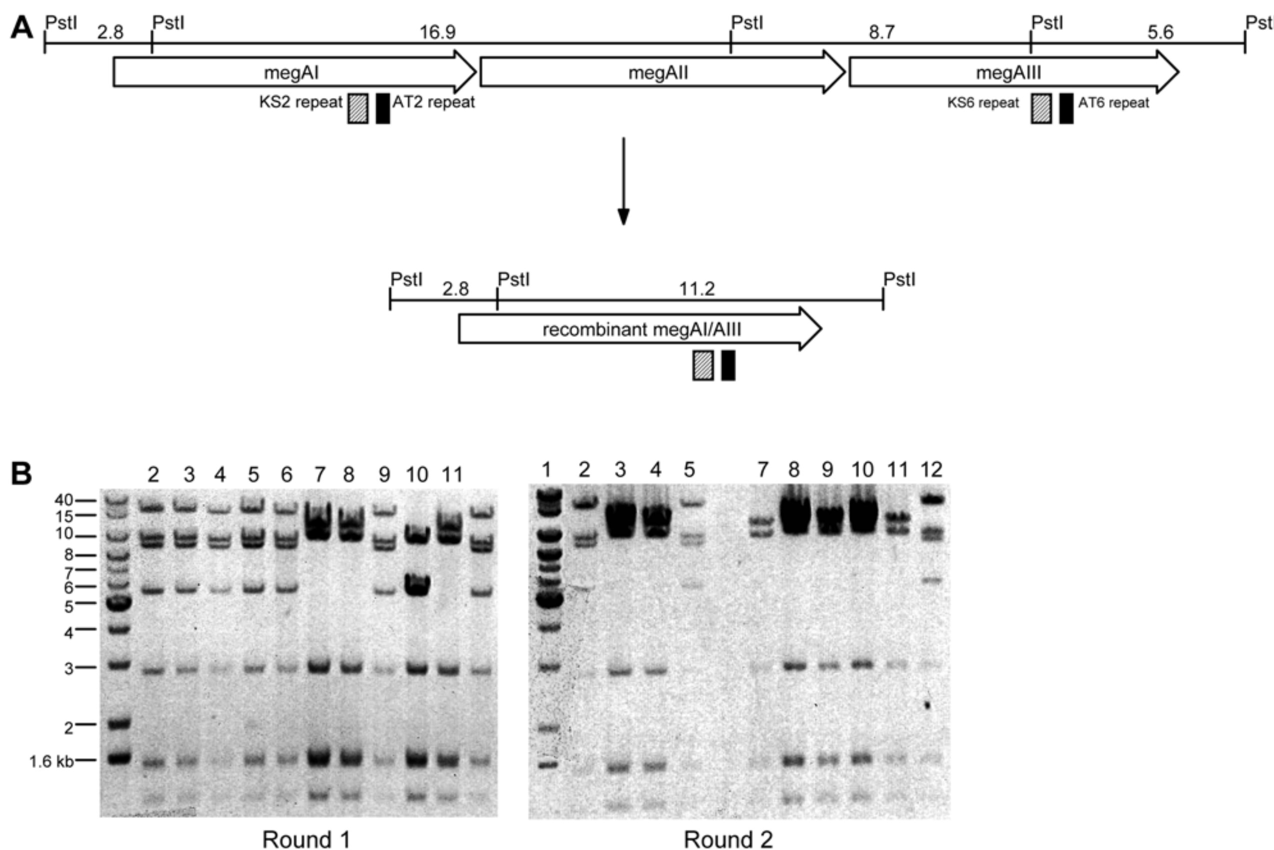

Fig. 5A, B Instability of the *meg* DEBS expression plasmid in *S. coelicolor*. **A** Recombination between the module 2 and 6 repeats of *megAI* and *megAIII* results in the collapse of the 16.9-kb, 8.7-kb, and 5.6-kb *Pst*I fragments to a single 11.2-kb *Pst*I fragment. **B** *Pst*I restriction analysis of plasmids rescued from *S. coelicolor* CH999/pKOS108-06 after one and two rounds of propagation, indicating a recombination event between modules 2 and 6 in some of the plasmids (lanes 7, 8, 11 in Round 1; lanes 3, 4, 7–11 in Round 2). Lane 12 contains control DNA from plasmid pKOS108-06 prior to transformation. A low frequency of unknown rearrangement (lane 10 in Round 1) was also observed but did not increase with time and is therefore thought to occur during the rescue process

DEBS and *ery* DEBS but were prohibited by the plasmid instability of *meg* DEBS. The above strains, all containing the same KS1 mutation as *ery* DEBS (KS1°) and under the same regulatory elements [8], now afforded an opportunity to evaluate diketide precursor incorporation by *meg* DEBS.

Shake-flask fermentations were conducted with the engineered *meg* DEBS and *meg*/*ole* hybrid in the presence of racemic diketide-SNPC substrate (diketide **1**, Fig. 1 and its **2**,**3** enantiomer). Results are summarized in Table 1. Both strains produced 15-methyl-6dEB, with the *meg*/*ole* hybrid PKS consistently yielding the highest titers. The higher titer observed with the hybrid PKS complex [(CH999/pKOS108-14 + pKOS39-133) compared with the wild type *meg* DEBS complex (CH999/pKOS97-152a)] cannot be currently rationalized, although considerable differences in expression of the wild-type *megAI*, *megAII*, and *megAIII* genes have been observed in *E. coli* (S. Murli, personal communication). However, the titers of both strains are comparable with those of *ery* DEBS (KS1°) in the same host and under the same conditions (Table 1) and suggest that the diketide incorporation efficiency of *meg* DEBS is similar to that of *ery* DEBS.

The stability and high titer of the *S. coelicolor* CH999/pKOS108-14/pKOS039-133 strain allowed us to examine production from large-scale cultivation. Peak titers of 15-methyl-6dEB in 5-l fermentations conducted with glucose feeding and maintenance of diketide concentration were similar to those obtained in the shake-flask experiments. This experiment was performed in duplicate and suggests that expression of the PKS is reproducible and the plasmids are stable.

Table 1 Comparison of polyketide titers for polyketide synthase (*PKS*) expressed in *Streptomyces coelicolor* CH999. Fermentations were conducted in shake-flasks fed with diketide **1** in Fig. 1. See Materials and methods for PKS subunit details

Plasmid(s)	PKS subunits	15-Methyl-6-dEB titer (mg/l)
pKOS097-152a	*meg* DEBS1(KS1°), *meg* DEBS2, *meg* DEBS3*	22
pKOS108-14 + pKOS039-133	*meg* DEBS1(KS1°), *meg* DEBS2, OleAIII	43
pJRJ2	*ery* DEBS1(KS1°), *ery* DEBS2, *ery* DEBS3	30

Conclusions

Two distinct methods are described for engineering PKSs in order to prevent potential homologous recombination events in expression plasmids; and both techniques appear to be useful for such purposes. The high titer of the *meg/ole* hybrid PKSs further reinforces this previously described technique as a beneficial tool for PKS engineering [14]. However, since not all PKSs have known or isolated homologues that can be utilized for efficient subunit complementation, the approach of codon reengineering may be of more general utility.

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