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Analysis of Type II Polyketide β-Ketoacyl Synthase Specificity in *Streptomyces coelicolor* A3(2) by *trans* Complementation of Actinorhodin Synthase Mutants

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Complementation of defined actinorhodin β-ketoacyl synthase (KS) mutants by various other KS genes suggested that the ORF1-encoded KS may be relatively generalized in function, whereas the ORF2-encoded KS component may provide specificity in polyketide chain construction. Evidence for differential temporal-spatial expression of the actinorhodin and spore pigment KSs in *Streptomyces coelicolor* was obtained.

Significant recent progress in the cloning and analysis of polyketide synthase (PKS) genes from members of the genus *Streptomyces* and related bacteria (11, 12) has been made. This includes type I (multifunctional) and type II (multicomponent) PKS systems. The type I PKSs specify the construction of macrolide antibiotics and can utilize a range of different precursor starter and extender unit carboxyl coenzyme A (CoA) esters for carbon chain assembly (4, 7, 12). In contrast, the type II PKS systems so far investigated specify the construction of a diverse class of aromatic metabolites, which are derived from a more limited range of starter units (usually acetyl-CoA) and seven, eight, or nine malonyl-CoA extender units (2, 3, 5, 8, 11, 16, 23). Molecular genetic analysis of several type II PKS systems has shown that the earliest biosynthetic steps (i.e., carbon chain construction) are catalyzed by three gene products, named *actI*-ORF1, *actI*-ORF2, and *actI*-ORF3 in the actinorhodin model system of *Streptomyces coelicolor* A3(2) (Fig. 1). One of these (the ORF1 product) resembles closely the *Escherichia coli fabB* KS, including an appropriately positioned cysteine active site residue (13). Another related gene product (that of ORF2) appears to be translationally coupled in most type II PKSs and also resembles the *E. coli fabB* KS, but the active-site Cys has been replaced by another amino acid (glutamine). It has been suggested that a heterodimeric KS consisting of the ORF1 and ORF2 products is required for polyketide biosynthesis in *Streptomyces* spp. (2, 23). An acyl carrier protein (ACP), the product of ORF3, is also required for a functional type II PKS. This protein also closely resembles its *E. coli* fatty acid synthase (FAS) counterpart, in addition to a variety of plant type II FAS ACPs (11).

In a previous study, we showed that it is possible to complement, in *trans*, PKS mutations corresponding to *actI*-ORF1 and *actI*-ORF2, as well as mutations in *actIII* (ORF5) and *actVII* (ORF4), which encode the polyketide ketoreductase and cyclase, respectively, by the corresponding homologous genes from the *gra* cluster (Fig. 1), cloned onto low-copy-number plasmids (22). Additional studies involving in *cis* replacements of the *actI*-ORF3 gene showed that its gene product could be successfully replaced as well by the products of several other type II ACP genes, to form functional hybrid PKSs (14, 15). Here, we describe further studies, using the *trans* complementation strategy, of type II KS genes (Fig. 1) from the *Streptomyces rimosus* oxytetracycline (*otc*) (16) and *S. coelicolor* spore pigment (*whiE*) (5) PKSs, the FAS of *E. coli* (*fabB* [13]), and the type I rat FAS (KS gene domain) (1, 21). The results provide further insight into the functional specificity of type II PKS KS components and may provide a basis for defining the functional relationships between FAS and PKS KSs.

Transformation of *act* KS mutants by low-copy plasmids containing PKS KS genes. In order to determine the ability of the *otcY*-ORF1, *otcY*-ORF2, *whiE*-ORFIII, and *whiE*-ORFIV genes to complement *actI*-ORF1 or *actI*-ORF2 mutations, individual genes were cloned as described in Table 1. In each case, a DNA fragment containing the gene of interest (Fig. 1) was inserted into the single-copy SCP2*-derived *Streptomyces* vector pIJ941 (17) and introduced into *Streptomyces lividans* by transformation. The cloning sites and fragment orientations in pIJ941 had all been shown previously to allow expression of cloned genes from apparently constitutive vector promoters (1a, 22). Clones containing recombinant plasmids were identified by using colony hybridization (9). Following large-scale isolation of the plasmids, transformation of the *S. coelicolor actI*-ORF1 (B60) and *actI*-ORF2 (B78) mutant strains (22) was performed. Transformants containing the desired plasmid construct were identified by selection with hygromycin or thiostrepton (for the appropriate plasmid marker, see Table 1) and confirmed by restriction analysis of isolated plasmid DNA. Plates containing primary transformants were incubated at 30°C for 2 to 3 weeks, at which time individual colonies were scored for the presence of blue diffusible pigment (diagnostic for actinorhodin). These experiments showed that *otcY*-ORF1 and *whiE*-ORFIII were capable of replacing functionally the *actI*-ORF1 gene in the *S. coelicolor* B60 mutant strain, but not the *actI*-ORF2 mutant B78 (Table 2).

On the other hand, *otcY*-ORF2 and *whiE*-ORFIV gene constructs inserted into the *S. coelicolor* B60 (*actI*-ORF1) and B78 (*actI*-ORF2) mutant strains did not lead to detectable actinorhodin production (Table 2) even after prolonged incubation of the primary transformants. These results are significant because they suggest, along with our previous data on functional replacement of *actI*-ORF1 and *actI*-ORF2 with the corresponding *graI*-ORF1 and *graI*-ORF2 genes (in which both the *actI*-ORF1 and *actI*-ORF2 mutations were comple-

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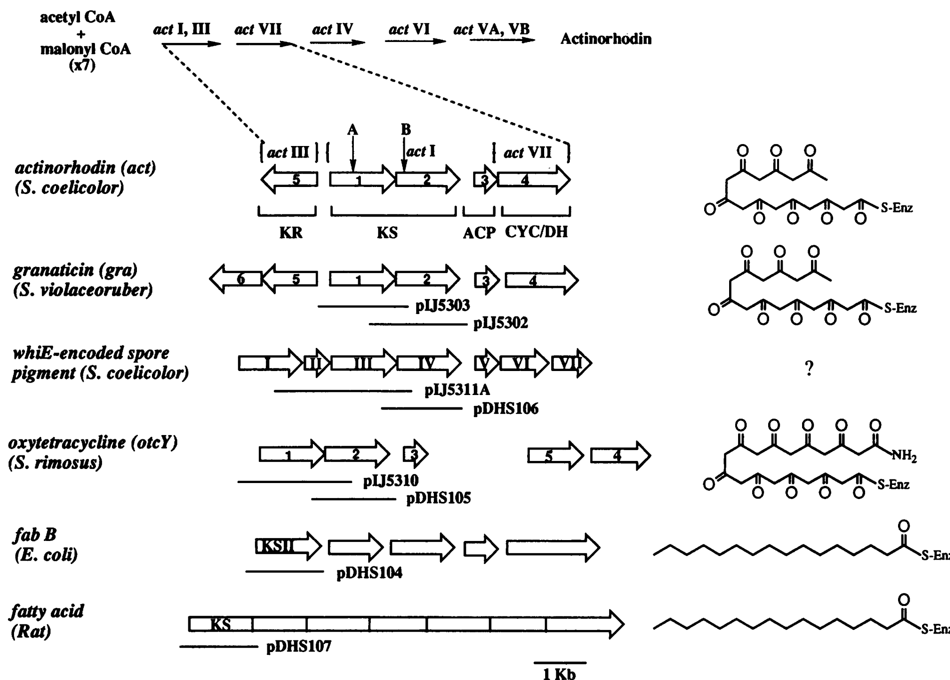

FIG. 1. DNA fragments of heterologous PKS and FAS genes used in the pIJ941-based constructs for *trans* complementation of *actI*-ORF1 (B60) and *actI*-ORF2 (B78) mutants. Open arrows refer to open reading frames (ORFs), and the lines refer to the specific segments of KS DNA used for individual constructs. A and B refer to the locations of the mutations in the *actI*-ORF1 (B60) and *actI*-ORF2 (B78) mutants of *S. coelicolor*, respectively (22). See Table 1 for additional details on the cloning of individual constructs. At the top is shown the sequence of action of the actinorhodin biosynthetic genes. To the right of each set of open arrows is shown the putative oligoketide or saturated fatty acid product: *act*, octaketide; *gra*, octaketide; *whiE*, unknown; *otcY*, decaketide; *fabB*, palmitic acid; Rat (*RATFAS*), palmityl-CoA. KR, ketoreductase; ACP, acyl carrier protein; CYC/DH, cyclase/dehydrase; Enz, enzyme.

mented by the corresponding *gra* open reading frame [ORF] [22]), that the *actI*-ORF1 gene product can be replaced by a range of type II PKS ORF1 homologs. In contrast, with the sample of PKS KS ORF2 genes used, *actI*-ORF2 can be replaced with *graI*-ORF2 only.

The finding of complementation of the *actI*-ORF1 mutation by the *whiE*-ORFIII gene is also significant in a developmental context. Under its natural developmental regulation in the *S. coelicolor* chromosome, the *whiE*-ORFIII product evidently cannot compensate for a mutation in *actI*-ORF1, because such mutants are devoid of actinorhodin and its pathway intermediates and shunt products (20). The result reported here establishes that this is not due to a lack of ability of the *whiE*-ORFIII protein to associate productively with the remaining components of the *act* PKS, and instead implicates a differential temporal-spatial control as the explanation.

Transformation of *act* KS mutants by low-copy plasmids containing FAS KS genes. Because of the relatively high degree of sequence similarity between *actI*-ORF1 and other PKS and FAS KS genes, we sought to determine whether *actI*-ORF1 could be functionally replaced by the *fabB* KS from *E. coli* (6, 13) or by the KS domain from rat FAS (1, 21). In order to clone the *fabB* gene into pIJ941, a product generated by PCR (24) that contained *Eco*RV termini for cloning into the thiostrepton resistance gene (*tsr*) of this SCP2*-derived vector (Fig. 1) was isolated. Initially, cloning of the *fabB* gene in pDHS104 was performed in *S. lividans*; following large-scale plasmid isolation and purification, pDHS104 was then intro-

TABLE 1. Mutant strains and plasmids

Strain or plasmid[a]	Description (plasmid selection marker)[b]
B60	*S. coelicolor* A3(2) 1190 *actI*-ORF1 mutant with a C deletion in the 5′ region of *actI*-ORF1 (bp 636)
B78	*S. coelicolor* A3(2) 1190 *actI*-ORF2 mutant with a GC deletion in the 5′ region of *actI*-ORF2 (bp 122)
pIJ5302	pIJ941 derivative containing the *graI*-ORF2 gene cloned into the *tsr Eco*RV site (*hyg*)
pIJ5303	pIJ941 derivative containing the *graI*-ORF1 gene cloned into the *Bam*HI-*Bgl*II site (*tsr*)
pIJ5310	pIJ941 derivative containing the *otcY*-ORF1 gene (344[b]/482[c])* cloned between the *Pst*I and *Eco*RI sites (*tsr*)
pIJ5311A	pIJ941 derivative containing the *whiE*-ORFIII gene (1155[b]/388[c])* cloned into the *Pst*I site (*tsr*)
pDHS104	pIJ941 derivative containing the *E. coli fabB* gene (40[b])* cloned into the *tsr Eco*RV site (*hyg*)
pDHS105	pIJ941 derivative containing the *otcY*-ORF2 gene (323[b]/62[c])* cloned into the *tsr Eco*RV site (*hyg*)
pDHS106	pIJ941 derivative containing the *whiE*-ORFIV gene (44[b]/72[c])* cloned into the *tsr Eco*RV site (*hyg*)
pDHS107	pIJ941 derivative containing the KS domain of the *RATFAS* gene (47[d])** cloned into the *tsr Eco*RV site (*hyg*)

[a] B60, B78, pIJ5302, and pIJ5303 are described in reference 22; the other plasmids were constructed in this study.

[b] In addition to the entire open reading frame, each construct marked with a single asterisk contains additional nucleotides upstream of the start codon ([b]) and downstream of the stop codon ([c]). A double asterisk indicates that, in addition to the entire predicted KS domain, additional nucleotides upstream of the start codon ([d]) are included.

TABLE 2. Complementation results

Construct used for *trans* complementation	Functional replacement of:	
	actI-ORF1 mutant (B60)	*actI*-ORF2 mutant (B78)
pIJ941 (plasmid vector)	−	−
pIJ5303 (*graI*-ORF1)	+	−
pIJ5310 (*otcY*-ORF1)	+	−
pIJ5311A (*whiE*-ORFIII)	+	−
pDHS104 (*E. coli fab*B)	− and +[a]	−
pDHS107 (*RATFAS*/KS)	−	−
pIJ5302 (*graI*-ORF2)	−	+
pDHS105 (*otcY*-ORF2)	−	−
pDHS106 (*whiE*-ORFIV)	−	−

[a] Denotes complementation observed in transconjugants of *S. coelicolor* B60 from a mating with *S. lividans*/pDHS104 (10).

duced by transformation into the *actI*-ORF1 (B60) and *actI*-ORF2 (B78) mutants of *S. coelicolor*. Transformants were selected by using hygromycin, and positive clones were confirmed by colony hybridization, plasmid isolation, and restriction mapping. For the rat FAS KS domain, the appropriate region of the complete rat FAS was generated by PCR from plasmids containing two segments of the KS gene (1, 25). The limits of the domain were predicted from computer-assisted comparison and alignment of the peptide sequences of several type II PKS KSs and the *fabB* KS, as well as the type I rat FAS (1, 21, 25) sequence. The reconstructed rat FAS KS domain was cloned into the *Eco*RV site of *tsr* in pIJ941 (Fig. 1). Following initial isolation from *S. lividans*, pDHS107 was isolated and used to transform the B60 and B78 mutant strains of *S. coelicolor*. The presence of the plasmids in the transformants was confirmed by using colony hybridization and plasmid DNA analysis. Neither pDHS104 nor pDHS107 transformants of B60 or B78 produced any blue diffusible pigment characteristic of actinorhodin even after 2 to 3 weeks of incubation (Table 2). The transformation result with pDHS104 was surprising since conjugation of an *S. lividans* strain bearing pDHS104 with the *S. coelicolor* B60 mutant had given transconjugants that produced blue diffusible pigment (10). We cannot explain the discrepancy in results between the transformation and conjugation methods with this plasmid. However, the transformation result was repeated several times, always with a clear negative result.

Comparison and analysis of KS protein sequences from PKS and FAS systems. We used the Intelligenetics program UPGMA to generate pairwise alignments between deduced protein sequences of PKS KS ORF1 and ORF2 and FAS KS gene products (Fig. 1 and 2). The PKS KS ORF1-encoded proteins are most closely related, with *act*, *gra*, and *otc* most similar to each other; these three are also the most similar of the ORF2 gene products. Another important aspect of this analysis is the significant divergence of the two FAS KS sequences (*fabB* and rat FAS KS domain) from the PKS (*act*, *gra*, *otc*, and *whiE*) KS sequences (as well as the divergence of the two FAS sequences from each other). It is clear that it is the most highly divergent KS sequences that fail to complement the *actI*-ORF1 or *actI*-ORF2 PKS genes.

The approach described herein, as well as those reported previously (22), depends on actinorhodin production (blue diffusible pigment) as the phenotypic marker for functional complementation. It is possible that nonpigmented aromatic molecules are being produced from hybrid polyketide synthases in the noncomplemented (and complemented) mutants generated in this study. However, the difficulty of purifying and

FIG. 2. Intelligenetics UPGMA tree analysis of PKS and FAS KS genes used for *trans* complementation of the *actI*-ORF1 and *actI*-ORF2 mutants. The deduced amino acid sequences of related ORFs that encode PKS (12) or FAS KS enzymes were compared by using the UPGMA algorithm. The primary nucleotide sequences encode the following products: *act*, actinorhodin (*S. coelicolor* [8]); *gra*, granaticin (*Streptomyces violaceoruber* [23]); *otc*, oxytetracycline (*S. rimosus* [16]); *whiE*, spore pigment (*S. coelicolor* [5]); *fabB*, FAS β-ketoacyl synthase II (*E. coli* [13]); and RATFAS, rat FAS KS domain (1, 21). The parameters for the UPGMA tree analysis have the following default values for protein: cost to open a gap, 5; cost to lengthen a gap, 25; minimum diagonal length, 4; and maximum diagonal length, 10.

quantifying actinorhodin from the complemented mutants precluded a complete analysis of polyketide metabolites produced in the *S. coelicolor* mutant strains transformed with plasmids containing PKS or FAS KS genes. Despite this limitation, it is possible to conclude that the type II PKS KS ORF1 gene product is more general in its ability to mediate polyketide chain construction in the *actI*-ORF1 mutant strain. Lack of complementation of the *actI*-ORF2 KS mutant by heterologous PKS KS ORF2 genes may suggest that this gene product plays a more highly specialized role in generating a functional condensing enzyme or a completely functional multicomponent PKS. Indeed, a recent study suggests (19) that the type II KS ORF2 gene product may play a role in determining polyketide chain length. However, additional studies are required to establish a complete set of general rules for polyketide chain construction and metabolite structural identity determined by the diverse range of multicomponent PKS systems.

A more penetrating approach that includes direct molecular analysis of metabolites produced by the construction of engineered PKS pathways using a replacement strategy in the actinorhodin (16a, 18, 19) and oxytetracycline (16) PKS systems is now being developed. This work provides a straightforward assay for functionality of hybrid PKSs by structural determination of novel products or by analysis of aloesaponarin II and its carboxylated analog, actinorhodin pathway shunt products which can be easily quantified. Ultimately, we expect these strategies to provide important information on the functional roles for individual genes, protein domains, and amino acid residues for starter unit choice, chain elongation, and polyketide processing in type II PKS complexes.

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