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Functional Replacement of Genes for Individual Polyketide Synthase Components in *Streptomyces coelicolor* A3(2) by Heterologous Genes from a Different Polyketide Pathway

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***Streptomyces coelicolor* A3(2) and *Streptomyces violaceoruber* Tü22 produce the antibiotics actinorhodin and granaticin, respectively. Both the aglycone of granaticin and the half-molecule of actinorhodin are derived from one acetyl coenzyme A starter unit and seven malonyl coenzyme A extender units via the polyketide pathway to produce benzoisochromane quinone moieties with identical structures (except for the stereochemistry at two chiral centers). In *S. coelicolor* and *S. violaceoruber*, the type II polyketide synthase (PKS) is encoded by clusters of five and six genes, respectively. We complemented a series of *S. coelicolor* mutants (*act*) defective in different components of the PKS (*actI* for carbon chain assembly, *actIII* for ketoreduction, and *actVII* for cyclization-dehydration) by the corresponding genes (*gra*) from *S. violaceoruber* introduced in *trans* on low-copy-number plasmids. This procedure showed that four of the *act* PKS components could be replaced by a heterologous *gra* protein to give a functional PKS. The analysis also served to identify which of three candidate open reading frames (ORFs) in the *actI* region had been altered in each of a set of 13 *actI* mutants. It also proved that *actI*-ORF2 (whose putative protein product shows overall similarity to the β-ketoacyl synthase encoded by *actI*-ORF1 but whose function is unclear) is essential for PKS function. Mutations in each of the four complemented *act* genes (*actI*-ORF1, *actI*-ORF2, *actIII*, and *actVII*) were cloned and sequenced, revealing a nonsense or frameshift mutation in each mutant.**

The polyketides are derived from simple carboxylic acid precursors by a biosynthetic mechanism similar to that of the fatty acids (3, 16). One of the most compelling reasons for studying polyketide biosynthesis is to elucidate the genetic and biochemical variables of the polyketide synthase (PKS) that determine the huge variety of product structures. Interestingly, the organization of the erythromycin PKS (a type I system) (4, 5) suggests an overall mechanistic strategy for the construction of macrolide chains. There appears to be a linear correspondence between the functional modules of the enzyme and the chemical reactions required on the growing acyl substrate along the three multifunctional PKS proteins. In type II systems, on the other hand, a clear mechanistic explanation for the construction of the polyketide chain does not exist. Unlike the erythromycin system, type II systems require that specific proteins (at least the β-ketoacyl synthase and also acyl carrier protein [ACP]) in the multicomponent PKS accept the products of successive steps in chain elongation as substrates during each round of chain building. It is this aspect of polyketide biosynthesis that has become one of the major challenges; i.e., which PKS components in type II systems contribute to the overall structure of the polyketide molecule, and how does this control (or programming) take place?

The benzoisochromane quinone class of antibiotics produced by *Streptomyces* spp. represents a system favorable for answering these questions. Several compounds of this structural type, including actinorhodin (*Streptomyces coelicolor*) (10), granaticin (*Streptomyces violaceoruber*) (30), frenolicin (*Streptomyces roseofulvus*) (6), and griseusin (*Streptomyces griseus*) (31), have been characterized. The actinorhodin half-molecule and the aglycone of granaticin are each derived from an identical set of precursor units (eight acetate equivalents), whereas frenolicin and griseusin contain additional carbon atoms compatible with derivations from nine and ten acetate equivalents, respectively; there are also differences in the patterns of reduction of the keto groups of the growing polyketide chains. We plan to construct hybrid clusters between members of the gene sets for these compounds in order to implicate specific PKS components in aspects of the programming. The PKS gene clusters for actinorhodin (8) and granaticin (20, 29) are highly related, consisting of five and six genes, respectively (Fig. 1). They have an overall nucleotide identity of 70 to 75% over the various open reading frames (ORFs), while the similarity of the deduced protein sequences varies considerably (*act*/*gra* ORF identities, 76.4, 59.5, 62.2, 54.2, and 73.3% for ORF1 through -5, respectively). These two gene clusters, therefore, provide an excellent starting point for determining whether combinations of PKS components from two different sets could associate to produce a functional hybrid enzyme without also requiring the PKS to generate a chemically hybrid product.

In this paper, we show that genes encoding components of the actinorhodin PKS can indeed be substituted by homologous genes from the granaticin system. The approach was to complement actinorhodin PKS mutations in *trans* by cloned genes from the granaticin PKS cluster. Of the set of 76 *S. coelicolor act* mutants isolated after UV irradiation on the basis of their failure to produce the characteristic blue color of actinorhodin, 13 were defined as *actI* mutants (Table 1), deduced to be blocked at the earliest steps of the actinorhodin biosynthetic pathway by their pattern of cosynthesis

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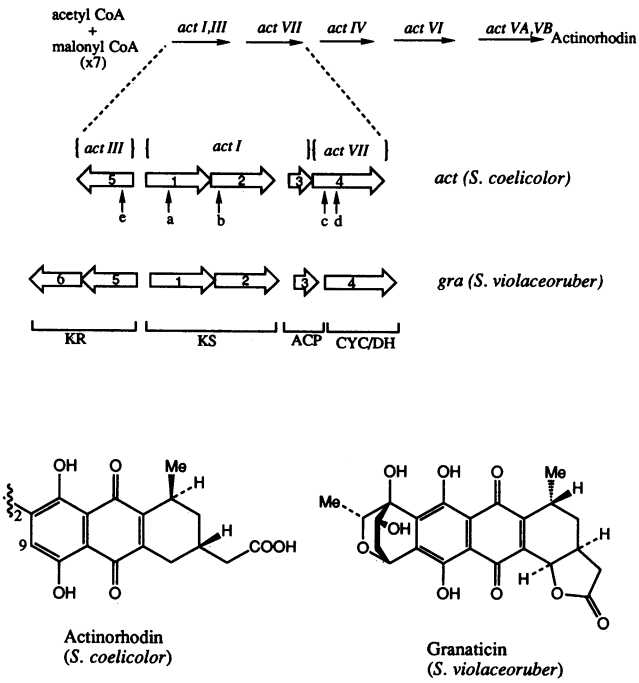

FIG. 1. Comparison of the organization and structure of the *S. coelicolor act* and *S. violaceoruber gra* PKS gene clusters and of the molecular structures of actinorhodin and granaticin. Horizontal black arrows, the sequence of blocks in the actinorhodin biosynthetic pathway identified by biochemical genetic analysis (21–23). Open arrows, genes identified previously (8, 29) that are involved in early steps of polyketide chain construction. CoA, coenzyme A; KS, β-ketoacyl synthase; CYC/DH, cyclase-dehydrase; KR, ketoreductase. Vertical black arrows, locations of mutations identified by DNA sequencing of the relevant *S. coelicolor actI*-ORF1 and -2, *actVII* (ORF4), and *actIII* (ORF5) genes. a, B60 frameshift mutation; b, B78 frameshift mutation; c, B140 frameshift mutation; d, B40 nonsense mutation; e, TK18 frameshift mutation.

with other mutant classes and failure to produce any pigmented actinorhodin precursors or shunt products (23). These *actI* mutants were proven by complementation analysis (22) to fall in a region recently shown to contain three ORFs (*actI*-ORF1 through -3) (Fig. 1) (8). A mutation in any one of these genes (*actI*-ORF1 and -2, encoding the putative heterodimeric β-ketoacyl synthase, and *actI*-ORF3, encoding ACP) would, in principle, result in an actinorhodin-negative phenotype representing that of the early blocked *actI* mutants. Previous work also characterized two *S. coelicolor actVII* mutant strains (B40 and B140) (23). Both produce a shunt product, mutactin (18a, 34), which contains the same number of carbon atoms (16 atoms) as the actinorhodin half-molecule but which has undergone an aberrant intramolecular aldol condensation and has failed to undergo dehydration in a separate region of the molecule. The characterization of mutactin (34) and subsequent molecular genetic analysis (28) revealed that the gene (ORF4) encoded in the *actVII* region presumably specifies two enzymatic steps: cyclization, to give the proper ring structure (catalyzed by the N-terminal region of the gene product), and dehydration, which allows complete aromatization of the nascent benzoisochromane quinone system (catalyzed by the C-terminal region). The remaining *act* PKS gene encodes a polyketide ketoreductase (DNA sequence analysis revealed an ORF whose product has high similarity to other oxidoreductases [12]) that specifies the reduction of the carbonyl group at C-9 of the actinorhodin polyketide chain (1). Seven *actIII* mutant strains were recognized among the original set of *act* mutants (23), and of these seven, a strain carrying the mutation *act-141* was chosen for detailed study.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids. *S. coelicolor actI*, *actVII*, and *actIII* mutants and *Streptomyces lividans* 66 (John Innes Institute strain 1326) or its derivative TK64 (*pro-2 str-6* SLP2$^-$ SLP3$^-$) (14) were used as hosts for plasmids derived from the low-copy-number vector pIJ941 (19). Agar media were R2 or R2YE (R2 with yeast extract) and minimal medium or, in some cases, minimal medium with mannitol (0.5%) instead of glucose as the carbon source to enhance sporulation (14). Conditions for culture, transformation, and conjugal transfer of *Streptomyces* plasmids were as described previously (14). *Escherichia coli* DH5α and JM101 were the hosts for the pUC plasmids and the M13 phages, respectively (24). *Streptomyces* plasmids were manipulated as described previously (14). pIJ5300 was constructed by isolating the 4.6-kb *Bam*HI fragment from pIJ5200 (29), making it blunt ended, and ligating it into the *Eco*RV site of pIJ941 (19). pIJ5301 was obtained by partial digestion of pIJ5200 with *Bam*HI-*Bgl*II and isolation of the 3.0-kb fragment containing *graI*-ORF1 through -3. This fragment was cloned into similarly digested pIJ941. pIJ5302 (*graI*-ORF2) was constructed by isolating the *Eco*RI-*Hin*dIII fragment from pIJ5206 (derived from cloning the blunt ended 1.3-kb *Nco*I fragment of pIJ5200 into the *Sma*I site of M13mp18), blunt ending, and ligating into the *Eco*RV site of pIJ941. pIJ5303 (*graI*-ORF1) was obtained by total digestion of pIJ5200 with *Bam*HI-*Bgl*II and isolation of the 2.2-kb

TABLE 1. Plasmids used for complementation of *actI*, *actVII*, and *actIII* mutants

Plasmid	Description[a]	Reference
pIJ5300	pIJ941 derivative containing *graI*-ORF1 through -3 and *graVII* (ORF4) cloned into the *tsr Eco*RV site	This study
pIJ5301	pIJ941 derivative containing *graI*-ORF1 through -3 cloned into the *Bam*HI-*Bgl*II site	This study
pIJ5302	pIJ941 derivative containing the *graI*-ORF2 gene cloned into the *tsr Eco*RV site	This study
pIJ5303	pIJ941 derivative containing the *graI*-ORF1 gene cloned into the *Bam*HI-*Bgl*II site	This study
pIJ5304	pIJ941 derivative containing the *graVII* (ORF4) gene cloned into the *tsr Eco*RV site	28
pIJ5305	pIJ941 derivative containing the *graI*-ORF3 gene cloned between the *Pst*I and *Eco*RI sites	This study
pDHS100	pIJ941 derivative containing the *graIII*-ORF5 and -6 genes cloned into the *tsr Eco*RV site	This study
pDHS101	pIJ941 derivative containing the *graIII*-ORF5 gene cloned into the *tsr Eco*RV site	This study
pDHS102	pIJ941 derivative containing the *graIII*-ORF6 gene cloned into the *Bam*HI site	This study

[a] *tsr*, thiostrepton resistance gene.

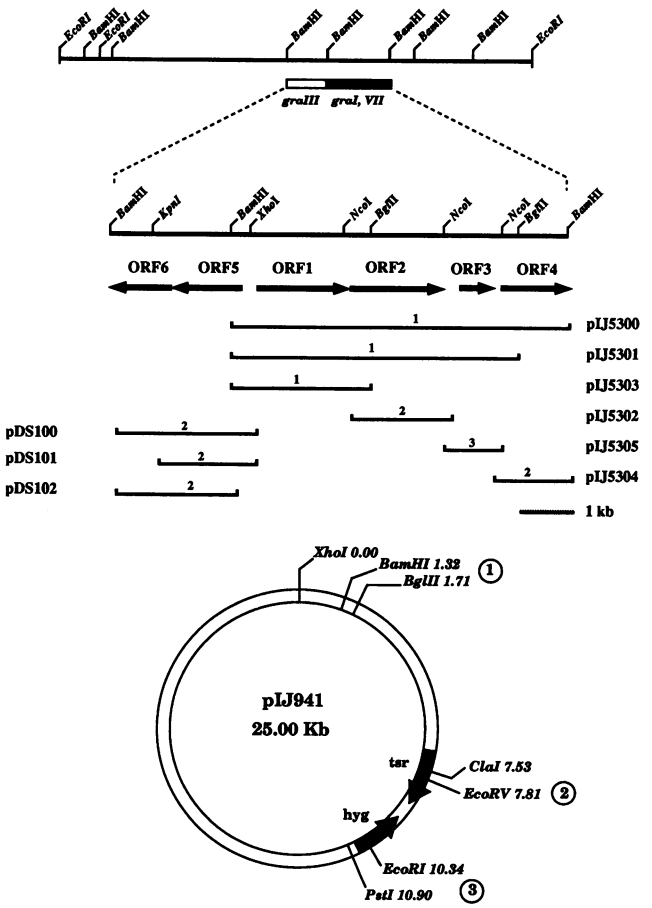

FIG. 2. DNA fragments from the *S. violaceoruber gra* cluster (20, 29) used in pIJ941-based constructs for *trans* complementation of *actI*, *actVII*, and *actIII* mutants. Numbers above the lines refer to the specific site into which each individual fragment was cloned in pIJ941. 1, *Bam*HI-*Bgl*II; 2, *Eco*RV; 3, *Pst*I-*Eco*RI). tsr, thiostrepton resistance gene; hyg, hygromycin resistance gene. See Materials and Methods for specific details on the derivation of each plasmid construct.

fragment containing *graI*-ORF1. This fragment was cloned into pIJ941 digested completely with the same enzymes. pIJ5304 (*graVII* [ORF4]) was obtained by isolating a 1.4-kb exonuclease III deletion fragment from pIJ5315, blunt ending, and ligating into the *Eco*RV site of pIJ941. pIJ5305 (*graI*-ORF3) was derived by isolating the 450-bp *Pst*I-*Eco*RI fragment from pIJ5205 (a blunt ended 450-bp *Nco*I fragment from pIJ5200 cloned into the *Sma*I site of M13mp18) and ligating into similarly digested pIJ941. pDHS100 (*graIII*-ORF5 and -6) was obtained by three-way ligation of the 2.4-kb *Bam*HI fragment from pIJ5201 (29) and the 0.3-kb *Xho*I-*Bam*HI fragment from pIJ5229 into *Sal*I-*Bam*HI-digested M13mp18 (33). The resulting clone was then prepared as a blunt-ended *Eco*RI-*Hin*dIII fragment and ligated into the *Eco*RV site of pIJ941. pDHS101 (*graIII*-ORF5) was constructed by isolating the *Xho*I-*Eco*RI fragment from pIJ5229 (containing a 3.0-kb *Bgl*II-*Eco*RI subclone of pIJ2361 [29]), blunt ending, and ligating into the *Eco*RV site of pIJ941. pDHS102 (*graIII*-ORF6) was derived from the 2.4-kb *Bam*HI fragment of pIJ5201 cloned into similarly digested pIJ941. Plasmids numbered 1 and 2 in Fig. 2 have the *gra* ORFs in the same orientation as the *tsr* gene, whereas plasmid 3 (Fig. 2) had *graI*-ORF3 in the same direction as *hyg*.

DNA isolation. *Streptomyces* plasmid DNA was prepared as described by Kieser (18). The alkaline lysis method (24) was used to prepare plasmids from *E. coli*. Small-scale preparation of *E. coli* plasmids and replicative-form M13 DNA was done by using a modification of the method of Ish-Horowitz and Burke (17).

DNA sequence analysis. Total genomic DNA from each of the representative mutant strains B60, B78, B40, B140, and TK18 was isolated by using the Kirby procedure (14) followed by restriction endonuclease digestion and size selection of the DNA from agarose gels (B60 and TK18 were digested with *Bam*HI for size selection at ~2.2 kb; B78, B40, and B140 were digested with *Pst*I for size selection at ~8.8 kb) and insertion into pUC18 to give minilibraries. Overlapping fragments of the *actI*, *actVII*, and *actIII* mutant genes were cloned in both orientations in either M13mp18 or M13mp19 (33). Oligonucleotide primers were constructed to obtain complete sequence information. Both strands of every clone were sequenced by the dideoxy method (25) to give the complete sequence for the relevant gene in each case. Regions of compression or sequence ambiguity were resolved by using Sequenase (U.S. Biochemicals).

Displacement of SCP2-derived plasmids. The SCP2* neomycin resistance plasmid pIJ80 was chosen for these experiments on the basis of its ability to cure resident SCP2*-based plasmids (27) and to be easily cured itself by virtue of an unstable Neo^r phenotype caused by absence of the partition region of SCP2* (2). Protoplasts from *S. coelicolor act* mutants containing SCP2 plasmids were transformed with pIJ80 and then regenerated and overlaid with neomycin or replica plated onto neomycin-containing R2YE plates after sporulation. Transformants were then replica plated onto a nonselective medium (R2YE) and allowed to sporulate. This procedure was repeated a second time on nonselective medium. After the second round of sporulation, transformants were replica plated onto R2YE containing either neomycin or hygromycin. Colonies that showed sensitivity to both antibiotics were then restreaked onto nonselective medium and tested for production of actinorhodin (blue diffusible pigment).

RESULTS

Complementation of the *S. coelicolor actI* PKS mutants with *S. violaceoruber graI* PKS genes. Our initial goal was to develop an effective method for mapping precisely the genetic lesions in the set of *actI* PKS mutants in *S. coelicolor* by complementation with defined *gra* PKS genes. Two recombinant plasmids carrying *gra* PKS DNA, pIJ5300 and pIJ5301 (Fig. 2), initially generated in *S. lividans* by using the SCP2-derived vector pIJ941 (19), were used to transform the 13 *S. coelicolor actI* mutant strains; both plasmids restored production of blue pigment (with the exception of strain B156, for unknown reasons; perhaps it has a complex mutation or deletion) to levels, as judged by visual inspection, approaching that of the *act*$^+$ strain *S. coelicolor* 1190. The set of three or four genes from the *S. violaceoruber gra* PKS could, therefore, operationally replace the corresponding *S. coelicolor act* PKS for production of an aromatic polyketide similar or identical to actinorhodin, and in the case of pIJ5301, the *graI*-ORF1 through -3 proteins must be able to function with (or at least pass on their products to) the *actIII* and *actVII* proteins.

In subsequent experiments, subclones of pIJ5301 were

TABLE 2. *trans* complementation of *actI*, *actVII*, and *actIII* mutants[a]

Mutant[b]	Results with *gra* ORF[c]:					
	1 to 4 (pIJ5300)	1 to 3 (pIJ5301)	1 (pIJ5303)	2 (pIJ5302)	3 (pIJ5305)	4 (pIJ5304)
actI						
B2 (*act*-102)	+	+	−	−	−	NT
B18 (*act*-118)	+	+	+	−	NT	NT
B60 (*act*-160)	+	+	+	−	NT	−
B65 (*act*-165)	+	+	−	−	−	NT
B78 (*act*-178)	+	+	−	+	−	NT
B89 (*act*-189)	+	+	−	−	−	NT
B91 (*act*-191)	+	+	+	−	NT	NT
B94 (*act*-194)	+	+	+	−	NT	NT
B101 (*act*-201)	+	+	−	−	−	NT
B115 (*act*-215)	+	+	+	−	NT	NT
B137 (*act*-237)	+	+	+	−	NT	NT
B156 (*act*-256)	+	−	−	NT	−	NT
B160 (*act*-260)	+	+	−	−	−	NT
actVII						
B40 (*act*-140)	+	−	−	NT	NT	+
B140 (*act*-240)	+	NT	−	NT	NT	+

[a] *actIII* mutant strain TK18 (genotype *argA1 uraA1 strA1 redE60* SCP1⁻ SCP2⁻) was complemented by *graIII*-ORF5 and -6 (pDS100), *graIII*-ORF5 (pDS101), and *actIII* (ORF5) (pIJ2314) but not by *graIII*-ORF6 (pDS102).

[b] All strains with the prefix B are *act* mutants derived from strain 1190 (*hisA1 uraA1 strA1* SCP1⁻ SCP2⁺ Pgl⁺).

[c] +, complementation (production of blue pigment); −, lack of complementation; NT, not tested.

generated so that individual ORFs of the *graI* region were cloned on separate plasmids (*graI*-ORF1 [pIJ5303], *graI*-ORF2 [pIJ5302], and *graI*-ORF3 [pIJ5305]) in sites and orientations appropriate for transcriptional read-through from vector promoters (Fig. 2). Transformation of the set of 13 *S. coelicolor actI* mutants with pIJ5303 resulted in complementation of 6 of the 13 strains (Table 2). A similar experiment was carried out with the *graI*-ORF2 construct (pIJ5302). In this case, a single *actI* mutant strain (B78) showed a clear restoration of blue-pigment production (Table 2). The *graI*-ORF3 construct (pIJ5305) was then used to transform the subset of *S. coelicolor actI* mutants which were not complemented by pIJ5301 or pIJ5302. No restoration of actinorhodin was observed among the collection of mutants, suggesting that none of the 13 *S. coelicolor actI* mutants contained a lesion in the ACP gene (or that the *gra* ACP could not function along with other *act* PKS proteins). For the work described above, the following set of controls was performed. First, transformation of all the *S. coelicolor actI* mutant strains with pIJ941 alone gave no detectable levels of blue-pigment production. Furthermore, transformation of *S. coelicolor* B60 (an *actI*-ORF1 mutant) with a construct containing *graVII* (ORF4) (pIJ5304) (28) resulted in no detectable production of actinorhodin.

Thus, at least two individual genes from the *S. violaceoruber graI* PKS cluster (ORF1 and -2) can relieve metabolic blocks caused by mutations in the *S. coelicolor actI* PKS. The positive results in these experiments also provided a method for preliminary mapping of specific lesions within the *act* cluster of PKS genes.

Complementation of *S. coelicolor actVII* mutants with *S. violaceoruber graVII* (ORF4). To confirm previous studies (22) that mapped the *actVII* gene to a region immediately downstream of the *actI* PKS cluster of genes (later shown to be in ORF4 [8]), we tested the *S. violaceoruber gra* construct containing ORF1 through -4 (pIJ5300) for its ability to complement the two available *S. coelicolor actVII* mutant strains, B40 and B140. pIJ5300 restored production of blue pigment to both mutants, in contrast to pIJ5301 (carrying ORF1 through -3 only), which caused no detectable change in phenotype (Table 2). To test further whether *S. violaceoruber graVII*-ORF4 alone could replace the homologous *actVII*-ORF4 gene in the *S. coelicolor actVII* mutants, a plasmid (pIJ5304) (28) which contained *graVII*-ORF4 (including the noncoding DNA between *graI*-ORF3 and *graVII*-ORF4) cloned into pIJ941 was constructed (Fig. 2). Transformation (or conjugative plasmid transfer from *S. lividans*) with pIJ5304 resulted in production of significant levels of blue pigment, showing that *graVII*-ORF4 alone was sufficient to relieve the block in the *S. coelicolor* B40 and B140 *actVII* mutant strains. As a control, transformation of B40 and B140 with pIJ941 (or pIJ5303) gave no detectable blue-pigment production (Table 2).

Complementation of the *S. coelicolor actIII* mutant with *S. violaceoruber graIII*. A further set of complementation experiments involved the *graIII* region which encodes the presumed granaticin ketoreductase. In contrast to *actIII*, which contains a monocistronic transcript (12), *graIII* includes two genes (ORF5 and -6) that show overlapping translational stop-start sites (29). Interestingly, the deduced protein sequences of ORF5 and ORF6 show a high degree of similarity to each other and to the deduced protein product of *actIII* (*actIII*/*graIII*-ORF5 identity, 73%), but while the deduced protein sequences of *actIII* (ORF5) and *graIII*-ORF5 have the characteristic NADPH-binding motif (Gly-X-Gly-X-X-Ala) (26, 32), the deduced protein sequence of *graIII*-ORF6 lacks this characteristic motif. Three plasmids which included *S. violaceoruber graIII*-ORF5 and -6 (pDHS100), *graIII*-ORF5 (pDHS101), and *graIII*-ORF6 (pDHS102) cloned into pIJ941 (Fig. 2) were constructed. Each plasmid construct was transferred by conjugation from *S. lividans* 1326 (in which the plasmids were constructed) to the *S. coelicolor actIII* mutant strain TK18. Apparently wild-type levels of blue-pigment production were observed with pDHS100, whereas pDHS101 produced a lower level (probably about half the amount) of pigment. TK18/pDHS102

transconjugants failed to produce detectable levels of blue pigment. As a positive control, pIJ2314, which carries the *actIII* gene (22), was transferred into TK18. As expected, this resulted in wild-type production levels of actinorhodin. A similar experiment with pIJ941 resulted in no detectable actinorhodin production.

Plasmids used to complement *S. coelicolor actI*, *actVII*, and *actIII* mutants can be displaced to restore the actinorhodin-negative phenotype. Since the DNA sequences for *S. coelicolor act* ORF1 through -5 and *S. violaceoruber gra* ORF1 through -5 showed only 70 to 75% overall homology, this should preclude the possibility of homologous recombination leading to replacement of the *act* mutant allele by the wild-type *gra* gene(s). In order to show directly that complementation was occurring in *trans* for each of the examples above, we eliminated the plasmid from a representative of each *S. coelicolor actI*, *actVII*, and *actIII* mutant class by displacing it with the segregationally unstable plasmid pIJ80 (see Materials and Methods). Loss of the antibiotic resistance markers of the plasmids provided a clear assay for successful curing of seven *graI*, *graVII*, and *graIII* pIJ941 plasmids from their hosts: B60/pIJ5303, B115/pIJ5303, B78/pIJ5302, B40/pIJ5304, TK18/pDHS100, and TK18/pDHS101. In all cases, curing of the resident plasmid resulted in restoration of the mutant (actinorhodin-negative) phenotype.

Cloning and sequence analysis of *act* PKS mutations from *S. coelicolor*. In order to determine the precise location and nature of genetic lesions in the *actI*, *actVII*, and *actIII* loci, we cloned and sequenced representatives of the *actI* (ORF1 and -2), *actVII* (ORF4), and *actIII* (ORF5) mutant classes. Size-selected genomic DNAs from B60 (*actI*-ORF1 mutant), B78 (*actI*-ORF2 mutant), B40 and B140 (*actVII* mutants), and TK18 (*actIII* mutant) were inserted into pUC18 (33) to give minilibraries. Screening of each of the minilibraries with the corresponding homologous wild-type gene probe led to positive clones which were then further subcloned into M13mp18 or M13mp19 (33) for DNA sequence analysis. This revealed the precise location and lesion for each of the mutations (Fig. 1). Thus, the mutation in the B60 *actI*-ORF1 is a C deletion in the 5′ region of the gene (bp 636) leading to frameshift and a premature stop codon 188 bp downstream (bp 824 through 826). The B78 *actI*-ORF2 lesion involves a GC deletion at bp 122 through 123 and would lead to a gene product unrelated to wild-type *actI*-ORF2 protein except for the first 40 amino acids. The new reading frame results in a translational stop codon 1,182 bp downstream of the mutation (bp 56 through 58 of the *actI*-ORF3 gene). The *actVII* mutant B40 contains a G-to-A transition at bp 294 of ORF4 which would result in a nonsense mutation in the N-terminal region (codon at bp 292 through 294) of the gene. The *actVII* mutation in B140, on the other hand, is a GC deletion at bp 57 through 58 resulting in a frameshift and a predicted gene product with no similarity to the wild-type *actVII* (ORF4) gene product. The TK18 *actIII* (ORF5) gene contains a G deletion at bp 183, causing a frameshift into a reading frame ending at a termination codon (bp 249 through 251) (Fig. 1).

DISCUSSION

This work introduces a method for the functional dissection of each component of type II PKS multienzyme complexes. Previous studies showed that heterologous complementation can relieve blocks in the actinorhodin biosynthetic pathway (15), but these studies involved imprecisely defined large segments of DNA and late steps in the pathway catalyzed by enzymes that modify the functional groups of the molecule rather than those that build the polyketide carbon chain itself. Here, we used individual genes from a PKS cluster to complement PKS mutations in *trans* in a heterologous host, showing that a functional PKS can be assembled from various mixtures of *act* and *gra* proteins even when these are expressed from different replicons and under different conditions of transcriptional regulation. The *act* genes are normally expressed under strict temporal control (8, 11), whereas in these experiments at least some of the *gra* PKS genes were expressed from unregulated pIJ941 promoters (either of SCP2* or of the cloned *tsr* gene); also, the *act* genes were expressed together in *cis*, whereas the cloned *gra* genes were expressed individually outside of the *act* gene cluster. It is still unclear to what extent the *actI*, *actIII*, and *actVII* PKS gene products and the equivalent *gra* PKS products form a tightly associated complex.

In the complementation experiments involving pIJ5301 (*graI*-ORF1 through -3) and pIJ5300 (*graI*-ORF1 through -3 and *graVII* [ORF4]), the extent of cooperation of the *gra* PKS proteins encoded by the plasmid and the *act* PKS components of the host is ambiguous, but certainly the *act* ketoreductase (the *actIII* product) and, in the case of pIJ5301, also the *act* cyclase-dehydrase (the *actVII* product) would have had to cooperate with the chain-building components of the *gra* PKS (the *actIII* product had already been shown to function heterologously in an anthracycline system [1]). In the other cases, pIJ5303 (*graI*-ORF1), pIJ5302 (*graI*-ORF2), pIJ5304 (*graVII* [ORF4]), pDHS100 (*graIII*-ORF5 and -6), and pDHS101 (*graIII*-ORF5), the only mechanism by which a polyketide product could be produced involved a functional hybrid PKS, in which one *gra* PKS component was operating in concert with the remaining *act* PKS components to construct the molecule. We have recently shown that PKS-condensing enzyme (ORF1) genes from acetate-derived but non-benzoisochromane quinone-producing systems can also complement *S. coelicolor actI*-ORF1 mutants (18a). Thus, the methodology described here should be broadly applicable to a variety of polyketide systems.

A second goal in these studies was to identify individual mutant genes within the early blocked *act* mutant strains (23) by using *trans* complementation with corresponding genes from the *gra* PKS (29). DNA sequence analysis of the mutant genes from each functional class (*actI*, *actVII*, and *actIII*) led to a precise characterization of the genetic lesions. Strikingly, all five of the chosen mutations turned out to be nonsense or frameshift mutations, perhaps reflecting a bias against missense mutations when colonies totally lacking blue pigmentation were originally selected (23). Thus, the *actI*-ORF1 frameshift mutation in *S. coelicolor* B60 would result in a premature stop codon and a truncated gene product. However, this mutation would not be expected to hinder translation of the *actI*-ORF2 gene in B60 because this gene has its own ribosome binding site (8). The *actI*-ORF2 mutation in *S. coelicolor* B78 is a deletion of two adjacent base pairs. The resulting frameshift would disrupt function of the ORF2 gene product but, again, should not hinder translation of downstream genes (ACP and cyclase-dehydrase) in the PKS operon because of their abilities to undergo independent translational initiation (ORF2 and ORF3 are not translationally coupled [8]). These arguments are functionally validated by the ability of *graI*-ORF1 to complement the B60 mutation and the ability of the *graI*-ORF2 gene to complement the B78 mutation. Importantly, these data also reveal that the *actI*-ORF2 product is essential for PKS function since B78 is clearly an ORF2 mutant. In addition, it is not possible to complement the *actI*-ORF2

mutation with the *graI*-ORF1 gene, which provides further evidence that the product of ORF2 is functionally distinct from that of ORF1.

In a separate study (8), 11 of the 13 *actI* mutants were tested for homologous complementation by segments of the *act* PKS DNA. These results can be related to those of the heterologous-complementation tests as follows. (i) The two sets of data agree in identifying no candidates for mutation in the *actI*-ORF3 (ACP) gene. This probably is a consequence of the small size of this gene as a mutational target. It is not due to failure of mutations in *actI*-ORF3 to exhibit an actinorhodin-negative phenotype because genetically engineered *actI*-ORF3 mutations have recently been shown to be Act$^-$ (17a). (ii) Three of the mutations were deduced by the homologous-complementation tests to lie in *actI*-ORF1, and all three are included in the set of six mutants classified as ORF1 mutations by complementation by *graI*-ORF1; another two of these six (B18 and B91) were not included in the tests with *act* DNA, while the sixth mutant gave no blue colonies in the complementation tests with *actI*-ORF1 (8), a discrepancy from the heterologous-complementation results. (iii) While only one clear example of heterologous complementation by *graI*-ORF2 was found (B78), seven other *actI* mutants (as well as B78, and including B156) were deduced to have mutations in ORF2 because they were complemented by *actI*-ORF1 and -2 but not by *actI*-ORF1 alone (8). A possible explanation for the failure of some *actI*-ORF2 mutants to be complemented by *graI*-ORF2 might be an interference of particular mutant ORF2 proteins with the formation of a hybrid PKS containing *actI*-ORF1 and *graI*-ORF2; alternatively, some of the mutants might carry polar mutations in ORF1 or structural changes involving both ORF1 and ORF2.

The complementation of *actVII* mutants B40 and B140 is particularly significant. Control of the intramolecular aldol reactions required for formation of the benzoisochromane quinone ring system, as well as formation of the related anthracyclines and tetracyclines, is an important issue in acetate-derived aromatic polyketide synthesis (13, 28). Previous results provided evidence that the production of mutactin by the *actVII* mutants resulted from spontaneous chemical reactions giving rise to an alternative cyclization product of lower internal energy than that of the normal actinorhodin intermediate (28, 34). This argument is further supported by sequence analysis of *actVII* (ORF4) from the mutant strains B40 and B140. In both cases, genetic lesions have resulted in nonfunctional proteins caused by a nonsense mutation (B40) or a frameshift (B140). Thus, the shunt product, mutactin, must arise by a failure of catalysis of the normal ring closure rather than a modified ring-closing mechanism. (Note that both mutations are in the N-terminal part of the protein and would abolish both functions of the presumed bifunctional enzyme.)

Results from the complementation of the *S. coelicolor* *actIII* mutant (TK18) with pDHS100, pDHS101, and pDHS102 support the prediction, on the basis of the absence of a putative NAD(P)H-binding motif, that the *graIII*-ORF6 gene cannot provide functional oxidoreductase activity on its own. It is interesting, however, that production of actinorhodin was clearly higher in the pDHS100 (*graIII*-ORF5 and -6) complementation of TK18 than in pDHS101 (*graIII*-ORF5). The reason for this difference is unclear but may suggest that a combined *graIII*-ORF5 and -6 gene product has greater catalytic activity than the *graIII*-ORF5 product alone. Sequence analysis of the *actIII* (ORF5) mutant gene from *S. coelicolor* TK18 revealed a single base pair deletion which would clearly disrupt normal function of the gene product by creating a grossly abnormal protein.

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