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Heterologous Expression of an Engineered Biosynthetic Pathway: Functional Dissection of Type II Polyketide Synthase Components in *Streptomyces* Species

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Polyketides are an extensive class of secondary metabolites with diverse molecular structures and biological activities. A plasmid-based multicomponent polyketide synthase expression cassette was constructed using a subset of actinorhodin (*act*) biosynthetic genes (*actI-orf1*, actI-orf2, *actI-orf3*, *actIII*, *actVII*, and *actIV*) from *Streptomyces coelicolor* which specify the construction of the anthraquinone product aloesaponarin II, a molecule derived from acetyl coenzyme A and 7 malonyl coenzyme A extender units. This system was designed as an indicator pathway in *Streptomyces parvulus* to quantify polyketide product formation and to examine the functional significance of specific polyketide synthase components, including the *act* β-ketoacyl synthase (β-KS; encoded by *actI-orf1* and *actI-orf2*) and the *act* cyclase/dehydrase (encoded by *actVII-orf4*). Site-directed mutagenesis of the putative active site Cys (to a Gln) in the *actI-orf1* β-KS product completely abrogated aloesaponarin II production. Changing the putative acyltransferase active-site Ser (to a Leu) located in the *actI-orf1* β-KS product led to significantly reduced but continued production of aloesaponarin II. Replacement of the expression cassette with one containing a mutant form of *actI-orf2* gave no production of aloesaponarin II or any other detectable polyketide products. However, an expression cassette containing a mutant form of *actVII-orf4* gave primarily mutactin with low-level production of aloesaponarin II.

Polyketide natural products represent a large class of complex organic molecules produced by bacteria, fungi, and plants (14, 16, 28). The mechanistic relationship of their biosynthesis to that of long-chain fatty acids has been evident for some time (5, 6). In contrast to the rather limited array of fatty acid structures used for maintaining cellular integrity (in the form of membrane lipids), polyketides are known for remarkable structural diversity, which results from variation in starter and extender units, carbon chain length, substituent functionalization, and stereochemistry. Two types of polyketide biosynthetic systems which are related structurally to analogous systems for production of fatty acids have been described elsewhere (14, 15a, 16). The type I multifunctional protein systems, first established for the 6-methylsalicylic acid synthase in *Penicillium patulum* (4) and subsequently for macrolide polyketide synthases (7, 9) (PKSs), bear a striking biochemical resemblance to fatty acid synthases (FASs) of higher eucaryotes (1, 30). Molecular genetic studies of the erythromycin PKS system indicate a linear correspondence between the genetic order of functional domains and their use in the stepwise synthesis of the aglycone moiety of erythromycin (9). In contrast, the 6-methylsalicylic acid synthase appears to operate more like a eucaryotic FAS in the ability of a single multifunctional enzyme to initiate carbon chain construction and transform acetyl coenzyme A (acetyl-CoA) and malonyl-CoA through a cycle of condensation and processing steps to 6-methylsalicylic acid (36).

The type II (multicomponent) PKS protein systems (typified conceptually by the *Escherichia coli* FAS) have been established as the biosynthetic machinery for numerous polycyclic aromatic natural products (14). Typically, in these systems an acetyl-CoA starter unit and malonyl-CoA extender units are used for carbon chain construction through a cycle of condensation steps. However, in selected cases, starter units other than acetyl-CoA are utilized to initiate polyketide biosynthesis (14, 21). The number of malonyl-CoA extender units used for construction of the carbon backbone can also vary in the aromatic polyketides, which is a key factor in determining the structural identity for these metabolites. Although the enzymatic transformations involved in post-polyketide processing may be studied in a more direct fashion (15), the instability of oligoketide structures (11) has stymied efforts to observe the stepwise construction, cyclization, and dehydration reactions leading to core aromatic structures (such as the benzoisochromanequinones, anthracyclines, and tetracyclines). Therefore, oligoketide chain assembly by the type II polyketide systems has remained largely undefined biochemically. Determining how the successive condensations by type II PKS occur and what molecular mechanisms control starter unit choice, oligoketide chain length and cyclization pattern in the aromatic polyketides are the current driving forces for this area of research. A more thorough understanding of these processes may provide a means to synthesize novel biologically active organic molecules through rational design of multicomponent PKS systems.

Functional analysis of individual components of type II PKS systems (3, 18, 19, 22, 33) is proving to be an effective strategy to elucidate the mechanisms of construction of aromatic polyketide metabolites. Considerable insight into the precise role of individual components of the polyketide synthase (including the *act* β-ketoacyl synthase [β-KS] *orf1* gene product [KS1], the β-KS *orf2* gene product [KS2], acyl carrier protein [ACP], cyclase/dehydrase [CYC/DH] and ketoreductase) has been obtained using *trans* complementation (22, 33), and *cis* replacement (18, 19) strategies in the actinorhodin (*act*) PKS

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(10, 24, 25). Although earlier studies relied on production of the characteristic blue pigment of actinorhodin as a phenotypic marker for functional replacement of individual PKS components (18, 19, 22, 33), more sophisticated systems are being developed to analyze compounds produced by replacement (26, 27) or modification of heterologous PKS genes. In addition, efforts to understand PKS function in the tetracenomycin biosynthetic pathway using biochemical approaches have been reported recently (31).

Here, we describe the construction and utility of a versatile indicator biosynthetic pathway to dissect the functional role of individual PKS genes at the molecular genetic level. The potential bifunctionality of the *actI-orf1* gene product was assessed by generating site-directed mutants in the presumed Cys active site of the condensing enzyme (β-KS) and Ser active site of the putative acyltransferase (AT) domain (10). In addition, this indicator pathway is used as a quantitative tool to study the roles of the *actI-orf2* gene product and of the *actVII-orf4*-encoded protein (CYC/DH) in mediating intramolecular aldol condensation of the nascent octaketide molecule to two possible cyclization products (32).

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Streptomyces parvulus* (ATCC 12434) and *Streptomyces lividans* 66 (or its derivative TK24) were used as the host for plasmids derived from the high-copy-number vector pIJ350 (20). Agar medium for *Streptomyces* species was R2YE, and liquid media were YEME or nitrate defined minimal (NDM) medium (8, 13). Conditions for *Streptomyces* culture and protoplast transformation have been described previously (13). *E. coli* DH5α and XL-1 blue (Stratagene, La Jolla, Calif.) were the hosts for pUC18 and pBluescript II (Stratagene), respectively.

DNA isolation and sequence analysis. *Streptomyces* plasmid DNA was prepared as described by Hopwood et al. (13). The alkaline lysis method (29) was used to prepare plasmids from *E. coli*. Single-stranded DNAs were used for dideoxy DNA sequencing with oligonucleotide primers. Preparation of single-stranded DNAs from pBluescript derivatives in XL-1 blue using helper phage has been described previously (29).

Construction of *act* PKS expression cassettes. To generate pDHS301, the 8.8-kb *Pst*I fragment from pANT12 was first subcloned into *Pst*I-digested pBluescript II. Two *Nde*I sites were introduced into the *actI-orf1* and *actI-orf2* genes by site-directed mutagenesis using PCR (12), resulting in pDHS301. The first *Nde*I site in pDHS301 was introduced at the start codon of the *actI-orf1* gene using two complementary oligonucleotide primers (no. 1 and 2) and two outside primers (no. 7 and 8), as shown in Fig. 2 (primer 1 [forward primer], 5′-gaaggagctgttc catatgaagcgcagagt-3′). Three upstream nucleotides and the start codon (a total of six nucleotides [GGATTG]) in the wild-type sequence were replaced by an *Nde*I (CATATG) site. The second *Nde*I site in pDHS301 was introduced immediately downstream of the stop codon of the *actI-orf2* gene by using two complementary oligonucleotide primers (no. 3 and 4) and two outside primers (no. 7 and 8), as shown in Fig. 2 (primer 3 [forward primer], 5′-ctccagttctcgcatatggt tacggggtcgg-3′). Six nucleotides (CGACCC) after the stop codon (TAA) of *actI-orf2* were replaced by an *Nde*I site (CATATG). Both *Nde*I sites in pDHS301 were verified by DNA sequencing. pDHS301 was then digested with *Sph*I into two fragments, and the *Sph*I fragment containing pBluescript II was replaced by the 7-kb *Sph*I fragment from pANT12 (3). To generate pDHS305, a unique *Nsi*I site was introduced between *actI-orf1* and *actI-orf2* in pDHS301. As for pDHS301, the insert was first subcloned into the pBluescript II for site-directed mutagenesis. A unique *Nsi*I (ATGCAT) site was introduced into the last two codons of the *actI-orf1* gene using two complementary oligonucleotide primers (no. 5 and 6) and two outside primers (no. 1 and 4) as shown in Fig. 2 (primer 5 [forward primer], 5′-cgggggcggatgcatgagcgtcctcatcac-3′). The sequence of GC CGCATGA (Ala-Ala-stop) in pDHS301 was replaced by GATGCATGA (Asp-Ala-stop) in pDHS305. The *Nde*I-*Nsi*I *actI-orf1* fragment in pDHS305 was then replaced by two PCR-generated *Nde*I-*Nsi*I *actI-orf1* fragments containing a glutamine codon (CAG) at the *act* β-KS active site (pDHS306) and a leucine codon (CTC) at the putative *act* β-KS AT active site (pDHS307), respectively. Two complementary oligonucleotide primers (forward primer 5′-ggtctccaccggccagac ctcgggcctgg-3′ for pDHS306 and forward primer 5′-gatggtcggccacctcctgggcgc gatcg-3′ for pDHS307) and two outside primers (no. 1 and 6) were used for site-directed mutagenesis. Both site-directed mutagenized codons in pDHS306 and pDHS307 were verified by DNA sequencing. To generate pDHS308, pDHS309, and pDHS310, the 8.8-kb *Pst*I fragments from *Streptomyces coelicolor* B78 (*actI-orf2* mutant), B40 (*actVII* mutant), and B140 (*actVII* mutant) were isolated (33) and cloned individually into the *Pst*I site in pIJ350.

Polyketide production, purification, and identification. Liquid cultures were inoculated from frozen spore suspensions to 250-ml flasks containing 50 ml of YEME medium and incubated for 3 days at 30°C with shaking. Two milliliters of the culture was then transferred to 50 ml of NDM medium for polyketide production. The entire culture was acidified to pH 3 with citric acid and then extracted with an equal volume of 10% MeOH/$CHCl_3$. The resulting emulsion was filtered and reextracted twice with the same solvent. Cells were dried on Whatman no. 1 filter paper and weighed to determine cell weight (dry weight). The total extract was dissolved in acetone and methylated using an excess of a solution of diazomethane in ether (2). The diazomethane-treated extract was dissolved in CH_2Cl_2, and the solution was loaded onto a 2-in. (ca. 5-cm) Whatman silica column which was eluted with 20 ml of 30% ethylacetate/hexane to obtain derivatized aloesaponarin II and 3,8-hydroxy-1-methyl-anthraquinone-2-carboxylic acid, while actinomycin D (produced endogenously by *S. parvulus*) was retained. The eluate was rotoevaporated to dryness in small vials. Polyketide compounds were visualized by thin-layer chromatography (TLC) or high-pressure liquid chromatography, as described previously (3). Individual polyketide compounds were further purified using a Chromatotron (Harrison Research, Palo Alto, Calif.) and were characterized using ^1H nuclear magnetic resonance spectroscopy, gas chromatography (GC), and mass spectroscopy (MS).

GC-MS analysis of polyketide products. To quantify and compare the production of aloesaponarin II and 3,8-hydroxy-1-methyl-anthraquinone-2-carboxylic acid produced in *S. parvulus* by various expression cassettes, total extracts were directly derivatized with diazomethane and were resuspended in 20 μl of acetone. A 2-μl volume of each sample was injected onto a DB5M5 column (30 m by 0.25 mm; J. & W. Scientific, Fosom, Calif.) and analyzed using GC-MS (Kratos MS25; Dratos Analytical, Ramsey, N.J.); the column was programmed with 50°C to 120°C/min and 120 to 290°C at 8°C/min. Retention times of derivatized aloesaponarin II and 3,8-hydroxy-1-methyl-anthraquinone-2-carboxylic acid were approximately 14.5 and 18.5 min, respectively. Each peak was analyzed by identification of a characteristic MS fragmentation pattern; these were 250/268 for the methyl ether of aloesaponarin II and 311/326 for the methyl ester of 3,8-hydroxy-1-methyl-anthraquinone-2-carboxylic acid (23).

RESULTS

Development of a quantitative indicator system for functional dissection of a multicomponent PKS system in *S. parvulus*. An 8.8-kb *Pst*I fragment containing the *actI*, *-III*, *-VII*, and *-IV* genes of *S. coelicolor*, which was cloned into the high-copy-number *Streptomyces* vector pIJ350 (pANT12) (3), conferred the ability to produce significant levels of aloesaponarin II when it was introduced into *S. parvulus*, a host that produces no known polyketide metabolites (3, 37). This work and subsequent DNA sequence analysis of the *act* biosynthetic pathway (10) showed that four genetic loci containing six open reading frames are required to generate a molecule derived from one acetyl-CoA starter unit and seven malonyl-CoA extender units (Fig. 1). Thus, this set of genes encodes protein products that are responsible for cyclization of the nascent oligoketide chain to give an anthraquinone ring system. Our strategy was designed to generate pANT12 derivatives containing mutant *actI-orf1*, *actI-orf2*, or *actVII-orf4* genes to study their effect on the production of polyketide metabolites in *S. parvulus*. Identification and quantification of polyketide compounds produced by the individual expression cassettes provide further insight into the mechanistic significance of specific components in the *act* PKS.

To facilitate the construction of expression cassettes, two background constructs were generated. The first was a derivative of pANT12 which contains unique restriction sites for replacement of the endogenous *actI-orf1* and *actI-orf2* genes (Table 1) (Fig. 2). Two *Nde*I (CATATG) sites were introduced by site-directed mutagenesis (12) to generate pDHS301; one was incorporated into the start codon of *actI-orf1*, and the second one was generated immediately downstream of the *actI-orf2* stop codon. These mutations did not alter the translation products; however, the start codon of *actI-orf1* in pDHS301 was changed from TTG to ATG. The second construct (pDHS305) was generated as a derivative of pDHS301. An *Nsi*I site was introduced into the last two codons of *actI-orf1* without interfering with the presumed translational coupling between *actI-orf1* and *actI-orf2* (Fig. 2). The plasmids

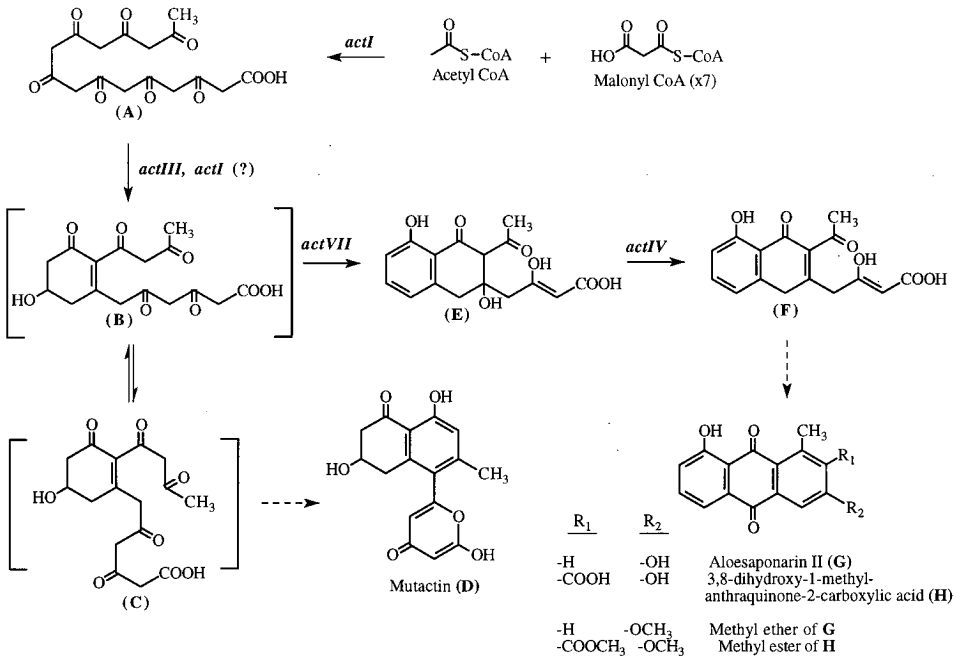

FIG. 1. Proposed biosynthetic pathway for actinorhodin shunt products, mutactin, and aloesaponarin II. Solid and dotted arrows represent presumed enzymatic and spontaneous steps, respectively. Structures in the brackets are hypothetical intermediates.

pANT12 (3), pDHS301, and pDHS305 were introduced separately into *S. parvulus* and subsequently grown in NDM medium (7). All three strains produced an orange fluorescent compound, shown by TLC, ^{1}H-nuclear magnetic resonance spectroscopy, and MS to be aloesaponarin II (3). Treatment of the extracts from these three strains with diazomethane gave two compounds, which were shown to be the methyl ether derivative of aloesaponarin II and the methyl ester derivative of 3,8-hydroxy-1-methyl-anthraquinone-2-carboxylic acid (Fig. 1). The molar ratio of aloesaponarin II to its acidic form was approximately 1:1, as determined by GC-MS. Production levels of aloesaponarin II in *S. parvulus* from the parent construct (pANT12), pDHS301, and pDHS305 were about 1 to 2 μg/ml, which was consistent with levels previously reported for *S. parvulus* (3).

Site-directed mutagenesis of the putative bifunctional *actI-orf1* gene. The deduced amino acid sequences of type II PKS KS1-condensing enzymes all show high similarity with that of the *E. coli fabB* gene product (16, 35). The active site in *E. coli* FabB has been shown to contain a Cys residue that is selectively labeled and inactivated by cerulenin (17). A presumed Cys active site has been found in the deduced protein sequence of all reported type II KS1-condensing enzymes. In contrast, KS2 has a Gln residue in the analogous position (35). To determine if Cys-169 in the *actI-orf1* gene product is essential for oligoketide chain assembly, site-directed mutagenesis was employed to make a Cys→Gln change. pDHS305 was used to generate pDHS306 incorporating the Cys-169→Gln mutation. *S. parvulus*/pDHS306 failed to produce aloesaponarin II or any other detectable polyketide-derived compounds, as shown by TLC and GC-MS (Table 2). This result strongly supports the idea that Cys-169 in the *actI-orf1* gene product plays a critical role in the catalytic activity of the presumed heterodimeric PKS β-KS.

Sequence comparison of known type II PKS KS1 gene products shows a highly conserved domain that appears to resemble an AT active site, specifically a Ser residue in a GHS motif (10). The recognition of this motif led originally to the supposition that KS1 is bifunctional, with the AT domain involved in catalyzing transfer (via an ester linkage) of the nascent acyl chain from the ACP to Cys-169. To test whether the Ser residue in the presumed AT encoded by *actI-orf1* is necessary for activity, pDHS307, which has a Ser-347→Leu change was generated. When grown in liquid culture, *S. parvulus*/pDHS307

TABLE 1. Plasmids used in this study for functional dissection of type II PKS components

Construct	Description	Reference
pIJ350	pIJ101 derivative; *Streptomyces* high-copy-number plasmid	12
pANT12	pIJ350 derivative containing the 8.8-kb *Pst*I fragment including *actI*, *actIII*, *actVII*, and *actIV* loci	2
pDHS301	pANT12 derivative containing two *Nde*I sites at the *actI* region; one is at the start codon of *actI-orf1* and the other one is right after the stop codon of *actI-orf2*	This study
pDHS305	pDHS301 derivative containing the *Nsi*I site between *actI-orf1* and *actI-orf2*	This study
pDHS306	pDHS305 derivative containing Gln-169 (not Cys-169) at the presumed β-KS active site in *actI-orf1*	This study
pDHS307	pDHS305 derivative containing Leu-347 (not Ser-347) at the presumed AT active site in *actI-orf1*	This study
pDHS308	pIJ350 derivative containing the 8.8-kb *Pst*I fragment from *S. coelicolor* B78 (*actI-orf2* mutant)	This study; 33
pDHS309	pIJ350 derivative containing the 8.8-kb *Pst*I fragment from *S. coelicolor* B40 (*actVII* mutant)	This study; 33
pDHS310	pIJ350 derivative containing the 8.8-kb *Pst*I fragment from *S. coelicolor* B140 (*actVII* mutant)	This study; 33

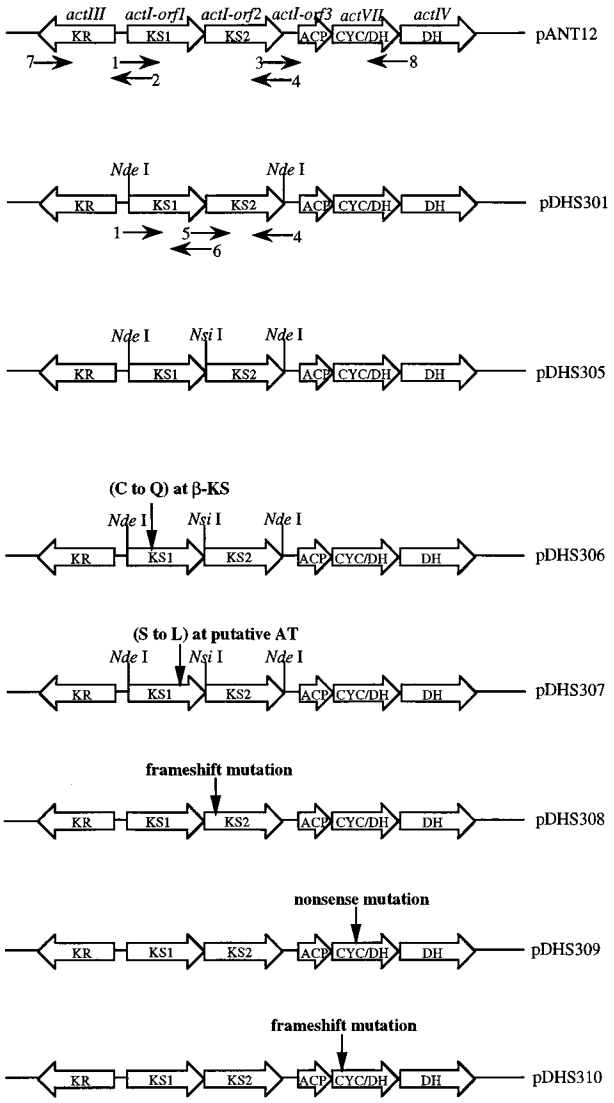

FIG. 2. The 8.8-kb *Pst*I fragments containing various *act* gene cassette constructs. Short arrows with a number in pANT12 and pDHS301 represent PCR primers (see Materials and Methods for details). Open arrows represent individual genes. KR, keto reductase.

gave no apparent production of aloesaponarin II. However, substantial levels of this polyketide product were detectable by TLC and GC-MS when cells were grown on agar plates over a 2- to 3-week period (Table 2). This result indicates that Ser-347 in the *actI-orf1* gene product does not function directly as an AT active-site residue.

Construction of expression cassettes containing either an *actI-orf2* or an *actVII* mutant gene. Previously, it was shown that the *actI-orf2* gene, whose putative protein product shows overall similarity to the β-KS component encoded by *actI-orfI*, is essential for PKS function (33). Complementation of an *S. coelicolor actI-orf2* mutant (B78) by various other type II PKS β-KS *orf2* genes also indicated that this component might provide specificity in polyketide chain construction (22, 33). Recently, it was suggested that the *orf2*-encoded β-KS component plays a role in determining polyketide chain length (26, 27). To quantify the effect of a nonfunctional *actI-orf2* gene on polyketide construction, the 8.8-kb *Pst*I fragment from *S. coelicolor*

TABLE 2. Polyketide production by *S. parvulus* expression systems containing various *act* gene cassette constructs

Expression system	Polyketide product(s)[a]	Relative production of aloesaponarin II (%)[b]
S. parvulus/pANT12	G and H	100 and 100
S. parvulus/pIJ350	NP	0 and 0
S. parvulus/pDHS301	G and H	ND
S. parvulus/pDHS305	G and H	71 and 68
S. parvulus/pDHS306	NP	0 and 0
S. parvulus/pDHS307	(G, H)[c]	0 and 0
S. parvulus/pDHS308	NP	0 and 0
S. parvulus/pDHS310	D, G, and H	11 and 24[d]

[a] Polyketides were produced from *S. parvulus* transformants grown in NDM medium as described in Materials and Methods. NP, no product; D, mutactin; G, aloesaponarin II; H, 3,8-hydroxy-1-methyl-anthraquinone-2-carboxylic acid.

[b] The amounts of methyl ester derivative of 3,8-hydroxy-1-methyl-anthraquinone-2-carboxylic acid were measured from two separate fermentations by GC-MS as described in Materials and Methods. ND, not determined.

[c] *S. parvulus*/pDHS307 grown in NDM medium did not produce any detectable polyketides. However, both aloesaponarin II and its acidic form were detected from the culture grown on R2YE agar plates.

[d] *S. parvulus*/pDHS309 was used instead of *S. parvulus*/pDHS310 in the second fermentation.

actI-orf2 mutant strain B78 was cloned into pIJ350 to generate pDHS308. The genetic lesion in B78 *actI-orf2* mutant is a deletion of 2 nucleotides at bp 122 to 123 (33) to give a gene product unrelated to the wild-type *actI-orf2* protein beyond the first 40 amino acids. As expected, *S. parvulus* transformants containing pDHS308 grown either on plates or in liquid culture failed to produce any detectable polyketides, as determined by TLC and GC-MS (Table 2).

Another important aspect of polyketide construction involves cyclization of the nascent oligoketide chain to give fused aromatic ring systems. Previous studies using *trans* complementation of *S. coelicolor actVII* mutants revealed that *actVII-orf4* encodes a CYC/DH (31), which is believed to generate the second ring and catalyze dehydration of C-9 hydroxyl in the first ring of the actinorhodin benzoisochromane quinone system. To probe this function further, the 8.8-kb *Pst*I fragments from *S. coelicolor actVII* mutant strains B40 and B140 were cloned in pIJ350 to generate pDHS309 and pDHS310, respectively (Table 1). The B40 *actVII-orf4* mutant had a single base pair substitution (G to A) at position 294, resulting in a nonsense mutation which would lead to a severely truncated gene product (33). In the B140 *actVII-orf4* mutant, a deletion of positions 57 and 58 caused a frameshift which would result in the production of a protein lacking similarity to the wild-type CYC/DH (33). Introduction of these two plasmids, pDHS309 and pDHS310, into *S. parvulus* gave the expected product, mutactin (D). However, in addition to this aberrantly cyclized product, small amounts of aloesaponarin II and its acidic form were produced (Table 2). This result showed that the preferred route of cyclization in the absence of the *actVII-orf4* gene leads to mutactin, although low levels of the octaketide do cyclize via the aloesaponarin II route (Fig. 1).

DISCUSSION

The actinorhodin biosynthetic pathway represents a paradigm for the multicomponent PKSs (14, 16, 34). Complete nucleotide and deduced amino acid sequence information revealed that these systems operate by an ACP-mediated mechanism similar to those of some prokaryotic and plant FASs (10, 14). The roles of individual PKS gene products involved in carbon chain assembly have been suggested on the basis of

molecular genetic analysis (14, 16), deduced protein sequence relationships (35), and in vitro studies (15, 31). However, direct experimental evidence to prove the identity of specific active-site residues has been lacking.

To extend our studies of the functional role of individual PKS components, we developed a quantifiable plasmid-based indicator biosynthetic pathway for expression in *S. parvulus* and other *Streptomyces* spp. This system was designed to provide a versatile gene replacement strategy in which to test the function of specific mutant genes and amino acid residues in the *actI*, *actIII*, *actVII*, and *actIV* loci. To facilitate cloning, *Nde*I sites, including the translational start site of *actI-orf1* and the *actI-orf2* termination codon, were introduced into the coding sequence. In addition, an *Nsi*I site was introduced at the overlapping termination-initiation codon of *actI-orfI* and *actI-orf2* to allow individual replacements of *actI-orf1* or *actI-orf2* without disturbing translational coupling between the gene products. Comparison of all known PKS and FAS β-KSs reveals a Cys active-site residue within a highly conserved amino acid motif. Our results show that polyketide production in *S. parvulus* was completed abrogated when Cys-169 was changed to Gln at the presumed β-KS active site in pDHS306. Although this result is consistent with an effect on a KS1 active site, it is not possible to rule out other roles for the Cys-169 residue in polyketide chain assembly.

S. parvulus/pDHS307, which included a Ser-347→Leu change at the putative KS1 AT active site, still produced (albeit at low levels) aloesaponarin II and its acidic form when it was grown on agar plates over extended periods. It is particularly important to consider that KS1 remains catalytically active even in the presence of Leu-347, which represents a significant amino acid alteration. This result indicates that Ser-347, which is located in the GHS motif of the *actI-orf1* gene product, is not an active site per se; however, the reduced levels of polyketide production suggest that it may play another role in type II AT activity or in maintaining the overall catalytic integrity of the KS1 protein. Although these site-directed mutagenesis studies cannot exclude the possibility that the type II PKS KS1 product is bifunctional (catalyzing condensation as well as acyl transfer of the nascent oligoketide chain from the ACP to the KS1 Cys active site), it is clear that AT activity is not mediated through an ester linkage at Ser-347, as was previously assumed.

In a previous study of the *act* PKS, an early blocked mutant was shown to have a lesion in the *actI-orf2* gene, which established its role as an essential element for β-KS activity (33). Although there have been recent suggestions that the KS2 product is uniquely involved in polyketide chain length determination (26, 27), the precise mechanistic role of this protein remains unclear. The construction of pDHS308 was designed to corroborate the role of *actI-orf2* in a system that allowed quantitative analysis as opposed to visual inspection of pigmented metabolites produced in *S. coelicolor act* mutants. The current analysis shows that polyketide chain construction cannot occur (to produce pigmented or nonpigmented polyketides) in the absence of the PKS KS2 component. Further molecular genetic and biochemical analyses are now required to probe the nature of the catalytic role of the β-KS and how it interacts with other biosynthetic enzymes in multicomponent PKSs.

The production of mutactin by *S. coelicolor actVII* mutants (B40 and B140) has provided valuable insight into the role of *actVII-orf4* in type II polyketide chain cyclization (32, 33). After completion of successive carbon-carbon condensation which was followed by ketoreduction at the C-9 position, it is believed that the polyketide intermediate can exist as two hypothetical reactive conformations (B and C). It has been shown that structure C is the lower-energy conformer, which supports the idea that mutactin is produced spontaneously by *S. coelicolor actVII* mutants (32). *S. parvulus* containing pDHS309 or pDHS310 produced primarily mutactin; however, low levels of aloesaponarin II (as well as of its acidic form) were detected as minor products by GC-MS. This result supports our previous analysis (32) that the preferred route of polyketide second-ring cyclization in the absence of the *actVII* gene leads to mutactin formation. Interestingly, *S. coelicolor* B40 and B140 *actVII* mutants fail to produce aloesaponarin II. Therefore, it is suggested that the low levels of product that undergo normal cyclization to the anthraquinone system would serve as a substrate in *S. coelicolor* mutants containing a complete *act* biosynthetic pathway. Therefore, it is possible that trace (visually undetectable) levels of actinorhodin are being produced in the *S. coelicolor* actVII mutant strains. Alternatively, the small amount of aloesaponarin II production by *S. parvulus*/pDHS309 or *S. parvulus*/pDHS310 could be due to *trans* complementation by an *actVII*-like gene product which might be present in *S. parvulus*. Southern blot hybridization showed that *S. parvulus* chromosomal DNA contains sequences which could weakly hybridize with an *actVII* gene probe (22a). Although the *actVII* gene product was assigned as a bifunctional CYC/DH (14, 32), its actual function, including the identity of an active-sites residue(s), remains unclear. It has been shown by in vitro biomimetic synthesis (11) that linear polyketide chains are very unstable and cyclize spontaneously in an aqueous environment. Therefore, it is also possible that the *actVII* gene product is essential to facilitate proper polyketide chain cyclization by providing conformational specificity to the oligoketide chain rather than by carrying out a specific catalytic reaction.

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