Genetic Construction and Functional Analysis of Hybrid Polyketide Synthases Containing Heterologous Acyl Carrier Proteins

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The gene that encodes the acyl carrier protein (ACP) of the actinorhodin polyketide synthase (PKS) of *Streptomyces coelicolor* A3(2) was replaced with homologs from the granaticin, oxytetracycline, tetracenomycin, and putative frenolicin polyketide synthase gene clusters. All of the replacements led to expression of functional synthases, and the recombinants synthesized aromatic polyketides similar in chromatographic properties to actinorhodin or to shunt products produced by mutants defective in the actinorhodin pathway. Some regions within the ACP were also shown to be interchangeable and allow production of a functional hybrid ACP. Structural analysis of the most abundant polyketide product of one of the recombinants by electrospray mass spectrometry suggested that it is identical to mutactin, a previously characterized shunt product of an *actVII* mutant (deficient in cyclase and dehydrase activities). Quantitative differences in the product profiles of strains that express the various hybrid synthases were observed. These can be explained, at least in part, by differences in ribosome-binding sites upstream of each ACP gene, implying either that the ACP concentration in some strains is rate limiting to overall PKS activity or that the level of ACP expression also influences the expression of another enzyme(s) encoded by a downstream gene(s) in the same operon as the actinorhodin ACP gene. These results reaffirm the idea that construction of hybrid polyketide synthases will be a useful approach for dissecting the molecular basis of the specificity of PKS-catalyzed reactions. However, they also point to the need for reducing the chemical complexity of the approach by minimizing the diversity of polyketide products synthesized in strains that produce recombinant polyketide synthases.

Polyketides include antibiotics such as oxytetracycline and erythromycin made by actinomycetes, fungal toxins such as aflatoxin, and plant anthocyanins. Chemical and biochemical evidence (21, 30) has long pointed to a similarity between (i) the mechanism of fatty acid synthesis, in which the fatty acid synthase (FAS) assembles a long carbon chain by successive decarboxylative condensation of simple carboxylic acids, with a round of β -ketoreduction, dehydration, and enoylreduction after most or all of the condensation steps, and (ii) the mechanism of polyketide synthesis by polyketide synthases (PKS). The chief difference is in the omission of many of the reductive steps in polyketide assembly. The recent sequencing of many polyketide-biosynthetic genes has shown that this similarity actually reflects a clear evolutionary relationship between PKS and FAS genes (see references 13, 14, and 22 for reviews). In particular, there are striking end-to-end similarities in the amino acid sequences of β -ketoacyl-acyl carrier protein (ACP) synthase (condensing enzyme) domains or subunits between and within groups of PKSs and FASs. One also observes a high degree of sequence similarity around the 4' phosphopantetheinylation site of ACP domains and subunits, as well as conserved sequences for ketoreductase, dehydrase, enoylreductase, and acyltransferase active sites in most cases, when their existence is expected from the chemical structure of the product. (The only exceptions

appear to be plant chalcone and resveratrol synthases, which lack obvious resemblances to other PKSs and may well represent a separate line of evolution [17].)

Notwithstanding this sequence conservation across the PKSs and FASs, a fundamental difference has emerged in the mechanisms by which the enzyme determines the structure of its product between the synthases for fatty acids and simple aromatic polyketides on the one hand and those for complex polyketides, such as macrolides, on the other. In the macrolide case, exemplified by the erythromycin PKS of *Saccharopolyspora erythraea*, each round of carbon chain assembly and modification (ketoreduction, dehydration, and enoylreduction, as appropriate) is catalyzed by a distinct active site (6). Thus, the sequence of biosynthetic reactions is overtly "programmed" into the DNA that encodes the PKS. In contrast, for aromatic polyketides, such as granaticin and tetracenomycin produced by *Streptomyces* spp. (3, 26), a single set of reiteratively used active sites of the PKS must determine the choice of a carbon chain starter unit, the number of extender units used to build the chain, and the extent of ketoreduction. The mechanism of programming of this class of PKS is obscure.

We and others are attempting to elucidate the programming mechanism of aromatic PKSs by constructing recombinant *Streptomyces* strains carrying hybrid PKS gene clusters and analyzing their metabolic capabilities. In one study, mutations in each of four genes that encode components of the actinorhodin PKS of *Streptomyces coelicolor* A3(2) were complemented in *trans* by the homologous genes for the

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granaticin PKS of *S. violaceoruber* Tü22 (25). In a second, the actinorhodin ACP was replaced in *cis* by a presumed FAS ACP from *S. erythraea* to produce an active PKS (16). Here we describe the application of the same gene replacement technology to construct hybrid polyketide synthases in which the ACP gene of the actinorhodin PKS is replaced by ACP genes from gene clusters involved in the biosynthesis of four aromatic polyketides that are structurally related to but distinct from actinorhodin (see Fig. 1). The choice of ACP genes for these first experiments was dictated in part by prior biochemical findings on the key role of the ACP. Studies on FAS function (reviewed in reference 30) revealed that the ACP holoprotein includes a pantethenic cofactor, which is the site at which individual malonate units (the extenders) are sequentially thioesterified to be used for generation of nucleophiles in the condensation reaction catalyzed by the ketosynthase. Thus, ACP (together with malonyl coenzyme A) is the substrate for the malonyltransferase reaction, and malonyl-ACP is the substrate for the ketosynthase. Furthermore, the substrate of the thioesterase or acyltransferase responsible for release of the completed carbon chain from the synthase is fatty acyl ACP. It is assumed that the ACPs of PKSs also share both of these features: one of the conclusions of our earlier study was that a highly conserved serine residue (S-42 in the actinorhodin ACP), as the putative site of pantethenylation, is indeed essential for the function of the PKS (16). We report here that the actinorhodin ACP can be replaced by any one of the four other available aromatic PKS ACPs to produce a functional PKS. However, we also found interesting differences in the total and relative amounts of the polyketide metabolites produced by the various recombinant strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *S. coelicolor* CH1 (*proA1 argA1 redE60 SCP1⁻ SCP2⁻*) (16) was used as the parent strain. CH2 is a derivative of CH1 in which the *actI*-open reading frame 3 (ORF3) gene (which encodes the actinorhodin ACP) and flanking sequences (including the 3' end of the upstream gene and the 5' end of the downstream gene in the *act* PKS cluster) were replaced by the *ermE* gene, which confers resistance to erythromycin or lincomycin. CH5 was constructed from CH2 by *in vivo* recombination and contains the entire wild-type *act* cluster, except that the translational coupling between the ACP gene and the downstream *actVII* gene has been disrupted via introduction of a restriction site. The construction of both CH2 and CH5 was described elsewhere (16). B40 and B17 are *actVII* and *actIV* actinorhodin-biosynthetic pathway mutants, respectively, which also carry the markers *hisA1 uraA1 strA1* (7, 23, 25). DH5 α was used as the *Escherichia coli* host for all DNA manipulation experiments, while *E. coli* ET12567 (*dam dcm hsdS Cm^r*) was used to generate unmethylated plasmid DNA suitable for transformation of *Streptomyces* strains, such as *S. coelicolor* A3(2), that restrict methylated DNA (18). The *gra*, "*fren*," *otc*, and *tcm* ACP genes were obtained from pIJ5200 (26), pIJ5214 (2), pIJ5210 (24), and pELE37 (10), respectively. (The DNA cloned from the frenolicin-producing strain, *S. roseofulvus*, has not been shown to encode the "*fren*" gene cluster via gene disruption or mutant complementation. Hence, although the gene organization and sequence homology suggest that the DNA probably encodes an aromatic polyketide synthase such as frenolicin synthase, the function of this genetic locus is still tentative.) The construction of pIJ5609

(which carries the *act* ACP gene flanked by upstream *XbaI* and downstream *PstI* sites) was also described earlier (16). pGM160 is a broad-host-range shuttle vector for *E. coli* and *Streptomyces* spp. with temperature-sensitive replication in *Streptomyces* spp. (20). pBR322 (5) and pUC118 (29) were used as cloning vectors in *E. coli*. *E. coli* strains were grown under standard culture conditions (19), while the procedures for *Streptomyces* growth were as described by Hopwood et al. (12). The colors of the various strains (reflecting metabolite production) were assessed on R2YE plates.

Manipulation of DNA and organisms. The polymerase chain reaction was carried out under standard conditions (10 mM Tris-Cl [pH 8.3], 50 mM KCl, 2.5 mM MgCl₂, 0.1% gelatin, 200 μ M deoxynucleoside triphosphates) with Ampli-taq (Perkin-Elmer Cetus). All of the DNA obtained via polymerase chain reaction was either verified by sequencing with Sequenase (U.S. Biochemicals) or replaced with wild-type DNA (from a primary clone) by using standard restriction site cloning methodology (19). For other *in vitro* DNA manipulations, standard procedures were used (19). *E. coli* strains were transformed in accordance with the protocol of Silhavy et al. (27), while *S. coelicolor* was transformed as described by Hopwood et al. (12). *S. coelicolor* transformants were selected by using an overlay of 2 ml of 500- μ g/ml thiostrepton in water.

Construction of strains containing heterologous and recombinant ACP genes. Heterologous ACP genes were amplified via polymerase chain reaction by using the DNA templates described above. For a list of the engineered sequences, see Fig. 5. In each case, the gene was cloned with flanking *XbaI* and *PstI* sites at its 5' and 3' ends, respectively. Following initial cloning into pUC118, the *XbaI-PstI* fragments were used to replace the corresponding fragment carrying the *act* ACP gene in pIJ5609. The *HindIII-EcoRI* fragments from the resultant plasmids were then used to construct shuttle delivery plasmids based on pGM160, exactly as in the construction of pIJ5714 from pIJ5609 itself (16). These shuttle delivery plasmids were introduced by transformation into CH2, and the desired clones were identified by screening for double-crossover events (Lin^s) and loss of the delivery plasmid (Thio^s) as described earlier (16). For each recombinant strain, a candidate clone was chosen that was typical of several candidates in its pigmentation phenotype. The presence of the altered ACP gene in the genome of each final clone was also confirmed by amplification via genomic polymerase chain reaction by using the same oligonucleotides referred to above.

Isolation of metabolites and thin-layer chromatography (TLC). Liquid or agar plate media were extracted with an equal volume of ethyl acetate (pH 3). Following concentration of the ethyl acetate phase, the samples were spotted on silica gel F254 plates (E. Merck) and developed with benzene-methanol (9:1). Patterns of ordinarily visible and UV-visible spots were recorded for each sample.

Polyketide purification and analysis. Following extraction of the medium with ethyl acetate and concentration as described above, the extracts were applied to a silica gel (40 μ m) column, which was developed first with ethyl acetate and subsequently with acetonitrile. A fraction that had properties similar to those of a product characteristic of the *actVII* mutant B40 was concentrated and purified via preparative reverse-phase (C18) high-performance liquid chromatography with an acetonitrile-water gradient (25 to 100% acetonitrile). This product was then analyzed via electrospray mass spectrometry (Hewlett-Packard).

RESULTS

Construction of strains that express hybrid polyketide synthases. To facilitate the construction of recombinant PKS gene clusters, it was considered desirable to disrupt the presumed translational coupling between the ACP gene (*actI*-ORF3) and the *actVII* gene immediately downstream that exists in the wild-type *S. coelicolor* A3(2) chromosome. The resulting strain, CH5, appeared to have a partial blockage of the actinorhodin pathway, producing several shunt products normally produced by mutants blocked in post-PKS steps of actinorhodin biosynthesis, in addition to actinorhodin itself (16). It was reasoned that from the viewpoint of analysis of the structure and function of actinorhodin PKS, any product possessing the complete C-16 polyketide backbone with the correct site and extent of ketoreduction (Fig. 1) should be equally useful as a reporter molecule. Consequently, the studies described here were done with the decoupled strain, CH5, as a reference.

PKS genes have recently been cloned from strains that produce four other structurally related aromatic polyketides besides actinorhodin (Fig. 1): granaticin (26), frenolicin (2), oxytetracycline (24), and tetracenomycin (3). The amino acid sequences of the ACPs from these five PKS gene clusters can be aligned as shown in Fig. 2. Each heterologous ACP gene was used to construct a recombinant strain in which the corresponding ACP gene (together with its own ribosome-binding site [RBS]) was inserted into the chromosomal *act* cluster in place of the *act* ACP gene of *S. coelicolor* CH5. The resulting strains were designated CH7 (granaticin ACP), CH8 (putative frenolicin ACP), CH9 (oxytetracycline ACP), and CH10 (tetracenomycin ACP). All of the strains produced diffusible pigments, indicative of functional polyketide synthases (Fig. 3). However, both the color and the intensity of pigmentation differed from strain to strain. Qualitatively, each strain produced a diffusible brown pigment (similar to that produced by an *actIV* mutant as well as CH5) and a diffusible blue pigment (similar to the predominant product of CH1 and also produced to some extent by CH5). Quantitatively, CH7 and CH8 appeared to produce more pigmented products than the other strains, while the intensity of color produced by CH9 was comparable to that of CH5. CH10 produced the smallest amount of pigmented products.

Chemical analysis of products from hybrid polyketide synthases. The metabolites produced by CH1, CH5, CH7, CH8, CH9, and CH10 were analyzed by TLC. CH2, the null mutant carrying a deletion of the *act* ACP gene and parts of the upstream and downstream *act* PKS genes (16), was used as a negative control. *S. coelicolor* B40 (an *actVII* mutant) and B17 (an *actIV* mutant) were also included in the study as reference strains. The principal product of B40 has been identified as mutactin (Fig. 4; 31), whereas the precise structures of the brown pigment produced by B17 and the blue pigment produced by CH1 are unknown. (Although the blue pigment is likely to be very closely related to actinorhodin [9], it differs from the reported compound in several physicochemical properties [8].)

Three main conclusions could be drawn from the chromatographic analysis. (i) All of the strains produced several (typically, three to six) presumptive aromatic polyketide products, albeit in different ratios. (An aromatic polyketide product here is defined as a colored and/or UV-fluorescent product that is absent from CH2.) This confirmed the above-described finding, based on visual inspection of colonies on agar, that a functional polyketide synthase is produced in

FIG. 1. Programming of aromatic PKSs. Hypothetical polyketide precursors synthesized by the actinorhodin, granaticin, frenolicin, oxytetracycline, and tetracenomycin PKSs are shown on the left. The structure of each of the five final products is shown on the right. The bold bonds on the left connect carbon atoms derived from individual monomeric building units. For some compounds, this monomer incorporation pattern has been defined through isotopic labeling studies. For others, this is tentative. For example, the first four carbon atoms in the frenolicin backbone could be derived from a single butyrate, and the starter monomer in oxytetracycline could be an acetate that is subsequently derivatized into a malonamide. The sequence of reductions and dehydrations relative to chain termination and cyclization has also not been unambiguously established in these systems. S-Enz indicates the site of thioester linkage of the nascent polyketide to the polyketide synthase. (These data were adapted from reference 13.)

each recombinant strain. (ii) Differences in the overall levels of aromatic polyketide biosynthesis detected by TLC analysis correlated well with differences in the intensity of pigmentation of the various strains growing on R2YE plates. (iii) For every major polyketide product produced by CH7, CH8, CH9, and CH10, a corresponding product (as judged by R_f , fluorescence, and color) was seen in CH5 and/or in B40 and B17. This suggests that the strains carrying heterologous ACPs produce polyketides that are structurally very similar (perhaps identical) to the blue compound produced by CH1 and shunt products of its biosynthesis (which also have the same polyketide backbone as actinorhodin; Fig. 4).

The above-described TLC studies showed that both CH7 (carrying the *gra* ACP) and CH8 (carrying the "*fren*" ACP)

FIG. 2. Alignment of protein sequences of ACPs from five aromatic PKSs. A computer-generated alignment of the amino acid sequences of the ACPs of the actinorhodin (*act*), granaticin (*gra*), putative frenolicin ("fren"), oxytetracycline (*otc*), and tetracenomycin (*tcm*) PKSs is shown. The numbers above the sequences represent the positions of these residues in the *act* ACP. The sequences were obtained from the following references: *act*, 7; *gra*, 26; *tcm*, 4; *otc*, 24; "fren," 2. Symbols: ~, highly conservative changes, such as those within the groups LIV, FY, and DNEQ; |, position of the fusion between the *act* and *tcm* ACPs in CH16; *, perfectly conserved residues.

produced significant quantities of a compound that was characteristic of the B40 mutant. A sample of this compound was purified from CH8. The compound was colorless and had a fluorescence color similar to that of a reference sample of mutactin (kindly donated by H. Floss, University of Washington, Seattle) under UV illumination. Electrospray mass spectrometry revealed two major peaks in this sample, with molecular weights of 302 ± 1 and 284 ± 1 , respectively (data not shown). These values correspond to the molecular weights of mutactin and its dehydration product, dehydromutactin (in which, presumably, the tetralone moiety has been converted into a substituted naphthalene ring system; Fig. 4). Similar peaks were obtained with the mutactin reference sample under identical conditions of analysis. This confirms that a polyketide backbone synthesized by the hybrid polyketide synthase from CH8 is identical to that produced by the actinorhodin polyketide synthase. Since granaticin has the same polyketide backbone structure as actinorhodin (Fig. 1), it is reasonable to expect that the corresponding product of CH7 is also identical to mutactin.

Strains CH9 and CH10 produced relatively very small amounts of the compound similar to mutactin, and isolation of this product from these strains proved unsuccessful. As judged by TLC, their predominant products were similar to the diffusible brown product produced by the *actIV* mutant, B17, and the diffusible blue product produced by CH1. Since the structures of these compounds are unknown, further chemical characterization of these strains was not pursued.

Effects of mutations in the RBS of the ACP on polyketide biosynthesis. The genetic differences between strains CH5, CH7, CH8, CH9, and CH10 are confined to the RBSs and the structural genes of their respective ACPs. Differences in the intensity of pigmentation (which was found to be a good

indicator of the total amount of polyketide synthesized by each strain) from strain to strain could be due to either structural differences in the ACPs, differences in levels of expression of the ACPs and/or their downstream gene products (Fig. 4), or a combination of these reasons. To explore these alternatives, two additional recombinant strains were constructed in which the RBSs of the ACP genes in CH5 and CH10 were altered. As shown in Fig. 5, CH14 contained a fusion between the RBS of the *gra* ACP gene and the structural gene that encodes the *act* ACP. In CH15 (Fig. 5), the RBS of the *tcm* ACP gene was altered to be identical to the RBS of the *act* ACP in CH5. As seen in Fig. 3, both mutations led to significant increases in levels of pigmentation compared with the mutants carrying the ACP genes with their native RBSs (i.e., CH5 and CH10, respectively). TLC comparison of these strains also showed that the levels of biosynthesis of all of the major polyketide products were increased in the RBS mutants. Taken together, these results suggest that changing the RBS of ACPs in strains that produce lesser amounts of polyketides can alleviate one or more bottlenecks in the polyketide biosynthesis pathway, leading to enhanced polyketide production.

Construction and analysis of a fusion ACP. Alignment of the amino acid sequences of the *act* and *tcm* ACPs revealed strong end-to-end homology (40% identity), suggesting that the tertiary structures of these two proteins are very similar. The same is true of any pair of the ACPs of the five PKSs studied, as seen in Fig. 2. Hence, assuming that heterologous gene replacement will eventually be found to give rise to polyketides with novel backbone structures, the construction and analysis of fusion proteins might be a potentially powerful way to identify a domain(s) that controls the specificity of individual enzyme-catalyzed reactions. To explore the feasibility of generating functional protein fusions between different ACPs, a gene fusion was constructed at the *Bgl*II site, which is positioned at codon 46 of the 84 codons of the *tcm* ACP, to generate a product with the 5' portion of the *tcm* ACP gene and the 3' portion of the *act* ACP gene. The fusion protein was expressed by using the RBS of the *tcm* ACP gene and was delivered into the *S. coelicolor* chromosome as described above. The resulting mutant was designated CH16 (Fig. 5). On the basis of both visual inspection of colonies (Fig. 3) and TLC assessment, the PKS in this mutant was functional and gave products qualitatively and quantitatively similar to those of the PKS of CH10.

DISCUSSION

The results described here strongly endorse the suitability of a method (16) for genetic engineering of the actinorhodin PKS gene cluster of *S. coelicolor* A3(2) through in-cis replacement of native sequences by heterologous genes, hybrid genes constructed by in vitro recombination, or PCR-modified DNA. Encouragingly for the goal of trying to understand the basis of the programming of PKSs for aromatic polyketides through construction and analysis of recombinants carrying hybrid synthases, every replacement involving the actinorhodin ACP—by four heterologous PKS ACPs as described here and by a FAS ACP as described earlier (16)—led to a functional PKS. Thus, all of the foreign ACPs so far studied are competent to interact with the other components of the actinorhodin PKS. Even a hybrid ACP derived by a fusion between the actinorhodin and tetracenomycin ACP genes was competent in this system.

Analysis of the metabolites produced by the recombinant

FIG. 3. Appearance of the various strains carrying genetically engineered PKS gene clusters. CH1 is the *act*⁺ parent strain, and CH2 is the *act* mutant culture carrying the *ermE* gene replacing the *act* ACP gene and parts of the neighboring genes. For relevant details of the other eight strains, see Fig. 5. The cultures were grown on R2YE agar for 7 days.

iment described here is a first step in this direction and supports the idea that polypeptide subdomains from different PKSs may be interchangeable without loss of function.

The strong influence of DNA sequences upstream of the ACP-coding sequences on the levels of polyketide synthesized is a second feature of this study (Fig. 5). Many of these effects may well be due to the RBS itself, although the correlation between the complementarity with the 16S rRNA and the level of metabolite production is by no means perfect. The existence of these strong quantitative variations in total aromatic polyketide production implies either that the ACP concentration is rate limiting in at least some strains or that some level of translational coupling still exists (despite the introduction of an intervening DNA sequence including a restriction site) between the ACP gene and downstream genes (*actVII* and *actIV*), one of whose products may be expressed at rate-limiting levels, resulting in the production of pathway shunt products (Fig. 4). The former hypothesis is also supported by the finding that overexpression of the *tcm* ACP in *S. glaucescens* gives rise to elevated levels of polyketide production (15). Furthermore, the latter hypothesis is less likely, since it would merely result in redistribution of relative ratios of mutactin, the *actIV* shunt product, and the blue pigment without influencing total polyketide biosynthesis, unless the activity of one (or more) of these post-PKS enzymes is subject to product inhibition. In any event, these results underscore the importance of taking posttranscriptional effects into consideration in the design of metabolic engineering strategies for improving polyketide titers and thus aiding the task of deducing the full biosynthetic capabilities of each hybrid PKS.

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