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Expression of a Functional Fungal Polyketide Synthase in the Bacterium *Streptomyces coelicolor* A3(2)

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The multifunctional 6-methylsalicylic acid synthase gene from *Penicillium patulum* was engineered for regulated expression in *Streptomyces coelicolor*. Production of significant amounts of 6-methylsalicylic acid by the recombinant strain was proven by nuclear magnetic resonance spectroscopy. These results suggest that it is possible to harness the molecular diversity of eukaryotic polyketide pathways by heterologous expression of biosynthetic genes in an easily manipulated model bacterial host in which prokaryotic aromatic and modular polyketide synthase genes are already expressed and recombined.

Polyketides are structurally diverse natural products that have a wide range of biological activities, including antibiotic and pharmacological properties. Biosynthesis of the carbon chain backbone of polyketides is catalyzed by polyketide synthases (PKSs). Like the structurally and mechanistically related fatty acid synthases, PKSs catalyze the repeated decarboxylative condensations between acyl thioesters that build the carbon chain two carbons at a time (33). However, unlike fatty acid synthases, PKSs generate structural variability in the carbon chain by varying the reductive cycle of ketoreduction, dehydration, and enoyl reduction on the β-keto group formed after each condensation (19). Molecular genetic analysis has revealed several kinds of PKSs. Aromatic PKSs consist of iteratively used active sites that are contained either on a group of separate proteins encoded by clustered genes (bacterial PKSs [3, 10, 37]) or on a single protein (fungal PKSs [2, 26]). In contrast, modular PKSs, so far confined to bacteria, contain a separate active site for every enzyme-catalyzed reaction in the pathway, with active sites distributed in modules over one or more large proteins (4, 9, 32).

A system for the expression and analysis of PKS genes has been developed in the gram-positive actinomycete *Streptomyces coelicolor* A3(2) (strain CH999) (28, 29). This system has facilitated functional analysis of both aromatic and modular PKSs from *Streptomyces* spp., which are a particularly rich source of polyketides, as well as from other actinomycetes (21, 28, 29). Both classes of bacterial PKSs are amenable to genetic engineering for the production of novel polyketides (8, 11–14, 21, 22, 27–31), and there is considerable interest in the possibility of generating useful novel polyketides through rational design and/or via combinatorial libraries of genetically engineered PKSs (31). Apart from actinomycetes, a potentially rich source of PKS genes for such approaches are filamentous fungi, which produce a wide array of aromatic polyketides (33). The potential for combinatorial biosynthesis with PKS genes will be greatly enhanced if it is possible to express such fungal genes in *S. coelicolor* CH999.

6-Methylsalicylic acid is an extensively studied polyketide from *Penicillium patulum*. The PKS responsible for its biosynthesis, 6-methylsalicylic acid synthase (6MSAS), catalyzes the three successive condensations required to make the eight-carbon molecule (7) (Fig. 1). All of the active sites required for a total of 11 transformations are carried on a single multifunctional protein (2), and catalysis involves repeated use of these active sites. Here, we describe the heterologous expression of the 6MSAS gene from *P. patulum* in *S. coelicolor* CH999 and the production in vivo of significant amounts of 6-methylsalicylic acid.

Reconstruction of the 6msas gene. The 6MSAS gene from *P. patulum* consists of two exons (exons 1 and 2) of 29 and 1,745 codons, respectively, separated by an intron of 69 bp (Fig. 2a). To allow optimal expression in *S. coelicolor*, a modified 6MSAS gene was constructed. The starting material was the cloned fragments (M1 and M41) that contain between them all of exon 2. Exon 1 was synthesized as a fragment containing suitable restriction sites for cloning. To facilitate high-level expression of the reconstructed gene in *Streptomyces* spp., a Shine-Dalgarno sequence complementary to the 3′ region of *S. coelicolor* 16S rRNA (1) was included in the fragment and four of the first seven codons of exon 1 were changed to synonymous codons most frequently used in *Streptomyces* spp. (40) (ATG CAT TCC GCT GCA ACT TCT was changed to ATG CAT TCC **GCC GCC ACC TCC**).

The modified 6MSAS gene was reconstructed as follows. A fragment containing all of exon 1 together with restriction sites for cloning was synthesized by using two 146-mers with complementary nucleotide sequences (G1638 and G1639; Fig. 2c). G1638 and G1639 were synthesized (with the trityl group on) with a 0.2 μM scale polystyrene support and reagents from Cruachem, Glasgow, Scotland, and a GeneAssembler Plus oligosynthesis machine (Pharmacia LKB) and purified with an oligonucleotide purification cartridge (Applied Biosystems). G1638 and G1639 (100 pmol each) were denatured in the same tube at 90°C for 2 min in 50 μl of restriction enzyme digestion buffer M (Boehringer) and then annealed by allowing the mixture to cool to room temperature to make a 146-bp double-stranded fragment (Fig. 2c). After digestion with *Bgl*II, the resulting 140-bp fragment was ligated into *Bgl*II-digested

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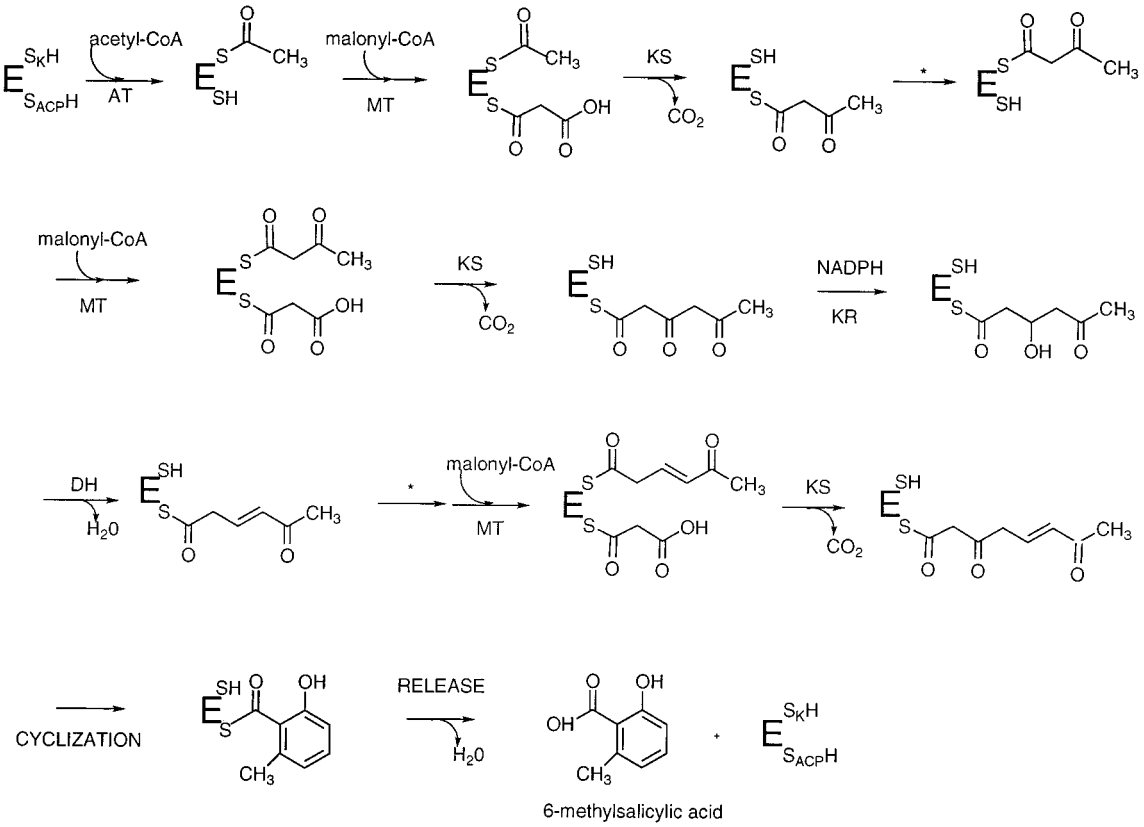

FIG. 1. Hypothetical reaction pathway for the formation of 6-methylsalicylic acid by *P. patulum* according to Dimroth et al. (7), Beck et al. (2), and O'Hagan (33). The loading of acetyl and malonyl groups onto PKS (E) on the active-site cysteine of the ketosynthase (S_K) and the prosthetic group of the ACP (S_{ACP}), respectively, is followed by condensation. Three decarboxylative condensations of malonyl chain extender units with the acetyl starter unit are followed by cyclization of the polyketide chain. Ketoeduction (KR) occurs only on the β-carbon formed after the second condensation. Dehydration (DH) may occur as indicated or following cyclization. Release of 6-methylsalicylic acid from the enzyme occurs by deacylation. AT, acetyl transfer; MT, malonyl transfer; KS, ketosynthesis; ∗, transacylation from ACP to ketosynthase active site.

pIJ2925 (20) and introduced into *Escherichia coli* DH5α by transformation (17). Sequence analyses of eight of the resulting clones showed that one of them (named pDB100) contained the intact 140-bp synthesized *Bgl*II fragment containing exon 1 (Fig. 2b). (The remaining seven clones contained deletions of between 1 and >100 bp from the center of this fragment.)

Exon 2 was constructed and joined with exon 1 in two stages (Fig. 2b). First, the 1.622-kb *Eco*RI-*Sal*I fragment of plasmid M41 (Fig. 2a, sites 3 to 5) was cloned into pDB100 digested with *Eco*RI and *Sal*I to make pDB101. Second, the 3.86-kb *Sal*I-*Sac*I fragment from plasmid M1 (Fig. 2a, sites 5 to 8) was cloned into pDB101 digested with *Sal*I and *Sac*I to make pDB102. pDB102 was passaged through *E. coli* ET12567 (*dam dcm hsdM* [24]) to remove Dam methylation that prevented digestion at the engineered *Xba*I cloning site, and then the 5.6-kb *Pac*I-*Xba*I fragment containing the entire modified 6MSAS gene was removed from pDB102 and cloned into pKAO18′ (20a) digested with *Pac*I and *Xba*I to make pDB106 (Fig. 3). pDB106 was passaged through *E. coli* ET12567 to overcome the methyl-specific restriction barrier of *S. coelicolor* (23) and then introduced into *S. coelicolor* CH999 (28, 29) by transformation, with selection of thiostrepton-resistant transformants (18).

pDB106 is a derivative of the expression vector pRM5 (28). In it, the modified 6MSAS gene is under the control of the *act*I promoter, which is positively regulated by the activator protein encoded by *act*II-ORF4, also carried on pDB106. Therefore, 6MSAS gene expression was expected to occur at the onset of the stationary phase, when the natural polyketide actinorhodine is produced in *S. coelicolor* (15). (pDB106 also carries the *act*III gene, encoding a ketoreductase from the actinorhodin biosynthetic pathway; this gene product was not expected to associate productively with 6MSAS or to interfere with the formation of 6-methylsalicylic acid.) In addition to the features derived from pRM5, pDB106 has the special advantage of pKAO18′, namely, a kanamycin resistance gene inserted into the *Hin*dIII site of pRM5 to allow selection for plasmid stability in *E. coli* by culturing on both kanamycin and carbenicillin (Fig. 3).

6-Methylsalicylic acid analysis. Three transformants of *S. coelicolor* CH999 containing pDB106 and a control transformant containing pKAO18′ were analyzed for production of 6-methylsalicylic acid. A confluent lawn of each transformant was grown on an R5 agar plate (18) at 30°C for 6 days. The agar plus mycelium was chopped and extracted with 100 ml of ethyl acetate–1% acetic acid. The solvent was removed under vacuum, and the product was resuspended in methanol. Thin-layer chromatography analysis in ethyl acetate–1% acetic acid was used to reveal a UV-fluorescent compound in the three CH999/pDB106 transformants; no such compound was present in the control CH999/pKAO18′.

To characterize the UV-fluorescent compound, one pDB106 transformant was grown on 12 agar plates. The product was

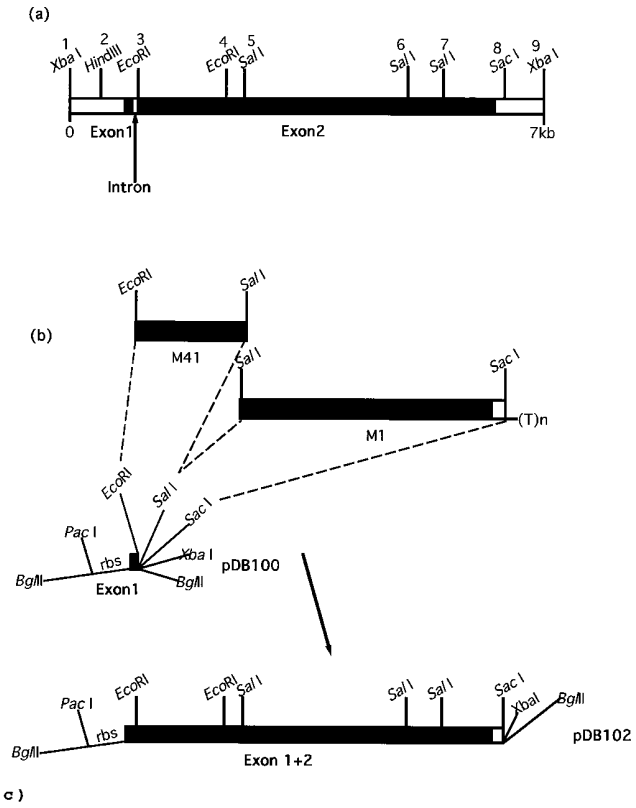

FIG. 2. Structure of the 6MSAS gene and clones. (a) Schematic of the 6MSAS gene of *P. patulum* and surrounding DNA according to Beck et al. (2). Filled boxes represent coding sequences (exons 1 and 2); open boxes represent noncoding sequences, including the intron separating exons 1 and 2. (b) Reconstruction of the 6MSAS gene. Plasmids M41 and M1 were used to reconstruct exon 2. M41 contains the 1.622-kb *Eco*RI-*Sal*I fragment (sites 3 to 5) cloned into pUC18. M1 contains the 3.86-kb *Sal*I-*Sac*I fragment (sites 5 to 8) cloned into pUC19. M41 is actually cDNA and contains short sequences upstream of *Sal*I site 5 and downstream of *Sac*I site 8, including a poly(T) sequence. rbs, ribosome binding site. (c) Synthesized fragment (146 bp) comprising exon 1 and engineered cloning sites. Oligonucleotides G1638 and G1639 were used to make the top and bottom strands, respectively.

extracted as described above and purified by preparative C_{18} reverse-phase high-pressure liquid chromatography (Beckman) (mobile-phase acetonitrile, 15 to 60% gradient, in water and in the presence of 1% acetic acid for 50 min), and spectra were determined at wavelengths of 254 and 280 nm. Fractions corresponding to the major absorbance peak (20 to 22 min) were pooled, the solvent was extracted, and the product was dissolved in 0.6 ml of deuterated dimethyl sulfoxide (DMSO-d_6). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) analyses confirmed the presence of 6-methylsalicylic acid. (^{1}H NMR [DMSO-d_6, 400 MHz] δ [ppm] 2.43 [s, 3H], 6.79 [d, J = 7.6 Hz, 1H], 6.83 [d, J = 8.24 Hz, 1H], 7.28 [dd, J = 7.92 Hz/7.92 Hz, 1H], with tetramethylsilane as the internal reference.) (^{13}C NMR [DMSO-d_6, 100 MHz] δ [ppm] 20.9, 113.9, 119.0, 121.2, 131.4, 137.7, 157.5, and 171.1, with DMSO-d_6 as the internal reference.) The ^{1}H NMR spectrum was consistent with that reported by Hamada et al. (16). Approximately 20 mg of 6-methylsalicylic acid was produced from 12 agar plates (a total of 300 ml of medium), an amount comparable to the highest yields for actinomycete polyketide production in the plasmid expression system in CH999.

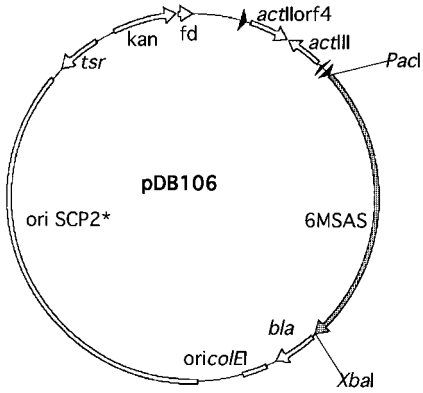

FIG. 3. Expression plasmid pDB106. Arrows represent coding sequences. *tsr*, *Streptomyces azureus* thiostrepton resistance gene; kan, kanamycin resistance gene of pUC4K (Pharmacia); *bla*, β-lactamase gene encoding carbenicillin resistance; 6MSAS, the engineered 6MSAS gene. Filled arrowheads represent *act* gene promoters. SCP2* and *colE*I origins (ori) of replication for plasmid replication in *S. coelicolor* and *E. coli*, respectively, are indicated by open boxes. fd, transcription terminator from phage fd. pDB106 was derived from pKAO18′ by replacing the *act*I gene between the *Pac*I and *Xba*I sites with the engineered 6MSAS gene.

***S. coelicolor* as a host for fungal PKS expression.** Expression of the 6MSAS gene in *S. coelicolor* CH999 has several important implications. The production of a significant amount of 6-methylsalicylic acid demonstrates that the enzyme, which probably acts as a tetramer (39), was folded into the correct tertiary structure. Furthermore, posttranslational addition of the 4′-phosphopantetheine prosthetic group to the acyl carrier protein (ACP) segment of 6MSAS must have occurred. Functional expression of a heterologous PKS in a similar expression plasmid in *S. coelicolor* CH999 has been demonstrated for the erythromycin PKS genes from *Saccharopolyspora erythraea* (21). In contrast, the erythromycin PKS proteins isolated from *E. coli* could not direct chain extension in vitro (although acyl transfer occurred) because of the lack of 4′-phosphopantetheine group addition (25, 35). The type II *Streptomyces glaucescens* PKS ACP component from the tetracenomycin C antibiotic gene cluster also lacked significant addition of the 4′-phosphopantetheine group when it was expressed in *E. coli* (36). On the other hand, the recently reported expression of *S. coelicolor* actinorhodin ACP in active form in *E. coli* under specific culture conditions suggests that it is possible to use *E. coli* as a host for the production of active heterologous PKSs (5).

Production of erythromycin PKS and 6MSAS in active forms in *S. coelicolor* CH999 augurs well for the exploitation of this prokaryotic expression system for further analysis of polyketide biosynthesis in general. In particular, successful expression of an active fungal PKS gene suggests that the wide range of polyketide structures available from fungi should be accessible for this combinatorial approach. The diversity among PKSs

from fungi is indicated by several unusual features of fungal polyketides. Aromatic fungal polyketides have a wide range of chain lengths, with all lengths between 6 and 18 carbons found abundantly (38). Whereas bacterial polyketides typically derive methyl side groups from the incorporation of propionate chain extender units from methyl malonate, in fungal polyketides propionate is used only as a starter unit and methyl side groups are added from *S*-adenosyl-L-methionine (34). Indeed, sequence analysis of lovastatin PKS from *Aspergillus terreus* has revealed a potential methyltransferase segment within this multifunctional PKS (6). Some fungal polyketides are wholly or largely reduced (for instance, lovastatin and compactin), and in some cases, they undergo unusual chain cyclization (for instance, bikaverin) (33).

In addition to the diverse nature of fungal PKS enzymology, the fact that fungal PKSs are found as single proteins facilitates both heterologous gene expression and structural analysis. The inherent stability of a multienzyme system encoded by a single protein is one reason why the only complete PKSs purified to homogeneity as active enzymes are fungal PKSs (34). Expression of such PKSs in genetically well-characterized *S. coelicolor* will allow the use of genetic manipulations (such as site-directed mutagenesis) to dissect PKS structure-function relationships, an approach that is not yet possible for the majority of polyketide-producing fungi.

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