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Heterologous Expression of Hybrid Type II Polyketide Synthase System in *Streptomyces* Species

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Abstract Polyketides are an extensive class of secondary metabolites with diverse molecular structures and biological activities. A plasmid-based minimal polyketide synthase (PKS) expression cassette was constructed using a subset of actinorhodin (*act*) biosynthetic genes (*actI-orf1*, *actI-orf2*, *actI-orf3*, *actIII*, *actVI*, and *actIV*) from *Streptomyces coelicolor*, which specify the construction of an orange-fluorescent anthraquinone product aloesaponarin II, a type II polyketide compound derived from one acetyl coenzyme A and 7 malonyl coenzyme A extender units. This system was designed as an indicator pathway in *S. parvulus* to generate a hybrid type II polyketide compound via gene-specific replacement. The *act* β-ketoacyl synthase unit (*actI-orf1* and *actI-orf2*) in the expression cassette was specifically replaced with oxytetracycline β-ketoacyl synthase (*otcY-orf1* and *otcY-orf2*). This plasmid-based hybrid PKS cassette generated a novel orange-fluorescent compound structurally different from aloesaponarin II in both *S. lividans* and *S. parvulus*. In addition, several additional distinctive blue-fluorescent compounds were detected, when this hybrid PKS cassette was expressed in *S. coelicolor* B78 (*actI-orf2* mutant), implying that the expression of plasmid-based hybrid PKS cassette in *Streptomyces* species should be an efficient way of generating hybrid type II polyketide compounds.

Key words: *Streptomyces*, hybrid polyketide, actinorhodin, oxytetracycline

Polyketide natural products represent a major class of complex organic natural products produced by bacteria, fungi, and plants [9, 11, 22]. Among various polyketide-producing organisms, *Streptomyces* species, a Gram-positive soil bacteria, have been recognized to be the most important bacterial species as a source of valuable polyketide compounds including antibiotics and anticancer drugs [3, 8, 9, 11, 14]. The mechanistic similarity of their biosynthesis to that of long-chain fatty acids has been well characterized and reviewed elsewhere [2, 4, 9]. In contrast to the rather limited array of fatty acid structures used for membrane lipids, polyketides are known for their remarkable structural diversity, which results from variations in their starter and extender units, carbon chain lengths, substituent functionalization, and stereochemistry [11]. Based on biosynthetic mechanisms, two types of polyketide biosynthetic systems (type I and type II), which are related structurally to analogous systems for production of fatty acids, have been identified [9, 10, 11]. Unlike modular-type multi-functional type I system, the multi-component type II polyketide synthase (PKS) systems have been established as the biosynthetic machinery for numerous polycyclic aromatic natural products [9]. Typically in these systems, an acetyl-CoA starter unit and malony-coA extender units are used for carbon chain construction through a cycle of condensation steps. Functional analysis of the individual components of type II PKS systems [1, 12, 13, 15, 23] has proved 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 [DYD/DH], and ketoreductase) has been obtained using *trans* complementation [15, 23], and *cis* replacement [12, 13] strategies in the actinorhodin (*act*) PKS [6, 18, 19]. Although earlier studies relied on the production of the characteristic blue pigment of actinorhodin as a phenotypic marker for the functional replacement of individual PKS components [12, 13, 15, 23], more sophisticated systems are being developed to produce and analyze novel polyketide compounds produced by replacement [20, 21] or the modification of heterologous PKS genes. Here, we describe the expression of a plasmid-based hybrid type II PKS expression cassette, a versatile indicator

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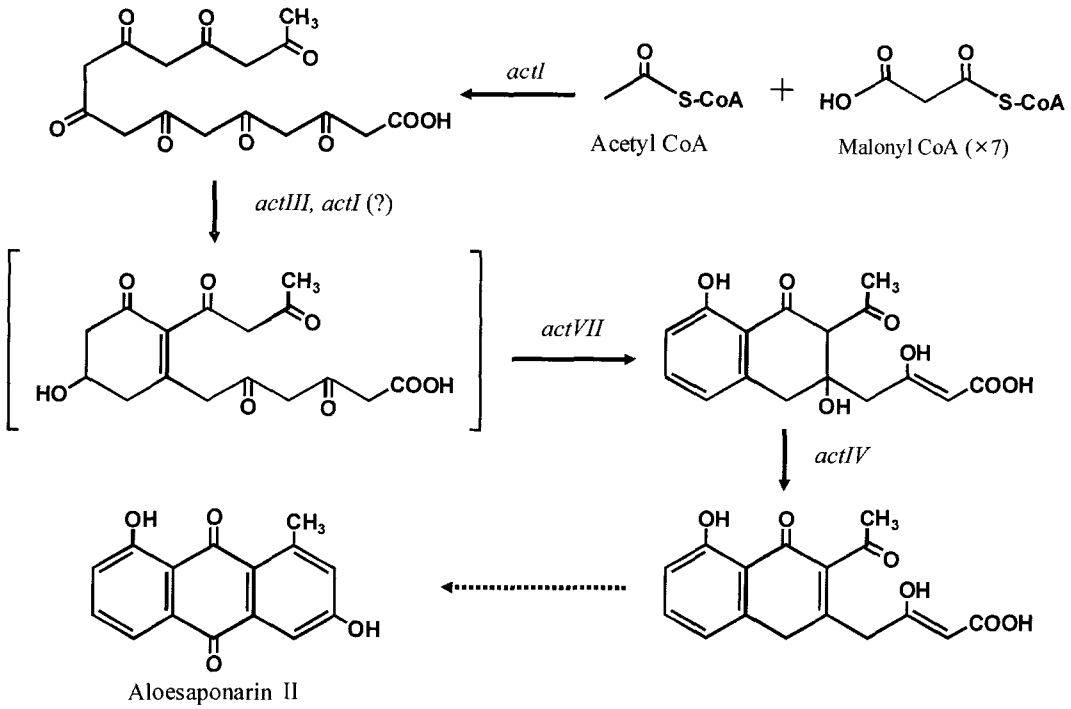

Fig. 1. Proposed biosynthetic pathway for actinorhodin shunt product, aloesaponarin II.
Solid and dotted arrows represent presumed enzymatic and spontaneous steps, respectively. Structures in the brackets are hypothetical intermediates.

biosynthetic pathway in several *Streptomyces* species, and evaluate the potential application of this system for hybrid polyketide production.

A plasmid-based minimal PKS expression cassette was previously 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 an orange-fluorescent anthraquinone product aloesaponarin II, a type II polyketide compound derived from one acetyl coenzyme A and 7 malonyl coenzyme A extender units [Fig. 1, 16]. This plasmid-based minimal PKS expression cassette, named pESK301, was also modified so as to contain two unique *Nde*I restriction sites at the start codon of *actI-orf1* and the stop codon of *actI-orf2* to facilitate the in-frame replacement of β-ketoacyl synthase genes [Fig. 2; 16]. The pESK301 was then used to test the effect of replacement of the *actI-orf1/orf2* with *otcY-orf1/orf2*, a homolog that mediates the construction of an oligoketide derived from a presumed malonamyl-CoA starter unit and 8 malonyl-CoA extender units for the construction of oxytetracycline in *S. rimosus* [Fig. 2; 17]. This replacement was designed to show the possibility of hybrid polyketide production as well as to determine whether the *otcY-orf1/orf2* gene products specify (or are limited to) the use of malonamyl-CoA as a starter unit and chain extension using malonyl-CoA units. The *Nde*I fragment of pESK301 was replaced by *Nde*I fragments containing the *otcY-orf1/orf2* genes, generated by PCR using modified primers containing *Nde*I sites at the 5' termini, resulting in pESK303 (primer-1 5'-GAAGGAGC-

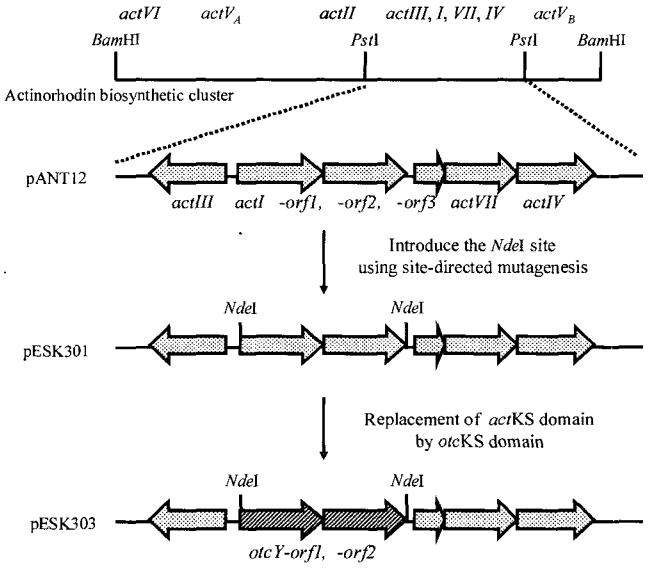

Fig. 2. Schematic presentation of plasmid-based hybrid PKS constructs.
a) Actinorhodin biosynthetic cluster in the chromosome of *S. coelicolor*; b) pANT12, a 8.8-kb *Pst*I fragment cloned into a pIJ350 [1]; c) pESK301, a pANT12 derivative containing two *Nde*I sites; d) pESK303, a pESK301 derivative containing heterologous oxytetracycline β-ketoacyl synthase genes (*otcY-orf1* and *otcY-orf2*) from the oxytetracycline pathway.

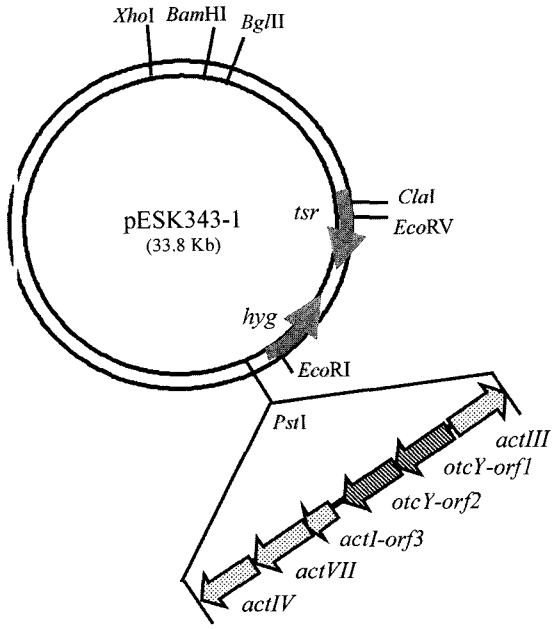

Fig. 3. Restriction map of pESK343-1, a hybrid construct containing heterologous oxytetracycline β-ketoacyl synthase genes (*otcY-orf1* and *otcY-orf2*) from the oxytetracycline pathway cloned into pIJ941.

'GTTCCATATGTCCAAG-3', primer-2: 5'-GGGACTG-ACATATGCGAGAA-3' (Fig. 2). The pESK303 was introduced into *S. parvulus* and subsequently grown in a nitrogen-defined medium (NDM) for polyketide production [5, 15]. *S. parvulus* containing pESK303, however, failed to produce aloesaponarin II or any other detectable new polyketide compound (data not shown). Because it was suggested that the expression cassette cloned in a low copy number plasmid produced about 10 times more polyketide product than when in a high copy number plasmid (personal communication with Prof. David Sherman), the 8.8-kb *Pst*I fragment of pESK303 was subcloned into the pIJ941, resulting in pESK343-1 (Fig. 3). The pESK343-1 was then introduced into *S. parvulus* and subsequently grown in NDM for polyketide production [16]. Total extract from *S. parvulus* containing pESK343-1 was then compared with that from *S. parvulus*/pIJ941 (negative control) to detect any new compound produced. As shown in Fig. 4a, a new orange fluorescence compound was detected by TLC only in the extract of *S. parvulus*/pESK343-1. A very similar spot was also detected from the extract of *S. lividans*/pESK343-1 (data not shown). However, the amount of the new compound produced by *S. parvulus*/pESK343-1 was extremely low and inconsistent. Although the exact chemical characteristics of the new compound need to be further determined, the TLC data clearly suggested that this new compound, a less polar orange fluorescent compound produced by the hybrid type II PKS system, could be a structurally similar yet distinct polyketide compound as compared to aloesaponarin II. Attempts to increase the production by *S. parvulus*/pESK343-1 using different growth conditions and media are currently under investigation.

To further evaluate the host-dependent expression of the plasmid-based hybrid PKS cassette, the pESK343-1 was used to transform another polyketide non-producer, *S. azureus*. Unlike *S. parvulus*, however, *S. azureus*/pESK343-1 failed to produce aloesaponarin II or any other detectable fluorescent compound (data not shown), suggesting that the expression of the hybrid PKS cassette depends on the specific *Streptomyces* species. Because the hybrid PKS expression cassette contains an inducible promoter system regulated by the *actII-orf4* gene present in the *S. coelicolor* chromosome [19], heterologous *Streptomyces* species such as *S. parvulus* (probably not *S. azureus*) might contain an *actII-orf4* homolog regulatory protein for the efficient expression of the hybrid PKS cassette. Finally, the pESK343-1 was also expressed in the *S. coelicolor* B78 mutant, which exhibited an actinorhodin nonproducing phenotype due to an *actI-orf2* mutation in the chromosome [16]. *S. coelicolor* B78/pESK343-1 cultured in an NDM medium produced several distinct blue-fluorescent compounds with more polar characteristics as compared to aloesaponarin II detected by TLC (Fig. 4b). Unlike other polyketide nonproducing *Streptomyces* species, *S. coelicolor* B78 contains functional genes of the entire actinorhodin biosynthetic pathway except *actI-orf2*, implying that the hybrid polyketide compound produced by the plasmid-based hybrid PKS cassette could

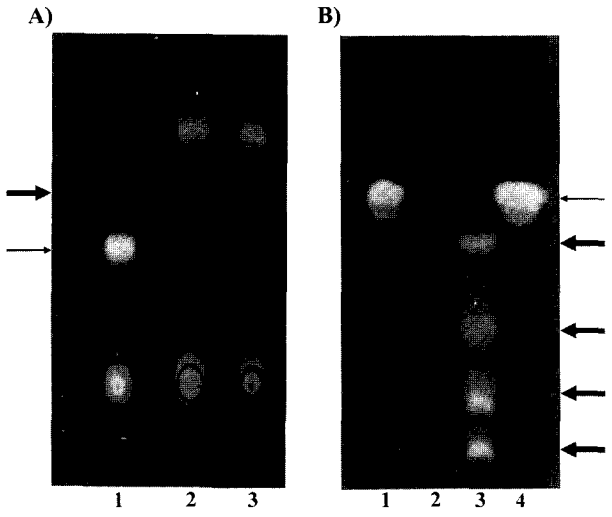

Fig. 4. TLC analysis of polyketide production by *Streptomyces* containing pESK343-1.

a) Lane 1, aloesaponarin II standard (indicated by thin arrow); lane 2, *S. parvulus*/pESK343-1; lane 3, *S. parvulus*/pIJ941. A new orange fluorescent compound (indicated by thick arrow) different from aloesaponarin II was detected in *S. parvulus*/pESK343-1. b) Lane 1 and lane 4, aloesaponarin II standard (indicated by thin arrow); lane 2, *S. coelicolor* B78/pIJ941; lane 3. *S. coelicolor* B78/pESK343-1. New blue fluorescent compounds (indicated by thick arrows) different from aloesaponarin II were detected in *S. coelicolor* B78/pESK343-1.

have been further modified by the late actinorhodin biosynthetic pathway genes in *S. coelicolor* B78. In conclusion, the construction of the plasmid-based hybrid type II PKS cassette along with a proper heterologous expression in *Streptomyces* species should provide an efficient way of generating novel type II polyketide compounds.

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