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Orthogonal Protein Interactions in Spore Pigment Producing and Antibiotic Producing Polyketide Synthases

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Abstract The actinomycetes produce antibiotics as well as spore pigments during their life cycle by using Type II polyketide synthases (PKSs). Each PKS minimally consists of a ketosynthase heterodimer and an acyl carrier protein. The acyl carrier protein has been shown to be interchangeable among different antibiotic producing Type II PKSs. Surprisingly, we have discovered a fundamental incompatibility between the ketosynthases and acyl carrier proteins from antibiotic producing pathways and those from spore pigment pathways. Although antibiotic PKSs can interact with acyl carrier proteins from spore pigment pathways, spore pigment PKSs are unable to recognize acyl carrier proteins from polyketide antibiotic pathways. This observation provides an insight into a critical mechanism by which natural product biosynthetic specificity is exercised by members of this bacterial family.

Keywords polyketide synthase, ketosynthase, acyl carrier protein, spore pigment, antibiotic

The actinomycetes, which include the genus *Streptomyces*, are soil bacteria that are well known for their exceptional ability to produce biologically active compounds [1]. Thousands of antibiotics have been identified from these microorganisms, which are frequently isolated as colored spores from soil samples [2]. The antibiotics and the spore pigments of the actinomycetes are biosynthesized as secondary metabolites by polyketide synthases (PKSs). Although the genes encoding numerous antibiotic producing PKSs have been studied to date [3~5], only a few spore pigment producing PKSs, including *whiE* gene cluster of *Streptomyces coelicolor*, have been analyzed [6~9]. In part, this difference is due to the difficulty of isolating and characterizing spore pigments, which are presumably covalently attached to macromolecular components of spores [10].

Although the precise structures of most spore pigments are unknown, their biosynthesis is catalyzed by type II PKSs, analogous to the biosynthesis of polyfunctional aromatic polyketides such as actinorhodin, tetracycline and daunorubicin. By expressing subsets of various spore pigment biosynthetic genes and characterizing the resultant products, Moore *et al.* were able to identify some of the salient structural features of spore pigments from *S. coelicolor* as well as *S. halstedii* [11] (Fig. 1). In turn, these findings yielded a range of new polyketides *via* combinatorial biosynthesis [11].

By analyzing mutant strains of *S. coelicolor*, which produces both actinorhodin and the *whiE* spore pigment, it has been suggested that the polyketide synthase components from antibiotic pathways and spore pigment pathways are interchangeable [7, 12]. Based on this observation, we attempted to engineer an alkylacyl-primed analog of aklanonic acid by co-expressing the C_{24} *sch* or *whiE* spore pigment PKSs and the genes encoding the R1128 initiation PKS module [13]. Unexpectedly, no products corresponding to a productive interaction between

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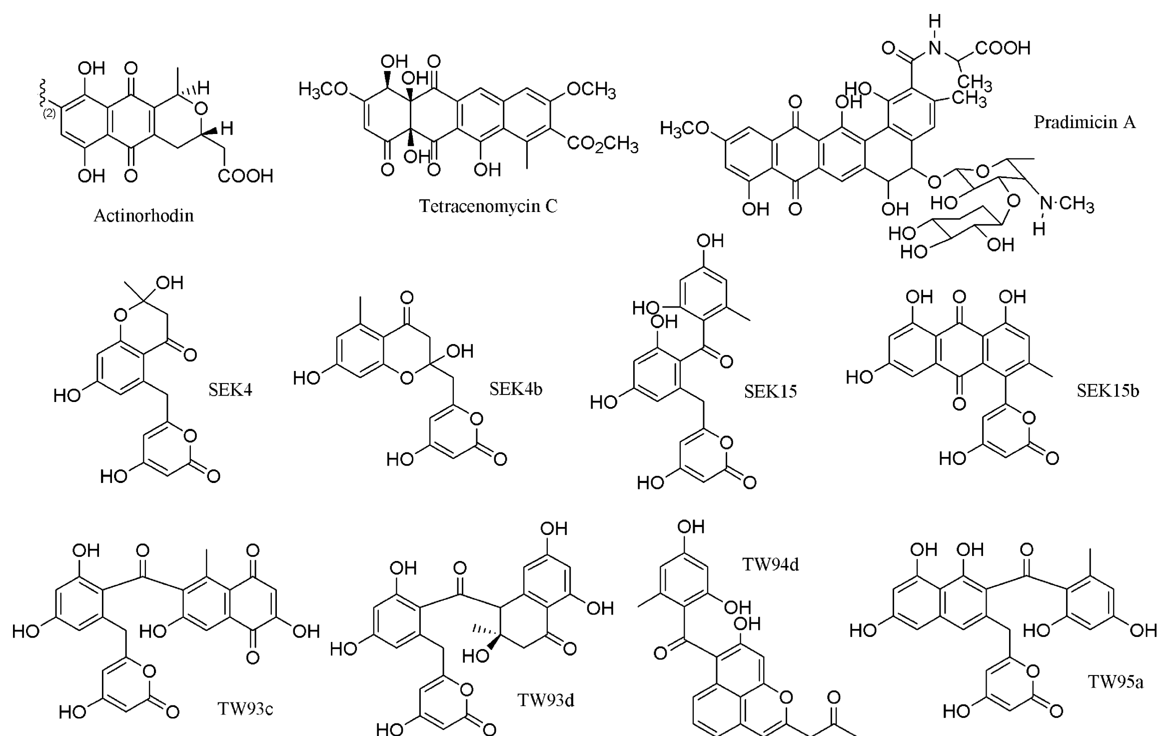

Fig. 1 Aromatic polyketides.

Actinorhodin, tetracenomycin C, and pradimicin A are antibiotics biosynthesized by natural polyketide synthases. SEK-compounds and TW-compounds are engineered polyketides biosynthesized from reconstituted antibiotic and spore pigments producing PKSs, respectively.

the R1128 initiation module and either spore pigment elongation module were observed. In contrast, when an equivalent C_{24} ketosynthase heterodimer from the pradimicin (*pms*) PKS was recombined with the R1128 initiation module, the engineered host was able to afford the expected products of the bimodular PKS. This observation suggested that there may be a fundamental incompatibility between PKS components from antibiotic and spore pigment pathways. Here, we test this hypothesis *via* a series of *in vitro* assays utilizing purified PKS proteins from different antibiotic and spore pigment pathways.

Although previous studies had shown that acyl carrier proteins (ACPs) from antibiotic biosynthetic pathways could be interchanged *in vivo* [14] and *in vitro* [15] without significant kinetic penalties, we speculated that the observed lack of crosstalk between heterologous spore pigment and antibiotic PKS subunits was primarily due to their inability to engage in catalytically productive protein-protein interactions. To test this hypothesis, we first investigated the properties of the ketosynthase-chain length factor (KS-CLF) heterodimer and ACP from the *sch* PKS, a representative dodecaketide synthase involved in spore pigment biosynthesis [8]. Purified *sch* KS-CLF was titrated *in vitro* with various ACPs (Fren N from frenolicin PKS, Zhu N from R1128 PKS, GraACP from granaticin PKS, WhiE ACP from whiE PKS, and Pms ACP from pradimicin PKS) [11, 15, 16]. Assays were performed at 30°C in 10 μl of reaction buffer containing 10% glycerol as previously described [15]. With 2 μM of the KS-CLF heterodimer, 0.5 μM malonyl-CoA : ACP transacylase (MAT), and 30 μM of different holo-ACPs in each reaction, the reaction was initiated by adding 2 mM ^{14}C-malonyl-CoA and quenched by adding 10 μl 12.5% SDS after 30 minutes. For comparison, *act* and *tcm* KS-CLFs were also assayed along with *sch* KS-CLF. Each quenched mixture was extracted with ethyl acetate and analyzed by thin-layer chromatography (TLC). The amount of product accumulated at the end of the reaction is shown in Fig. 2, and the kinetic parameters associated with each biosynthetic reaction are reported in Table 1. The identity of the product of each reaction mixture was verified by comparison with authentic standards of SEK4/4b, SEK15/15b, and TW93c/93d isolated from appropriate recombinant cell lines [11, 13, 17] (Fig. 1).

Our data revealed that the *sch* KS-CLF could synthesize polyketide in conjunction with the WhiE ACP but not any other heterologous ACP from antibiotic biosynthetic pathways, including the *fren*, *gra*, *dps*, and R1128 PKSs (Fig. 2). The absolute rate of dodecaketide synthesis *in vitro* by the *sch* KS-CLF outfitted with the WhiE ACP was

Table 1 Activities of various KS-CLFs toward ACPs

		FrenN	ZhuN	Gra ACP	WhiE ACP	Pms ACP
act KS-CLF	K_m (μM)	5.8±0.4	2.7±0.2	6.4±1.5	3.8±2.1	3.7±0.2
	k_{cat} (min^{-1})	0.27±0.005	0.18±0.002	0.17±0.01	0.13±0.02	0.11±0.02
	k_{cat}/K_m (min^{-1} mM^{-1})	47	66	27	34	31
	k_{rel}	1	1.40	0.57	0.72	0.66
tcm KS-CLF	K_m (μM)	2.3±0.3	1.4±0.2	1.6±0.3	3.0±1.2	2.8±0.6
	k_{cat} (min^{-1})	0.32±0.01	0.31±0.01	0.36±0.01	0.13±0.01	0.15±0.08
	k_{cat}/K_m (min^{-1} mM^{-1})	139	219	225	43	56
	k_{rel}	1	1.58	1.62	0.31	0.40
sch KS-CLF	K_m (μM)				2.1±1.2	
	k_{cat} (min^{-1})	No Activity	No Activity	No Activity	0.37±0.06	No Activity
	k_{cat}/K_m (min^{-1} mM^{-1})				176	
pms KS-CLF	K_m (μM)	1.6±0.5	1.7±0.6	2.8±0.6	2.3±0.7	2.7±0.4
	k_{cat} (min^{-1})	0.20±0.01	0.28±0.02	0.95±0.05	0.61±0.04	0.28±0.01
	k_{cat}/K_m (min^{-1} mM^{-1})	126	163	340	266	106
	k_{rel}	1	1.29	2.70	2.11	0.84

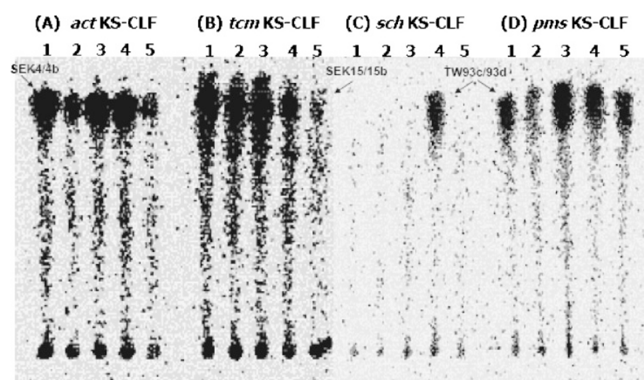

Fig. 2 Polyketide biosynthesis *in vitro* with heterologous combinations of ACP and KS-CLF from various antibiotic and spore pigment polyketide synthases (PKSs).

Radiolabeled polyketides synthesized by the minimal PKS from ^{14}C-malonyl-CoA are detected by thin layer chromatography, followed by phosphorimager counting. (A) *act* KS-CLF, (B) *tcm* KS-CLF, (C) *sch* KS-CLF, (D) *pms* KS-CLF. Five different ACPs were used, including (FrenN(1), ZhuN(2), Gra ACP(3), WhiE ACP(4), Pms ACP(5)) are used. Different solvent conditions were used for similar Rf of polyketide products. For experimental details, see text.

comparable to the rate of octaketide and decaketide synthesis by the *act* and *tcm* KS-CLF, respectively (Table 1). Thus, it appears that spore pigment KS-CLF heterodimers have exceptionally high specificity for ACPs from spore pigment pathways. In contrast, the *act* and *tcm* KS-CLFs were able to interact with the WhiE ACP fruitfully, producing the expected octaketides (SEK4 and SEK4b) and decaketides (SEK15 and SEK15b), respectively. Since the rate of malonylation of holo-ACP by MAT is much faster than that of condensation of the malonyl-ACP by KS/CLF [15], the MAT catalyzed reaction is unlikely to be the bottleneck in the above assay.

To rule out the possibility that chain length differences are responsible for the above observations, we extended these findings by assaying purified *pms* KS-CLF. The *pms* KS-CLF was cloned from *Actinomadura hibisca* [16], expressed in *S. coelicolor* and purified as described for the tcm KS-CLF [14] to >80% homogeneity. Consistent with *in vivo* observations [13], the purified *pms* KS-CLF synthesized the expected dodecaketides at wild-type or near wild-type rates in the presence of either heterologous antibiotic or spore pigment ACPs (Table 1).

At first glance our findings may appear somewhat contradictory with *in vivo* results of Yu and Hopwood [7]. These investigators reported that, not only could the *whiE* ACP gene complement a lesion in the *act* ACP gene to yield actinorhodin, but the *act* ACP gene could also partially complement a mutant *whiE* ACP gene to induce pale grey pigmentation of spores. The precise reasons for this difference are unclear. Perhaps it reflects a somewhat relaxed ACP specificity of the *whiE* KS-CLF as compared to the *sch* KS-CLF. This is unlikely because experiments in our own laboratory involving co-expression of genes encoding the *whiE* KS-CLF and either ACP from the R1128 PKS yielded no polyketide product (unpublished results). Alternatively, Yu and Hopwood's observation may not reflect true complementation, rather the spores may

have appeared pale grey due to diffusion of a small amount of actinorhodin from the substrate mycelium into spores. (A similar pale grey color was also observed when the *act* KS-CLF was used to complement a mutant *whiE* KS-CLF.) Further work will be required to understand the apparent partial complementation of the *whiE* spore pigment pathway *in vivo* by the *act* ACP.

In conclusion, we have uncovered a fundamental incompatibility at the level of protein-protein interactions between antibiotic producing PKSs and spore pigment producing PKSs. This finding may contribute to understand the biochemical logic of PKS specificity during contemporaneous antibiotic and spore pigment synthesis in the life cycle of an actinomycete bacterium. It could also pave the way to engineer new types of "hybrid" polyketides by rationally combining spore pigment and antibiotic biosynthetic pathways.

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