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The Hidden Steps of Domain Skipping: Macrolactone Ring Size Determination in the Pikromycin Modular Polyketide Synthase

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Summary

The pikromycin (Pik) polyketide synthase (PKS) from *Streptomyces venezuelae* comprises four multifunctional polypeptides (PikAI, PikAII, PikAIII, and PikAIV). This PKS can generate 12- and 14-membered ring macrolactones (10-deoxymethynolide and narbonolide, respectively) through the activity of its terminal modules (PikAIII and PikAIV). We performed a series of experiments involving the functional replacement of PikAIV in mutant strains with homodimeric and heterodimeric PikAIV modules to investigate the details of macrolactone ring size determination. The results suggest a new and surprising mechanism by which the penultimate hexaketide chain elongation intermediate is transferred from PikAIII ACP_5 to PikAIV ACP_6 before release by the terminal thioesterase domain. Elucidation of this chain transfer mechanism provides important new details about alternative macrolactone ring size formation in modular PKSs and contributes to the potential for rational design of structural diversity by combinatorial biosynthesis.

Introduction

Polyketides are a diverse class of natural products that possess antibiotic, immunosuppressive, anti-cancer, and other biological activities [1]. The synthesis of many of these compounds depends on the activity of modular polyketide synthases (PKSs), which are giant and complex multienzyme systems that catalyze the condensation of simple carboxylic acids such as acetate and propionate [2–5]. The enzymatic activities responsible for each successive condensation reaction are physically grouped and have been designated "modules" [3]. Each module contains an acyl transfer (AT) domain that loads an appropriate extender unit onto an acyl carrier protein (ACP). The ketosynthase (KS) domain catalyzes a Claisen condensation between the extender unit and the growing polyketide chain from the proceeding module, generating an ACP-bound β-carbonyl intermediate. The extent of the β-carbonyl group reduction that precedes the next chain extension is determined by the presence of a ketoreductase (KR), an enoyl reductase (ER), and a dehydratase (DH) within the module. A thioesterase (TE) domain covalently linked to the final module cleaves the acyl chain, presumably from the adjacent ACP domain, and this cleavage leads to the formation of a macrolactone.

Biochemical analysis of the erythromycin PKS (DEBS1, DEBS2, and DEBS3) resulted in the development of a model in which the DEBS multienzymes are dimeric and each module interacts with its complement in a helical arrangement that is both head-to-head and head-to-tail [6]. Mutagenesis studies with the DEBS1+TE protein that contains active site mutations in individual KS, AT, and ACP domains have been used to model the functional dimerization of polyketide synthases [7, 8]. Homodimeric complexes of these mutants failed to make the predicted triketide lactone product when provided with methylmalonyl-CoA as a substrate. However, specific heterodimeric complexes of these mutants were functional, indicating that the mutations disrupted the catalytic activity of the complex and not the structure or dimerization. These efforts have demonstrated that the KS domain within a module interacts with the ACP on the complementary polypeptide, whereas the AT domain can act on either ACP [7, 8].

Streptomyces venezuelae ATCC 15439 is known to produce the 12-membered ring macrolides methymycin, neomethymycin, and novamethymyin [9] as well as the 14-membered ring macrolides narbomycin and pikromycin (Figure 1). These 12- and 14-membered ring macrolides are derived from the aglycones, 10-deoxymethynolide and narbonolide, respectively. A single PKS containing four separate polypeptides (PikAI-PikAIV) is responsible for synthesis of the aglycone intermediates. Initiation of polyketide biosynthesis by PikAI and elongation through PikAIII provide hexaketide (**1**) and lead to 10-deoxymethynolide, whereas narbonolide production requires an additional extension of the hexaketide chain intermediate with methylmalonylCoA. This extension is catalyzed by PikAIV and leads to heptaketide (**2**).

The unique ability of the Pik PKS to generate 12- and 14-membered ring macrolactones presents an opportunity to explore the fundamental processes of polyketide synthesis, specifically the mechanistic details of the chain extension process and the flexibility of the TE domain. Previous complementation experiments with a mutant strain of *S. venezuelae* that expresses PikAIV lacking a TE domain demonstrated that alternative expression of the final module determines macrolactone ring size [10]. Expression of full-length PikAIV [the last module required for hexaketide (**1**) to heptaketide (**2**) chain elongation] generates the 14-membered ring macrolactone narbonolide, whereas expression of an N-terminally truncated form of PikAIV (in which the alternative translation start codon 600 amino acids downstream of the normal *pikAIV* start codon is used) results in synthesis of the 12-membered ring macrolactone 10-deoxy-

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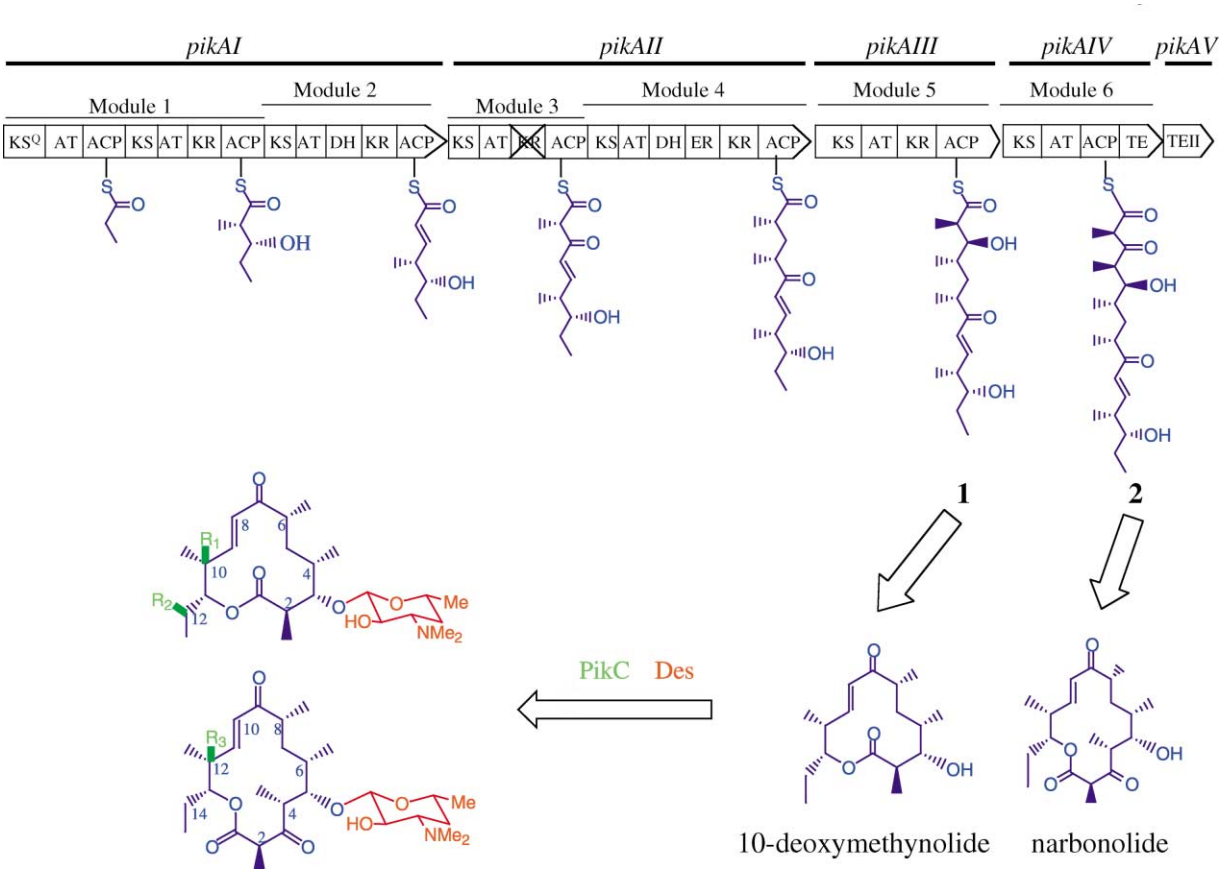

Figure 1. The Structure and Biosynthesis of Methymycin, Neomethymycin, Novamethymycin, Narbomycin, and Pikromycin in *S. venezuelae*

Methymycin: R_1 = OH, R_2 = H; neomethymycin: R_1 = H, R_2 = OH; novamethymycin; R_1 = OH, R_2 = OH; narbomycin R_3 = H; pikromycin R_3 = OH; the individual enzymatic domains in the multifunctional PKS protein. This figure uses the following abbreviations: ACP, acyl carrier protein; KS, β-ketoacyl-ACP synthase; KS^Q, a KS-like domain; AT, acyltransferase; KR, β-ketoacyl-ACP reductase (note the inactive KR domain in PikAII); DH, β-dehydratase; ER, enoyl reductase; TEI, thioesterase; TEII, type II thioesterase. Des represents all eight enzymes in desosamine biosynthesis and transfer. PikC catalyzes the final hydroxylation reaction in the synthesis of methymycin, neomethymycin, novamethymycin, and pikromycin.

methynolide. One hypothesis for this observation was that an interaction between PikAIII and an N-terminally truncated form of PikAIV enables the PikAIV TE to catalyze the release of hexaketide (**1**) from the ACP_5 domain of PikAIII. An alternative possibility is that the penultimate chain elongation intermediate (**1**) is transferred (without elongation) and released directly from PikAIV ACP_6 via the adjacent TE domain.

In a recent report based on studies with an engineered DEBS-based hybrid modular PKS, Leadlay and coworkers observed a process whereby the polyketide chain elongation intermediate bypasses or "skips" an entire module [11]. The Pik PKS might employ a similar process in which the hexaketide intermediate (**1**) undergoes transfer without extension or "skips" from PikAIII ACP_5 to PikAIV ACP_6 before its release. Herein, we report the results of an investigation designed to further elucidate the mechanism of macrolactone ring size determination in a *pikAIV* deletion mutant of *S. venezuelae* by functional replacement experiments with native and mutant forms of PikAIV (Pik module 6).

Results and Discussion

A PikAIV with Active KS_6, AT_6 and ACP_6, and TE Domains Is Required for 10-Deoxymethynolide Biosynthesis

Our initial investigation of the basis for macrolactone ring size determination by the Pik PKS involved observations from a complementation study in the *S. venezuelae* AX912 host strain. In AX912, the sequence encoding the TE domain of PikAIV was deleted (and replaced by 6X His-Tag), resulting in loss of antibiotic production [10]. Transformation of AX912 with plasmids expressing the Pik TE domain (pDHS704), the Pik ACP_6-TE domains (pDHS705), and the Pik AT_6-ACP_6-TE domains (pDHS708) resulted in the production of 10-deoxymethynolide and narbonolide and led to the hypothesis that 10-deoxymethynolide biosynthesis resulted from heterodimer formation between the chromosome-encoded PikAIV (lacking a TE domain) and the plasmid-encoded forms of PikAIV proteins (e.g., TE, ACP_6-TE, AT_6-ACP_6-TE).

In the current study, we conducted additional complementation studies with the *S. venezuelae* strain HYK954

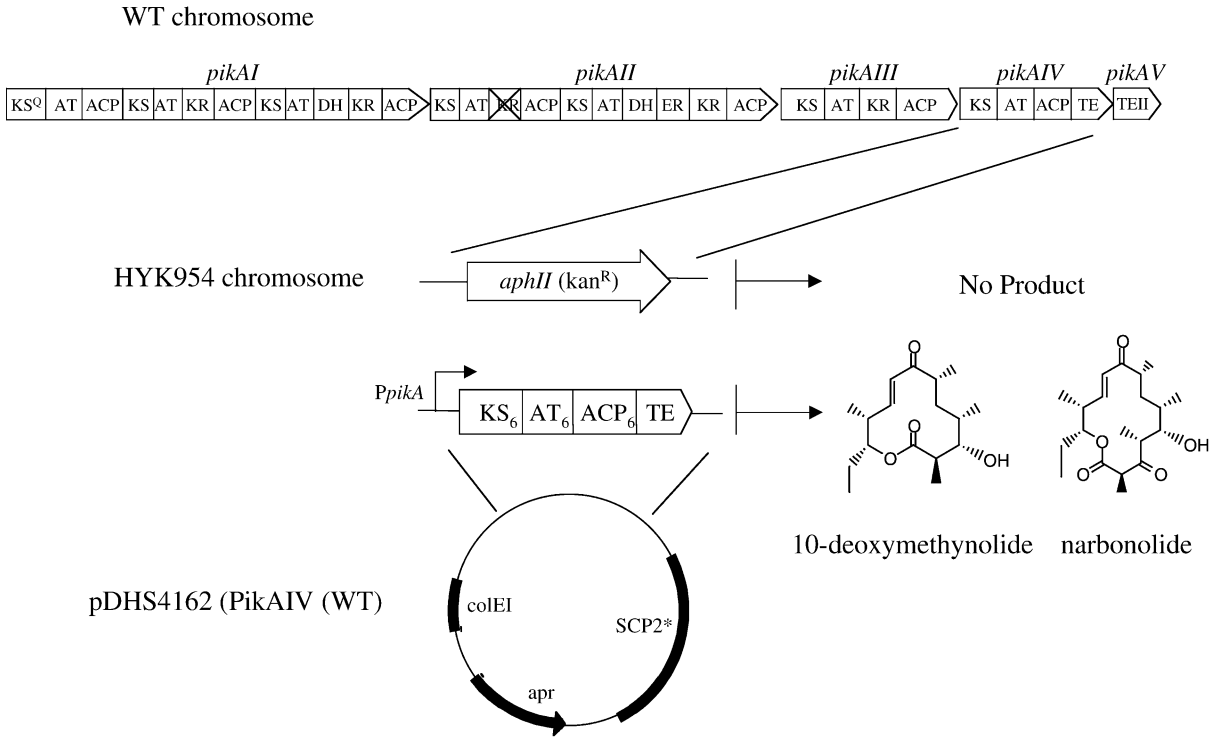

Figure 2. Chromosomal Arrangement of Pik PKS Genes in Wild-Type *S. venezuelae* and the *pikAIV*Δ Strain, HYK954

In HYK954, the *pikAIV* gene has been replaced with the kanamycin resistance gene (*aphII*). The *E. coli-Streptomyces* shuttle vector pDHS4162 expresses *pikAIV* under the control of the PikAI promoter (P*pikA*) and restores production of both 10-deoxymethynolide and narbonolide. This figure uses the following abbreviations: apr, apramycin resistance; SCP2*, *Streptomyces* origin of replication; colEI, *E. coli* origin of replication.

mutant in which the entire *pikAIV* gene was replaced by a kanamycin-resistance gene cassette (*aphII*) [12]. As predicted, the HYK954 mutant produced no detectable levels of macrolide antibiotics. Production is fully restored by transformation of the mutant strain with pDHS4162 (PikAIV(WT)) (Figure 2; [13]), but not by plasmids encoding the TE domain (pDHS704), ACP_6-TE domains (pDHS705), and AT_6-ACP_6-TE domains (pDHS708) of PikAIV (our unpublished data). *S. venezuelae* AX912 transformants containing pDHS704, pDHS705, and pDHS708 were grown under the same conditions as controls, and partial restoration of macrolide biosynthesis was observed as previously reported (Table 1; [10]).

We considered two explanations for the ability of these plasmids to complement AX912 but not HYK954. First, the plasmid-encoded truncated PikAIV proteins might require a unique interaction with AX912-encoded PikAIV [KS_6-AT_6-ACP_6] for TE-dependent release of hexaketide (**1**) from PikAIII ACP_5. Alternatively, the PikAIII hexaketide bound to ACP_5 may "skip" to ACP_6 of the AX912-encoded PikAIV protein [KS_6-AT_6-ACP_6], with the TE proteins (from the expression plasmids) catalyzing subsequent chain release. It is also important to note for these experiments and those reported previously [14] that the proposed heterodimeric forms of PikAIV are functional, whereas neither the N-terminally (AT_6-ACP_6-TE) nor the C-terminally truncated KS_6-AT_6-ACP_6 homodimeric forms of PikAIV are able to generate 10-deoxymethynolide.

In an effort to determine which Pik module 6 domain(s) are required for the 10-deoxymethynolide biosynthesis, we individually mutated the KS_6, AT_6, and ACP_6 active site residues of PikAIV. Specifically, the PikAIV KS_6 domain active site Cys (C207A), the AT_6 active site Ser (S652A), and the Ser phosphopantetheinylation site (S980A) in ACP_6 were individually inactivated by site-directed mutagenesis, yielding plasmids pDHS4176 (KS^-), pDHS4179(AT^-), and pDHS4163(ACP^-), respectively. In contrast to pDHS4162-expressing PikAIV (WT), which completely restored antibiotic production to wild-type levels in *S. venezuelae* HYK954, transformation of HYK954 with pDHS4176(KS^-), pDHS4179(AT^-), and pDHS4163(ACP^-) failed to produce significant levels of either 12- or 14-membered ring macrolide compounds by TLC or LC/MS analysis (data not shown). The inability of these mutant forms of PikAIV to produce 12-membered ring macrolide derivatives in the HYK954 *pikAIV* deletion mutant strain indicated an essential role for the KS_6, AT_6, and ACP_6 active site residues, consistent with a "domain skipping" mechanism.

Formation of Functional Heterodimeric PKSs between Domain-Inactivated PikAIV Polypeptides and a TE-Deleted PikAIV

Although the homodimeric forms of PikAIV(KS^-), PikAIV(AT^-), and PikAIV(ACP^-) were unable to restore 12-membered ring macrolactone formation in HYK954, we predicted that they would be capable of heterodimeriza-

Table 1. Aglycone Production in *S. venezulae* Strain Ax912

Line	Strain	Chromosomal PikAIV product	Plasmid Product	10-DML (mg/l)	NBL (mg/l)	Total (mg/l)
1	AX912	KS_6-AT_6-ACP_6		ND	ND	ND
2	AX912/pDHS704	KS_6-AT_6-ACP_6	TE	8±1	6±1	14±2
3	AX912/pDHS705	KS_6-AT_6-ACP_6	ACP_6-TE	3±1	5±1	8±2
4	AX912/pDHS708	KS_6-AT_6-ACP_6	AT_6-ACP_6-TE	7±2	11±4	18±6
5	AX912/pDHS4162	KS_6-AT_6-ACP_6	KS_6-AT_6-ACP_6-TE	11±7	68±5	79±12
6	AX912/pDHS4176	KS_6-AT_6-ACP_6	$KS_{6(C207A)}$-AT_6-ACP_6-TE	27±3	53±6	80±9
7	AX912/pDHS4179	KS_6-AT_6-ACP_6	KS_6-$AT_{6(S652A)}$-ACP_6-TE	73±8	19±5	92±13
8	AX912/pDHS4163	KS_6-AT_6-ACP_6	KS_6-AT_6-$ACP_{6(S980A)}$-TE	74±6	5±3	79±9
9	AX912/pDHS4182	KS_6-AT_6-ACP_6	$KS_{6(C207A)}$-AT_6-$ACP_{6(S980A)}$-TE	12±4	ND	12±4
10	AX912/pDHS3007	KS_6-AT_6-ACP_6	$[KS_7$-AT_7-KR_7-ACP_7-$TE]_{Tyl}$	trace	ND	trace
11	AX912/pDHS3016	KS_6-AT_6-ACP_6	$[KS_6]_{Pik}$-$[AT_7$-KR_7-ACP_7-$TE]_{Tyl}$	trace	ND	trace
12	AX912/pDHS3023	KS_6-AT_6-ACP_6	$[KS_6$-$AT_6]_{Pik}$-$[KR_7$-ACP_7-$TE]_{Tyl}$	trace	ND	trace

Production of aglycones 10-deoxymethynolide and narbonolide by strains grown on solid sporulation agar. Natural PikAIV domains are shown in red, and domains from the tylosin cluster (Tyl) are shown in blue. Brackets indicate the general fusion sites of the catalytic domains and are described in [12]. Yields were determined to be HPLC analysis with a standard curve derived for both 10-deoxymethynolide and narbonolide. ND, not detected.

tion with TE-deficient PikAIV expressed in AX912 and that restoration of 10-deoxymethynolide production would confirm the conformational stability of the site-directed mutants (Figure 3). Consistent with these predictions, pDHS4176 [PikAIV(KS^-)] restored production of 10-deoxymethynolide and narbonolide to levels that are similar to that observed with pDHS4162 [PikAIV (WT)]. Transformation of AX912 with pDHS4179 [PikAIV (AT^-)] and pDHS4163 [PikAIV(ACP^-)] also resulted in restoration of macrolactone levels, but with elevated 10-deoxymethynolide production relative to narbonolide (Table 1). The overall production of aglycones was significantly higher in AX912 strains complemented with pDHS4176 [PikAIV (KS^-)], pDHS4179 [PikAIV(AT^-)], and pDHS4163 [PikAIV(ACP^-)] than it was in AX912 strains complemented with plasmids pDHS704, pDHS705, pDHS708 that express N-terminally truncated forms of PikAIV (Table 1; compare lines 2–4 with lines 5–8).

In contrast, introduction of pDHS3007 expressing the terminal module (TylAV, module 7) from the tylosin PKS cluster (GenBank accession number U78289) into AX912 resulted in only trace production of 10-deoxymethynolide (Table 1, line 9). Similarly, pDHS3017 and pDHS3023 that provide the respective plasmid-based expression of hybrid modules in which Pik KS_6 and KS_6-AT_6 domains are substituted for the corresponding tylosin domains (e.g., KS_6-$[AT_7$-ACP_7-$TE]_{Tyl}$ and KS_6-AT_6-$[ACP_7$-$TE]_{Tyl}$) did not improve the production of 10-deoxymethynolide in strain AX912 (Table 1, lines 10 and 11).

Formation of Functional Heterodimeric PKSs between Domain-Inactivated PikAIV Polypeptides and an Inactive Engineered PikAIV Containing an AT-ACP Domain Switch

In a recent study of molecular recognition parameters in the Pik PKS, a series of hybrid modules comprising domains from Pik module 6 and modules 13 and 14 from the rapamycin (Rap) PKS cluster demonstrated that functional interactions could form between modules containing domains from these two systems [14]. For example, *S. venezuelae* strain SC1016 contains Rap ACP_{14} in place of the PikAIV ACP_6 domain, and the resulting hybrid module produces only the 12-membered ring macrolactone 10-deoxymethynolide (Table 2, Line 1). In light of the current study, this result suggests that substitution of the PikAIV ACP_6 for Rap ACP_{14} does not prevent the skipping of hexaketide (**1**) but does impair extension of the chain with methylmalonyl-CoA. Either the incorporation of a unique linker region between the AT_6 and ACP_{14} domains or the identity of the ACP domain disturbs the catalytic center formed by the dimerization of monomers such that the hexaketide chain can skip (e.g., transfer) to the ACP_6 domain via a flexible 4′-phosphopantetheine group but not be extended. It is notable that substitution of AT_{13} sequence from the Rap PKS into PikAIV produced a hybrid capable of synthesizing both 10-deoxymethynolide and narbonolide, thus highlighting the importance of domain linker regions and/or identity of the ACP in forming a fully functional module [14]. It is not clear if the extension reaction fails in the hybrid KS_6-$AT_6[$-$ACP_{14}]_{Rap}$-TE because the ACP domain remains unprimed with a methymalonyl extender unit as a result of poor communication between AT_6 and ACP_{14} or if the extender unit is loaded on the ACP but not capable of reacting with the hexaketide chain on the KS_6 active site. In contrast, *S. venezuelae* strain SC1017 in which PikAIV KS_6-AT_6-ACP_6-TE has been exchanged by Pik KS_6-$[AT_{14}$-$ACP_{14}]_{Rap}$-TE produces neither 10-deoxymethynolide nor the predicted 2-*des*-methylnarbonolide [14]. Plasmid-based expression of this hybrid PikAIV in strain AX912, however, does lead to restoration of macrolide production, demonstrating that this

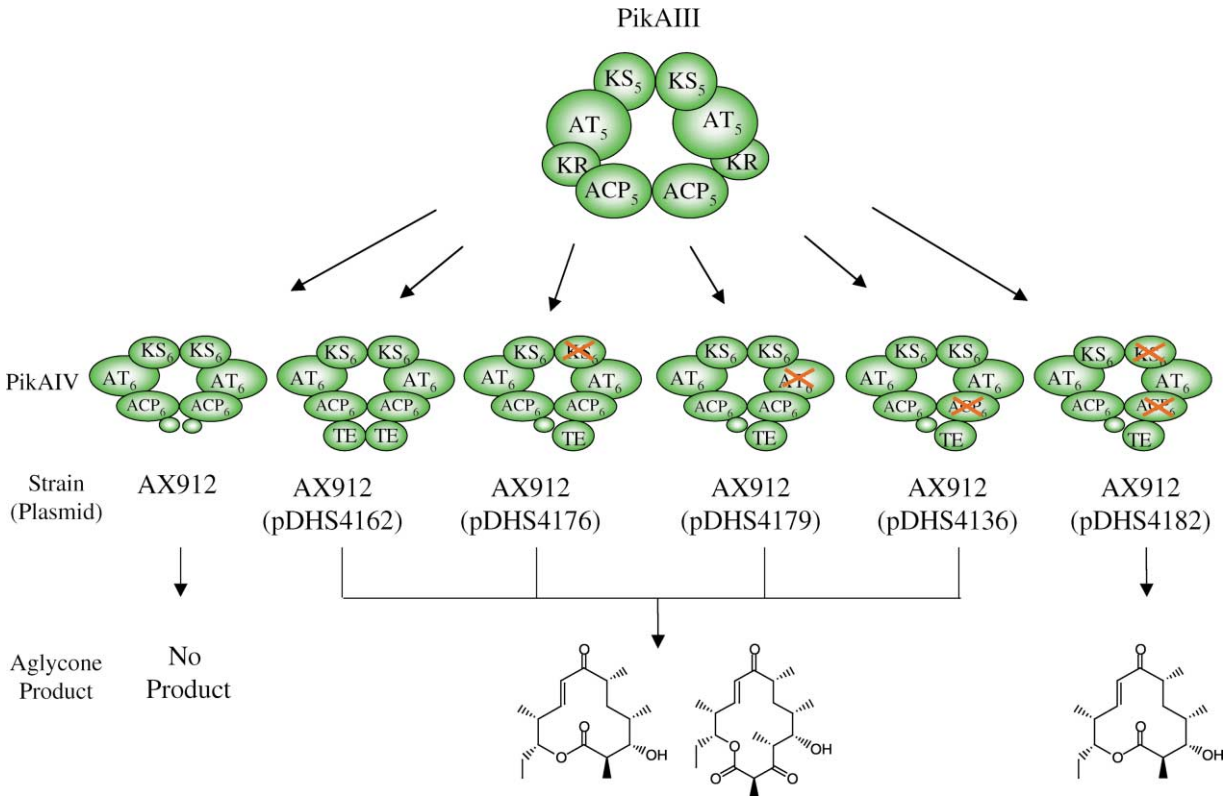

Figure 3. Proposed Interactions Leading to Functional Heterodimers

Plasmids expressing wild-type PikAIV or site-directed mutants were introduced into *S. venezuelae* strain AX912 that expresses an inactive PikAIV lacking the thioesterase domain.

apparent nonfunctional form of PikAIV can serve as a donor for complementation experiments to investigate the formation of active heterodimers from an inactive homodimeric module.

In the current study, the introduction of plasmids pDHS704, pDHS705, and pDHS708 into *S. venezuelae* SC1017 did not result in recombinants that produce significant levels of 10-deoxymethynolide or narbonol-

Table 2. Aglycone Production in *S. venezuelae* Strains SC1016 and SC1017

Line	Strain	Chromosomal PikAIV product	Plasmid Product	10-DML (mg/l)	NBL (mg/l)	Total (mg/l)
1	SC1016	KS_6-AT_6[-ACP_{14}]$_{Rap}$-TE		62±10	ND	62±10
2	SC1017	KS_6[-AT_{14}-ACP_{14}]$_{Rap}$-TE		ND	ND	ND
3	SC1017/pDHS704	KS_6[-AT_{14}-ACP_{14}]$_{Rap}$-TE	TE	10±2	ND	10±2
4	SC1017/pDHS705	KS_6[-AT_{14}-ACP_{14}]$_{Rap}$-TE	ACP_6-TE	trace	ND	trace
5	SC1017/pDHS708	KS_6[-AT_{14}-ACP_{14}]$_{Rap}$-TE	AT_6-ACP_6-TE	trace	ND	trace
6	SC1017/pDHS4162	KS_6[-AT_{14}-ACP_{14}]$_{Rap}$-TE	KS_6-AT_6-ACP_6-TE	17±4	65±8	82±12
7	SC1017/pDHS4176	KS_6[-AT_{14}-ACP_{14}]$_{Rap}$-TE	$KS_{6(C207A)}$-AT_6-ACP_6-TE	9±3	9±5	18±8
8	SC1017/pDHS4179	KS_6[-AT_{14}-ACP_{14}]$_{Rap}$-TE	KS_6-$AT_{6(S652A)}$-ACP_6-TE	55±3	ND	55±3
9	SC1017/pDHS4163	KS_6[-AT_{14}-ACP_{14}]$_{Rap}$-TE	KS_6-AT_6-$ACP_{6(S980A)}$-TE	25±2	ND	25±2
10	SC1017/pDHS4182	KS_6[-AT_{14}-ACP_{14}]$_{Rap}$-TE	$KS_{6(C207A)}$-AT_6-$ACP_{6(S980A)}$-TE	trace	ND	trace

Production of aglycones 10-deoxymethynolide and narbonolide by strains grown on solid sporulation agar. Natural PikAIV domains are shown in red, and the AT_{14} and ACP_{14} domains from the rapamycin cluster (Rap) are shown in green. Brackets indicate the general fusion sites of the catalytic domains and are described in [13]. Yields were determined to be HPLC analysis with a standard curve derived for both 10-deoxymethynolide and narbonolide. ND, not detected.

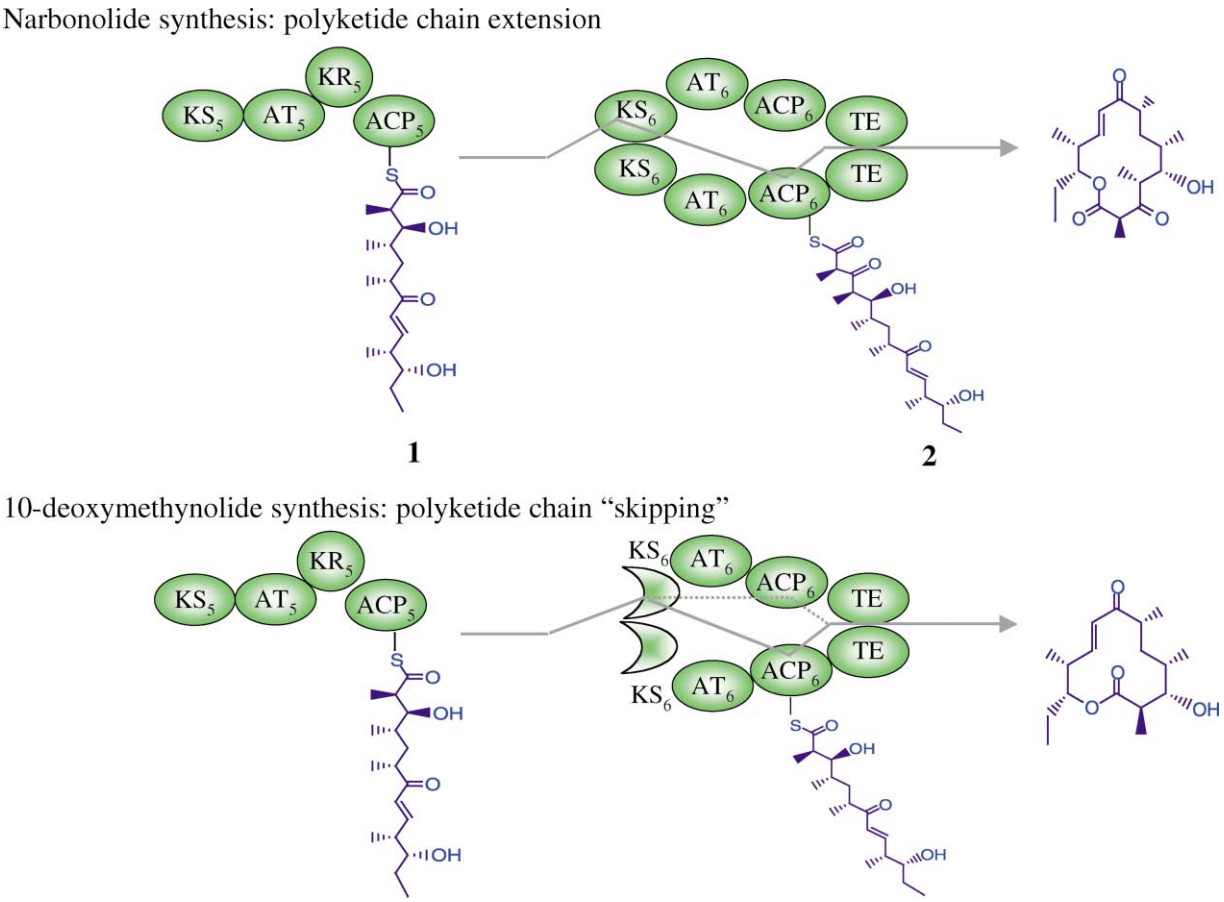

Figure 4. Proposed Mechanism of Narbonolide and 10-Deoxymethynolide Biosynthesis by PikAIV

The bold line indicates the major path of the polyketide chain through the individual domains of the terminal module. Transfer of the hexaketide (**1**) covalently bound to the ACP_5 domain of PikAIII to the KS_6 domain of the full-length PikAIV results in elongation to the heptaketide (**2**) and release as narbonolide. The N-terminally truncated form of PikAIV catalyzes the transfer of the hexaketide (**1**) from the ACP_5 domain of PikAIII to the catalytic cysteine residue in the PikAIV KS_6 domain. Translocation of the hexaketide (**1**) to the PikAIV ACP_6 domain on the complementary polypeptide leads to release and cyclization to 10-deoxymethynolide. The dashed line indicates the possible intrapolypeptide transfer of the chain elongation intermediate (**1**).

ide, in contrast with results from the AX912 strain. This observation is consistent with previous suggestions that KS_6 and AT_6 are important components for dimerization of PikAIV polypeptides and are thus required for generation of significant levels of active heterodimers. Substantial levels of macrolide biosynthesis were observed when SC1017 was transformed with plasmids that expressed both PikAIV(WT) (pDHS4162) and PikAIV containing mutations in KS_6 (pDHS4176), AT_6 (pDHS4179), or ACP_6 (pDHS4163) (Table 2). This result confirms that KS_6 is an important factor for dimerization and thus formation of functional PikAIV heterodimers. More importantly, these results demonstrate that the PikAIV proteins carrying mutations in the KS, AT, and ACP domains are able to generate active heterodimers both with the PikAIV in SC1017 (KS_6-$[AT_{14}$-$ACP_{14}]_{Rap}$-TE) and with AX912 (KS_6-AT_6-ACP_6). In SC1017, 10-deoxymethynolide and narbonolide production was comparable to that observed with AX912 containing pDHS4162 (compare Table 1, line 5 with Table 2, line 6). When pDHS4176 [PikAIV(KS^-)] was used to transform *S. venezuelae* SC1017, the resulting recombinant strain was able to produce both 10-deoxymethynolide and narbonolide, but at reduced levels. SC1017 transformants containing pDHS4179 [PikAIV(AT^-)] and pDHS4163 [PikAIV(ACP^-)] produced higher levels of 10-deoxymethynolide, but neither narbonolide nor 2-*des*-methylnarbonolide was detected (a similar shift compared with the same complementation experiment in AX912). Although these site-directed mutant forms of PikAIV were able to produce high levels of aglycones in AX912, the reduced production observed in SC1017 might reflect less efficient dimerization between PikAIV polypeptides containing a single point mutation and Pik KS_6-$[AT_{14}$-$ACP_{14}]_{Rap}$-TE than that between the same polypeptides and Pik KS_6-AT_6-ACP_6.

Mechanism of Skipping of the Hexaketide Chain Elongation Intermediate (1) in *S. venezuelae*

Our working model predicts that a homodimeric, N-terminally truncated form of PikAIV accepts the hexaketide intermediate from PikAIII and is required for the majority of 10-deoxymethynolide biosynthesis in a wild-type strain. The frequency of heterodimer formation between

Table 3. Bacterial Strains and Plasmids

S. venezuelae Strains	Relevant Genotype	Reference
WT (ATCC 15439)	Complete *pik* and *des* gene clusters	ATCC
HYK954	*pikAIV*Δ; complete *pikAV* and expression of *des* genes	this report
AX912	Deletion of sequence encoding the PikAIV TE domain and TEII. Deletion has polarity affect on *des* genes	[10]
SC1016	Chromosomal mutant in which ACP_{14} from Rap is substituted for ACP_6; *pikAV*Δ is polar on *des* genes	[14]
SC1017	Chromosomal mutant in which AT_{14}-ACP_{14} from Rap is substituted for AT_6-ACP_6; *pikAV*Δ is polar on *des* genes	[14]
Plasmids	**Description**	**Reference**
pDHS618	Low-copy number; *E. coli-Streptomyces* expression vector; Apr^R	[18]
pDHS702	Low-copy number; *E. coli-Streptomyces* expression vector; Amp^R Ts^R	[18]
pLITMUS 28	*E. coli* cloning vector; Amp^R	NEB
pLZ51	pNJ1 cosmid containing *pikAIV*	[5]
pDHS3007	pDHS618 derivative expressing TylGV, the terminal module from the tylosin cluster; Apr^R	[13]
pDHS3016	pDHS618 derivative expressing a hybrid module consisting of KS_6 from PikAIV and AT_7-KR_7-ACP_7-TE from TylGV, Apr^R	[13]
pDHS3023	pDHS618 derivative expressing a hybrid module consisting of KS_6 AT_6 from PikAIV and KR_7-ACP_7-TE from TylGV; Apr^R	[13]
pDHS4147	2.3 kb fo the 3′ of *pikAIV* cloned into pLITMUS28 as a EcoRI-BamHI fragment; Amp^R	This report
pDHS4172	2 kb of the 5′ of *pikAIV* cloned into pLITMUS28 as a BglII-EcoRI fragment; Amp^R	This report
pDHS4162	pDHS618 derivative expressing PikAIV(WT); Apr^R	This report
pDHS4163	pDHS618 derivative expressing PikAIV(ACP^-); Apr^R	This report
pDHS4175	pDHS702 derivative expressing PikAIV(WT); Amp^R Ts^R	This report
pDHS4176	pDHS702 derivative expressing PikAIV(KS^-); Amp^R Ts^R	This report
pDHS4179	pDHS618 derivative expressing PikAIV(ACP^-); Apr^R	This report
pDHS4182	pDHS618 derivative expressing PikAIV(KS^--AT-ACP^-); Apr^R	This report

full-length and truncated PikAIV and the ability of such a heterodimer to synthesize either narbonolide or 10-deoxymethynolide remains to be determined because the expression of each form in the fermentation experiments presented herein would be impossible to ascertain. In the current study, homodimers of the *pikAIV*-encoded site-directed mutants are nonfunctional, but the observed functionality of heterodimers allows us to assess the activity of each catalytic domain in 10-deoxymethynolide biosynthesis. A heterodimer between the chromosomally expressed PikAIV KS_6-AT_6-ACP_6 in AX912 and any of the three *pikAIV*-encoded site-directed mutants would comprise one thioesterase domain and only one functional active site for either the KS, AT or ACP domains. There would be two native copies of each of the remaining catalytic domains. Therefore, we constructed a mutant form of PikAIV with both C207A and S980A substitutions and used this in complementation studies with AX912. In this example, the heterodimer would contain one polypeptide with native active site residues for the KS, AT, and ACP domains but no thioesterase and the complementary polypeptide with a TE domain but inactive KS and ACP domains. When pDHS4182 was introduced into AX912, a decrease in overall yield (15%) of macrolides was observed relative to that observed in complementation experiments with the plasmids expressing single point mutations in PikAIV. This decrease in yield along with a shift toward 10-deoxymethynolide production (as observed in complementation experiments) resulted in no detectable levels of narbonolide (Table 1, line 9). In a skipping model for 10-deoxymethynolide biosynthesis, the observations of narbonolide biosynthesis require that the chain-elongated product on ACP_6 is cleaved by a TE domain on the opposite polypeptide strand. The model also requires that for some module skipping to occur, it is sufficient to have the KS, AT, and ACP domains present on just one polypeptide, whereas more efficient skipping occurs if domains on the other polypeptide are active (lines 5–8 versus line 9 of Table 1). As a further test for this hypothesis, we carried out a complementation experiment with pDHS4182. In this experiment, the plasmid-based KS-AT-ACP-TE would form a heterodimer with a hybrid PikAIV containing an inactive AT domain (AT_{14}). In this case, no significant levels of either 10-deoxymethyolide or narbonolide were observed.

The ability of the active site mutant forms of PikAIV to synthesize normal levels of 12-membered ring products in AX912 and SC1017, but not HYK954, indicates an essential role for the core catalytic domains in the synthesis of 10-deoxymethynolide and is consistent with the hexaketide being transferred to Pik ACP_6 prior to TE-dependent release. However, the mechanistic details of chain transfer from PikAIII ACP_5 to PikAIV ACP_6 remain unclear. Consideration of the normal activities of these domains and the complementation results presented herein and elsewhere [7, 8] suggests that in a model involving a dimeric modular PKS, an individual catalytic center consists of a KS domain and an ACP domain that are located on *either* the same *or* opposite polypeptide chains. Previously it has been suggested that the KS domain works only on the ACP domain of the opposite chain [8]. Elongation of the polyketide chain

requires translocation of the chain from the preceding ACP domain to the KS domain, where condensation with the extender unit attached to the 4′-phosphopantetheine prosthetic group of the ACP in the same module results in growth of the chain and its transfer between proteins in the dimer. In 10-deoxymethynolide biosynthesis, the interaction between PikAIII and the N-terminally truncated PikAIV allows the polyketide chain to be translocated via the flexible 4′-phosphopantetheine group from PikAIII ACP_5 to the catalytic cysteine of KS_6 (possibly exposed by the N-terminal truncation). Movement of the ACP_6 4′-phosphopantetheine group, presumably lacking the methylmalonyl CoA extender unit, toward the "loaded" cysteine of KS_6 may allow direct thiol transfer of the hexaketide to the ACP either on the same or on the opposite polypeptide in the dimer complex. The activity of the TE in an interpolypeptide-, and possible intrapolypeptide-, catalyzed reaction cleaves the ACP_6 bound hexaketide product to generate 10-deoxymethynolide (Figure 4). The translocation of the hexaketide from PikAIII ACP_5 through KS_6 to PikAIV ACP_6 appears to require the activity of the AT_6 domain. This result is consistent with previous conclusions that the identity and/or activity of the AT domain is an important structural determinant for facilitating TE-catalyzed production of 10-deoxymethynolide [14]. It is worth noting, however, that in a complementation study with SC1017 and pDHS4179, the heterodimer containing an AT_6 mutant and $RapAT_{14}$-containing PikAIV (which is not functional as homodimeric PikAIV) produces significant levels of 10-deoxymethynolide. This observation is consistent with a mechanism in which skipping of the hexaketide chain to the ACP_6 domain requires a 4′-phosphopantetheine group that is not loaded with a methylmalonyl extender unit. The structural or catalytic role of AT_6 in the production of 10-deoxymethynolide is an important component of our continued analysis of the specific biochemical details of the domain-skipping mechanism.

Significance

Polyketides are a structurally diverse class of compounds that have been developed as pharmaceuticals for the treatment of cancer and infection. The synthesis of many of these compounds depends on the activity of modular polyketide synthases (PKSs) that elongate simple carboxylic acids as intermediates bound to separate acyl carrier proteins (ACPs). The naturally occurring pikromycin polyketide synthase from *Streptomyces venezuelae* contains four polypeptides (PikAI-PikAIV) and has the unique ability to generate 12- and 14-membered ring macrolactones, 10-deoxymethynolide and narbonolide, respectively, by the activity of the thioesterase domain located in the terminal module, PikAIV. Extension of the hexaketide chain intermediate from PikAIII by PikAIV leads to formation of the 14-membered ketolide ring narbonolide, whereas release of the hexaketide results in the formation of 10-deoxymethynolide. While some interaction between PikAIII and an individual TE domain can allow for direct release of the hexaketide from the ACP_5 domain, experimental evidence suggests that more efficient release of the acyl chain is accomplished with a TE domain at the C terminus of PikAIV that contains catalytically competent KS, AT, and ACP domains. This data is consistent with the majority of the hexaketide chain intermediate being transferred to the PikAIV ACP_6 domain prior to release and cyclization to 10-deoxymethynolide. A more thorough understanding of this mechanism will contribute to our overall knowledge of polyketide chain transfer mechanisms as well as to the development of novel termination activities for combinatorial biosynthesis.

Experimental Procedures

Bacterial Strains and Culture Conditions

E. coli DH5α was used as a host for the manipulation of plasmid DNA. Wild-type *Streptomyces venezuelae* ATCC 15439 and the derivative mutant strains HYK954, AX912, SC1016, and SC1017 have been described previously [10, 13, 14]. Plasmids and *S. venezuelae* strains used as production hosts for the engineered expression plasmids in this study are summarized in Table 3. *S. venezuelae* transformants were grown on R2YE agar plates and then transferred to sporulation agar for production of spores and antibiotics.

Construction of Complementation Vectors

Complementation vectors are all derivatives of pDHS702 and pDHS618, which both contain *Streptomyces* SCP2* low-copy origin [15] and an *E. coli colE* origin and use the *pikAI* promoter to express gene inserts. Derivatives of these plasmids were transformed into *S. venezuelae* by the standard protoplast transformation procedure [16]. Plasmids were selectively maintained in *S. venezuelae* with thiostrepton (pDHS702 derivatives) and apramycin (pDHS618 derivatives) at a concentration of 25 μg/ml. Manipulation of plasmid DNA was performed by standard cloning procedures, and polymerase chain reaction (PCR) amplification was performed with the *pfu* polymerase (Stratagene) under conditions recommended by the manufacturer. The 3′ end of the *pikAIV* gene was cloned as a 2.3 kb EcoRI-BamHI fragment from pLZ51 into pLITMUS28 (New England Biolabs) to yield pDHS4147. The 5′ end of *pikAIV* was generated as a 2.4 kb BglII-EcoRI fragment, was amplified from pLZ51 with the primers 5′-AAA*AGATCT*GACCCGACCGCGGTCCTGCCCCA-3′ and 5′-GGCGGC*GAATTC*GGGTGAGCTGTC-3′, and was cloned into pLITMUS to yield pDHS4172. The BglII-EcoRI insert from pDHS4172 and an EcoRI-XbaI insert from pDHS4147 were cloned into pDHS4618 that had been digested with BamHI and XbaI to yield pDHS4162. The pDHS4175 plasmid was constructed in a similar manner except that the EcoRI-XbaI insert from pDHS4147 was first cloned into pUC118 so that the fragment could be digested with EcoRI-PstI and cloned into the NsiI site of pDHS702.

Site-directed mutagenesis of the active site residues in the *pikAIV* KS, AT, and ACP domains was accomplished by standard PCR methodologies and confirmed by DNA sequencing. The plasmid pDHS4163 expresses a mutant form of *pikAIV* in which the sequence encoding the ACP active site sequence has been changed to GAT GCA TTG, creating a diagnostic NsiI site and resulting in a S980A mutation. Plasmid pDHS4176 expresses a mutant form of PikAIV in which the sequence encoding the KS catalytic cysteine has been changed to GCG GCT ACG, creating a diagnostic NheI site and resulting in a C207A mutation. Plasmid pDHS4179 expresses a mutant form of PikAIV in which the sequence encoding the AT active site serine has been changed to CAT GCG CAG, creating a diagnostic FspI site that results in a S652A mutation. Plasmid pDHS4182 expresses a mutant form of PikAIV containing both C207A and S980A substitutions.

Purification and Characterization of Polyketide Production

Production of pikromycin-related polyketides was determined on solid phase medium. Spore suspensions were spread onto 25 ml of sporulation agar containing appropriate antibiotics and were grown at 30°C for 3 days. The agar was first extracted with 50 ml

of methanol for 1 hr. The dried extract was dissolved in 25 ml of water and then extracted with 25 ml of ethyl acetate. The dried ethyl acetate extract was resuspended in 500 μl of acetonitrile for analysis. For silica thin-layer-chromatography analysis, the aglycone compounds were separated with a chloroform: methanol (95:5) solvent, and the glycosylated/hydroxylated products were separated with a chloroform:methanol: 25% NH_4OH (90:10:1) solvent. All compounds were visualized by vanillin staining and heating [17]. HPLC analysis of extracts allowed quantitative comparison of polyketide production between strains. Sample extracts were injected into a C-18 reverse-phase HPLC analytical column (250 mm × 4.6 mm, 10 μm particle size) and eluted with a 30 min gradient of 50%–90% acetonitrile buffered with 10 mM ammonium acetate (pH 9.0). Peak areas were integrated compared to a standard curve generated with purified compounds. Antibiotic production was averaged from at least three separate *S. venezuelae* transformants.

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References

1. Katz, L. (1997). Manipulation of modular polyketide synthase. Chem. Rev. *97*, 2557–2575.
2. August, P.R., Tang, L., Yoon, Y.J., Ning, S., Muller, R., Yu, T.W., Taylor, M., Hoffmann, D., Kim, C.G., Zhang, X., et al. (1998). Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amycolatopsis mediterranei* S699. Chem. Biol. *5*, 69–79.
3. Donadio, S., Staver, M.J., McAlpine, J.B., Swanson, S.J., and Katz, L. (1991). Modular organization of genes required for complex polyketide biosynthesis. Science *252*, 675–679.
4. Schwecke, T., Aparicio, J.F., Molnar, I., Konig, A., Khaw, L.E., Haydock, S.F., Oliynyk, M., Caffrey, P., Cortes, J., Lester, J.B., et al. (1995). The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin. Proc. Natl. Acad. Sci. USA *92*, 7839–7843.
5. Xue, Y., Zhao, L., Liu, H.W., and Sherman, D.H. (1998). A gene cluster for macrolide antibiotic biosynthesis in *Streptomyces venezuelae*: architecture of metabolic diversity. Proc. Natl. Acad. Sci. USA *95*, 12111–12116.
6. Staunton, J., Caffrey, P., Aparicio, J.F., Roberts, G.A., Bethell, S.S., and Leadlay, P.F. (1996). Evidence for a double-helical structure for modular polyketide synthases. Nat. Struct. Biol. *3*, 188–192.
7. Gokhale, R.S., Lau, J., Cane, D.E., and Khosla, C. (1998). Functional orientation of the acyltransferase domain in a module of the erythromycin polyketide synthase. Biochemistry *37*, 2524–2528.
8. Kao, C.M., Pieper, R., Cane, D.E., and Khosla, C. (1996). Evidence for two catalytically independent clusters of active sites in a functional modular polyketide synthase. Biochemistry *35*, 12363–12368.
9. Zhang, Q., and Sherman, D.H. (2001). Isolation and structure determination of novamethymycin, a new bioactive metabolite of the methymycin biosynthetic pathway in *Streptomyces venezuelae*. J. Nat. Prod., *64*, 1447–1450.
10. Xue, Y., and Sherman, D.H. (2000). Alternative modular polyketide synthase expression controls macrolactone structure. Nature *403*, 571–575.
11. Rowe, C.J., Bohm, I.U., Thomas, I.P., Wilkinson, B., Rudd, B.A., Foster, G., Blackaby, A.P., Sidebottom, P.J., Roddis, Y., Buss, A.D., et al. (2001). Engineering a polyketide with a longer chain by insertion of an extra module into the erythromycin-producing polyketide synthase. Chem. Biol. *8*, 475–485.
12. Ward, J.M., Janssen, G.R., Kieser, T., Bibb, M.J., and Buttner, M.J. (1986). Construction and characterization of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn5 as indicator. Mol. Gen. Genet. *203*, 468–478.
13. Yoon, Y.-J., Beck, B.J., Kim, B.-S., Kang, H.Y., Reynolds, K.A., and Sherman, D.H. (2002). Generation of multiple bioactive macrolides by hybrid modular polyketide synthases in *Streptomyces venezuelae*. Chem. Biol., *9*, 203–214.
14. Chen, S., Xue, Y., Sherman, D.H., and Reynolds, K.A. (2000). Mechanisms of molecular recognition in the pikromycin polyketide synthase. Chem. Biol. *7*, 907–918.
15. Lydiate, D.J., Malpartida, F., and Hopwood, D.A. (1985). The *Streptomyces* plasmid SCP2*: its functional analysis and development into useful cloning vectors. Gene *35*, 223–235.
16. Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000). Practical Streptomyces Genetics. (Norwich, England: The John Innes Foundation).
17. Xue, Y., Wilson, D., Zhao, L., Liu, H., and Sherman, D.H. (1998). Hydroxylation of macrolactones YC-17 and narbomycin is mediated by the *pikC*-encoded cytochrome P450 in *Streptomyces venezuelae*. Chem. Biol. *5*, 661–667.
18. Xue, Y., and Sherman, D.H. (2001). Biosynthesis and combinatorial biosynthesis of pikromycin-related macrolides in *Streptomyces venezuelae*. Metab. Eng. *3*, 15–26.