Mechanisms of molecular recognition in the pikromycin polyketide synthase

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Background: Modular polyketide synthases (PKSs) produce a wide range of medically significant compounds. In the case of the pikromycin PKS of *Streptomyces venezuelae*, four separate polypeptides (PikAI–PikAIV), comprising a total of one loading domain and six extension modules, generate the 14-membered ring macrolactone narbonolide. The polypeptide PikAIV contains a thioesterase (TE) domain and is responsible for catalyzing both the last elongation step with methylmalonyl CoA, and subsequent release of the final polyketide chain elongation intermediate from the PKS. Under certain growth conditions this polypeptide is synthesized from an alternative translational start site, giving rise to an N-terminal truncated form of PikAIV, containing only half of the ketosynthase (KS_6) domain. The truncated form of PikAIV is unable to catalyze the final elongation step, but is able to cleave a polyketide chain from the preceding module on PikAIII (ACP_5), giving rise to the 12-membered ring product 10-deoxymethynolide.

Results: *S. venezuelae* mutants expressing hybrid PikAIV polypeptides containing acyl carrier protein (ACP) and malonyl CoA specific acyltransferase (AT) domains from the rapamycin PKS were unable to catalyze production of 12- or 14-membered ring macrolactone products. Plasmid-based expression of a hybrid PikAIV containing the native KS_6 and TE domains, however, restored production of both narbonolide and 10-deoxymethynolide in the *S. venezuelae* AX912 mutant that generates a TE-deleted form of PikAIV. Use of alternative KS domains or deletion of the KS_6 domain within the hybrid PikAIV resulted in loss of both products. Plasmid-based expression of a TE domain of PikAIV as a separate polypeptide in the AX912 mutant resulted in greater than 50% restoration of 10-deoxymethynolide, but not in mutants expressing a hybrid PikAIV bearing an unnatural AT domain. Mutants expressing hybrid PikAIV polypeptides containing the natural AT_6 domains and different ACP domains efficiently produced polyketide products, but with a significantly higher 10-deoxymethynolide/narbonolide ratio than observed with native PikAIV.

Conclusions: Dimerization of KS_6 modules allows in vivo formation of a PKS heterodimer using PikAIV polypeptides containing different AT and ACP domains. In such heterodimers, the TE domain and the AT_6 domain responsible for formation of the narbonolide product are located on different polypeptide chains. The AT_6 domain of PikAIV plays an important role in facilitating TE-catalyzed chain termination (10-deoxymethynolide formation) at the proceeding module in PikAIII. The pikromycin PKS can tolerate the presence of multiple forms (active and inactive) of PikAIV, and decreased efficiency of elongation by PikAIV can result in increased levels of 10-deoxymethynolide. These results provide new insight into functional molecular interactions and interdomain recognition in modular PKSs.

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Introduction

Modular polyketide synthases (PKSs) are responsible for generating the macrolide core of a diverse range of polyketide products with pharmaceutical, veterinary and agricultural applications [1]. These PKSs are comprised of numerous large multifunctional enzymes containing one or more discrete modules that catalyze the successive condensation of different extender units such as malonyl CoA and methylmalonyl CoA onto a starter unit (Figure 1) [2]. Each module contains an acyltransferase (AT) domain responsible for loading the appropriate extender unit onto an acyl carrier protein (ACP) domain [2]. A ketosynthase (KS) do-

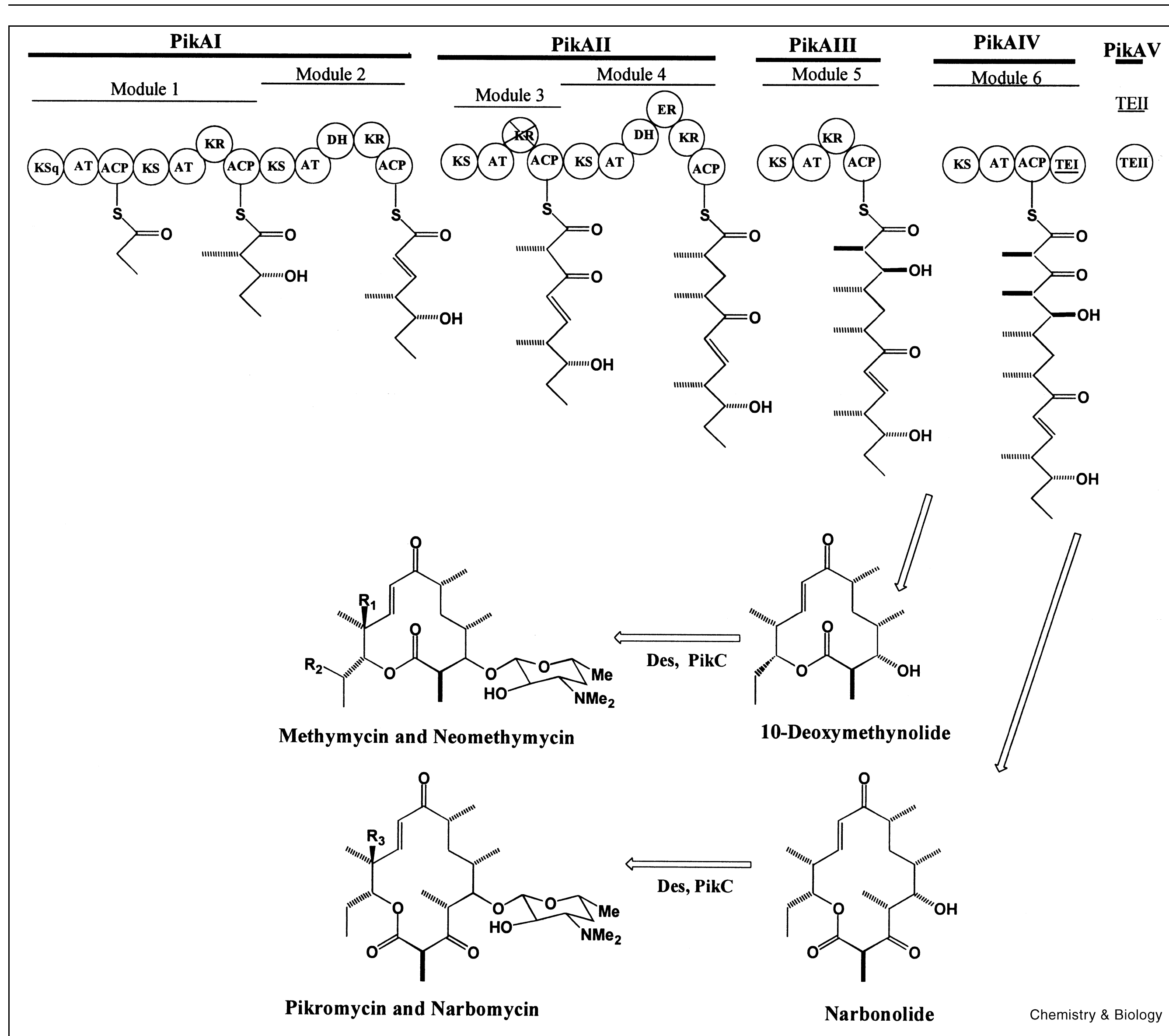

Figure 1. Biosynthesis of 10-deoxymethynolide and narbonolide by the pikromycin PKS (adapted from Xue and Sherman [12]). The system is comprised of six modules for elongation and one loading module (module L). Catalytic domains with the modules of the PKS are represented by circles (KS, ketosynthase; AT, acyl transferase; KR, ketoreductase; ACP, acyl carrier protein; DH, dehydratase, ER, enoyl reductase; KS^Q, a methylmalonyl decarboxylase; TE, thioesterase). Final products generated by the action of the desosamine biosynthetic enzymes (Des) and a cytochrome P_{450} monoxygenase (PikC) are methymycin (R_1, OH; R_2, H), neomethymycin (R_1, H; R_2, OH), pikromycin (R_3, OH) and narbomycin (R_3, H).

main at the beginning of the module catalyzes a decarboxylative condensation of this extender unit with a growing polyketide chain obtained from a proceeding module and generates an ACP-bound 3-ketoacyl product. The level of 3-keto group processing prior to the next chain extension step is determined by the presence of ketoreductase (KR), enoyl reductase (ER) and dehydratase (DH) domains within the module [3]. A thioesterase (TE) domain attached to the C-terminus of the last module is responsible for cleaving the acyl chain from the adjacent ACP domain and is presumed to catalyze cyclization to the macrolactone. The modular nature of the PKSs makes them particularly amenable to bioengineering approaches for designing catalysts capable of generating novel polyketide structures [4]. A number of hybrid and novel PKSs have been generated with alterations to the PKS ranging from deletion/insertion of domains and module switches and to combining complete PKS polypeptides from different systems [4–6].

Proteolytic analysis of the erythromycin PKS has shown that the different multienzymes exist as parallel (possibly helical) homodimeric complexes where identical modules interact closely across the dimer interface [7]. This arrangement allows identical modules to share active sites for chain extension and possible chain termination. Mutagenesis studies with the erythromycin PKS have clearly demonstrated that the KS domain within a module can interact with the ACP on the complementary chain [7,8] and that the AT domain can act on the ACP in either chain [9]. A number of different models for the structure of PKSs have been proposed based on these observations [8,10].

The pikromycin PKS of *Streptomyces venezuelae* has several unique attributes, including the ability to generate both 12- and 14-membered ring macrolactone products under different culture conditions [11]. This system generates a hexaketide product by using three polypeptides, PikAI, PikAII and PikAIII, to elongate a propionyl starter unit through five elongation steps (Figure 1). In PGM media, PikAIV containing just one module catalyzes an additional elongation step using methylmalonyl CoA, and generates a heptaketide product [12]. This product is then released from PikAIV by a TE domain and cyclized to produce narbonolide, which is subsequently converted to narbomycin and pikromycin. In SCM media polyketide chain extension is terminated at the end of PikAIII to generate 10-deoxymethynolide, which in turn is converted to methymycin and neomethymycin [12]. In SGGP media a mixture of both 12- and 14-membered ring macrolactone products are formed. The premature chain termination is catalyzed by the TE domain at the C-terminus of PikAIV [12]. It has recently been shown that under these conditions an alternative translational start codon 600 nucleotides downstream of the normal *pikAIV* start codon is used, giving rise to a N-terminal truncated PikAIV containing only half of the KS_6 domain and is unable catalyze the final chain elongation step [12]. It has been proposed that partial loss of this domain allows for a functional interaction between the TE domain of the truncated PikAIV and the hexaketide product bound to the ACP domain of PikAIII (an interaction that is blocked when the complete KS_6 is present in PikAIV) (Figure 2) [12]. This unusual chain termination mechanism generates multiple polyketide products from a single PKS and represents a potentially useful tool for combinatorial biosynthetic approaches for generating libraries of new natural products. The unusual nature of PikAIV to interact in two different forms in a

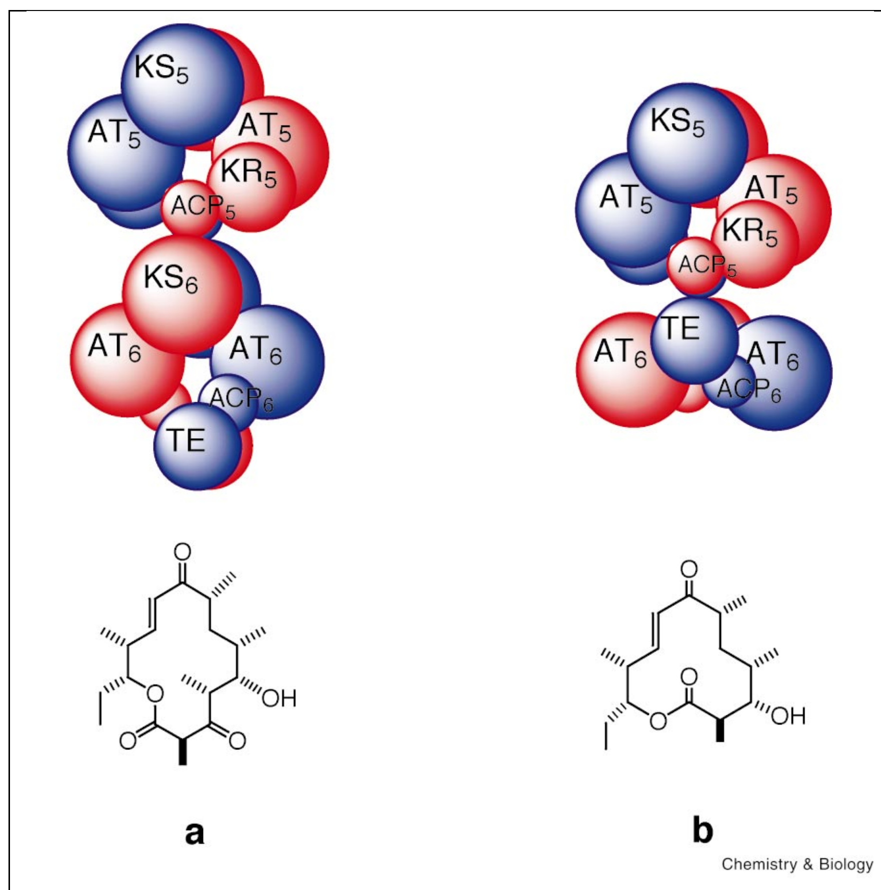

Figure 2. Proposed model for the role of PikAIV in production of narbonolide (**a**) and 10-deoxymethynolide (**b**) (adapted from Xue and Sherman [12]). Abbreviations for the catalytic domains (depicted as spheres) are provided in Fig. 1. Different shades of blue indicate the two polypeptide components of the homodimer.

catalytically distinct manner with PikAIII also offers a unique system for understanding the interactions and functional orientations of domains within a PKS dimer, and the interpolypeptide interactions that allow chain elongation intermediates to be transferred to subsequent modules. Here we describe the results of probing these interactions in vivo using a range of hybrid PikAIV polypeptides.

Results and discussion

Expression of PikAIV and the TE domain as separate polypeptides decrease narbonolide production but not 10-deoxymethynolide production

The mutant *S. venezuelae* AX912 in which both the TE domain of PikAIV and the TEII had been deleted was previously constructed, and has been shown to produce no detectable polyketides under culture conditions for 10-deoxymethynolide (SCM media) or narbonolide production (PGM media) [12]. Similarly, no products were obtained in this current study when this strain was grown in SGGP media, which allows for production of both 10-deoxymethynolide and narbonolide (Table 1). A new strain SC1022 with a complete *pikAIV* and an in-frame deletion of *pikAV* (encoding TEII) was generated and shown to produce both of these aglycone products (Table 1), clearly supporting the importance of the TE domain in termination of polyketide extension at both PikAIII and PikAIV. No glycosylated products were formed because the deletion of the *pikAV* encoding TEII affects expression of downstream genes involved in desosamine biosynthesis and transfer (Chen et al., unpublished results). Mutant BL3001 [13] impaired in desosamine biosynthesis produced similar levels of aglycone products as observed for the SC1022 strain (Table 1).

Table 1

Production of 10-deoxymethynolide and narbonolide in SGGP media by *S. venezuelae* strains producing either hybrid PikAIV polypeptides and/or PikAIV without a TE domain.

Strain	Chromosomal PikAIV product	Plasmid Product	10-Deoxymethynolide (mg/l)	Narbonolide (mg/l)	Total (mg/l)
AX912	KS$_6$-AT$_6$-ACP$_6$		ND*	ND	ND
SC1022	KS$_6$-AT$_6$-ACP$_6$-TEI		12±2	52±2	64±4
BL3001	KS$_6$-AT$_6$-ACP$_6$-TEI		18±2	32±2	50±3
AX912/pDHS704	KS$_6$-AT$_6$-ACP$_6$	TEI	12±5	4±2	16±7
AX912/pDHS707	KS$_6$-AT$_6$-ACP$_6$	KS$_6$-AT$_6$-ACP$_6$-TEI	2±1	27±3	29±4
AX912/pSC43	KS$_6$-AT$_6$-ACP$_6$	KS$_6$[-AT$_{14}$-ACP$_{14}$-]$_{rap}$TEI	16±2	3±1	19±3
SC1017	KS$_6$[-AT$_{14}$-ACP$_{14}$-]$_{rap}$TEI		ND	ND	ND
SC1017/pSC43	KS$_6$[-AT$_{14}$-ACP$_{14}$-]$_{rap}$TEI	KS$_6$[-AT$_{14}$-ACP$_{14}$-]$_{rap}$TEI	ND	ND	ND
AX912/pSC73	KS$_6$-AT$_6$-ACP$_6$	[-AT$_{14}$-ACP$_{14}$-]$_{rap}$TEI	ND	ND	ND
AX912/pSC51	KS$_6$-AT$_6$-ACP$_6$	K[S$_x$-AT$_x$-DH$_x$-KR$_x$-ACP$_x$-]$_{nap}$TEI	ND	ND	ND
AX912/pSC63	KS$_6$-AT$_6$-ACP$_6$	K[S$_7$-AT$_7$-KR$_7$-ACP$_7$-TE]$_{tyl}$	ND	ND	ND
SC1020	KS$_6$-[AT$_{14}$-ACP$_{14}$-]TEI		ND	ND	ND
SC1020/pDHS704	KS$_6$-[AT$_{14}$-ACP$_{14}$-]TEI	TEI	ND	ND	ND
SC1021	KS$_6$-A[T$_{14}$-ACP$_{14}$-]TEI		ND	ND	ND
SC1016	KS$_6$- AT$_6$[-ACP$_{14}$-]TEI		13±1	0	13±1
SC1015	KS$_6$- AT$_6$[-ACP$_{13}$-]TEI		48±2	15±2	63±4

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Natural PikAIV domains are shown in blue and the domains from the PKS module are shown in red (the same color schemes are used in Figures 3 and 4). Brackets indicate the general area where fusion of the catalytic domains of the various PKSs occurred (a more detailed description is provided in the text). The bracket in K[S and A[T indicate that the fusion point was made within the conserved catalytic domain. The dash between the different regions indicates the peptide linker region. The AT$_{13}$, ACP$_{13}$ and ACP$_{14}$ are domains of RapC of the rapamycin PKS. $_{nap}$AT$_x$-DH$_x$-KR$_x$-ACP$_x$ are from a module $_x$ of the putative naphthomycin PKS. $_{tyl}$AT$_7$-KR$_7$-ACP$_7$-TE is a module from the tylosin PKS. Yields were determined against a standard curve derived for both 10-deoxymethynolide and narbonolide. ND, not detectable.

A series of complementation experiments was then carried out using the AX912 strain. In these and subsequent complementation studies plasmid-based expression was accomplished using a low copy plasmid [12] and the *pikAI* promoter, P*pikAI* (allowing synchronized expression at similar levels as other components of the *pikA* cluster). Plasmid-based (pDHS707) expression of a complete PikAIV in the AX912 strain also gave both aglycones in SGGP media with overall lower yields (approximately 50%) and a significantly greater proportion of narbonolide relative to 10-deoxymethynolide than seen with SC1022 (Table 1). A similar increase in this ratio was observed when this strain AX912/pDHS707 is grown in SCM media, which typically produces predominantly 10-deoxymethynolide [12]. One possible explanation for the observed increase in narbonolide relative to 10-deoxymethynolide is that such plasmid-based expression of PikAIV may utilize the alternative translational start site (giving rise to the N-terminal truncated PikAIV) less readily as compared to chromosomal-based expression. The lower overall polyketide yields with the plasmid-based expression of PikAIV is presumably a reflection of the presence of the TE-deleted chromosomal copy of PikAIV.

Plasmid-based (pDHS704) expression of TE in the AX912 strain gave an apparent complete restoration of 10-deoxymethynolide production and low levels of narbonolide production (8%) as compared to the SC1022 strain. Similar results have previously been seen for this strain grown in SCM media (60% restoration of 10-deoxymethynolide) and PGM media (no detectable narbonolide production). The small variations in levels of antibiotic production (0% versus 8% for narbonolide production, for instance) presumably reflects the use of different media and the typical fluctuations observed for experiments performed using several randomly picked transformants. In all experiments the low levels of narbonolide product ($< 10\%$) are presumably a result of a poor functional association between the ACP domain of PikAIV and the TE, and is consistent with previous analyses of erythromycin where the TE domain functions efficiently only when covalently linked to ACP [14]. Conversely, substantial restoration of 10-deoxymethyonlide production (60–100%) in all of these experiments indicate that the TE domain is able to form a relatively efficient interaction with the PikAIII ACP_5 when the truncated form of PikAIV is present. This interaction appears to be facilitated in part by the AT_6 domain of PikAIV (see below).

A TE domain attached to a functionally inactive hybrid PikAIV releases narbonolide from a PikAIV module lacking a terminal TE domain

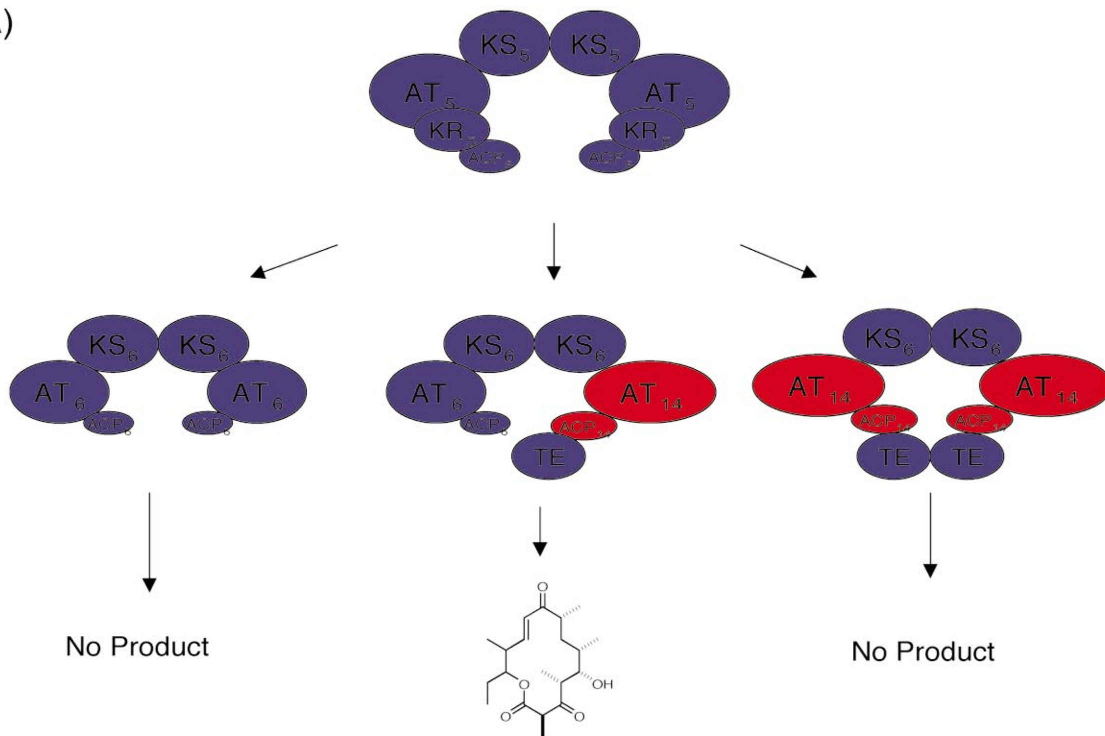
A plasmid (pSC43) expressing a hybrid PikAIV polypeptide (KS_6[-AT_{14}-ACP_{14}-]$_{rap}$TE) comprised of the PikAIV KS_6, TE domains and the $_{rap}AT_{14}$-ACP_{14} domains from RapsC of the rapamycin PKS [15] was constructed and introduced into the AX912 strain. The $_{rap}AT_{14}$ domain is responsible for incorporating a malonyl CoA extender unit during rapamycin biosynthesis and would be predicted to generate a 2-desmethylnarbonolide product when introduced into PikAIV. No evidence of this compound could be found suggesting that the hybrid PikAIV was unable to extend the polyketide chain. A chromosomal mutant (SC1017) containing only this hybrid PikAIV was generated and also shown to produce no detectable 14-membered ring macrolactone product, leading to the same conclusion (see below) (Table 1).

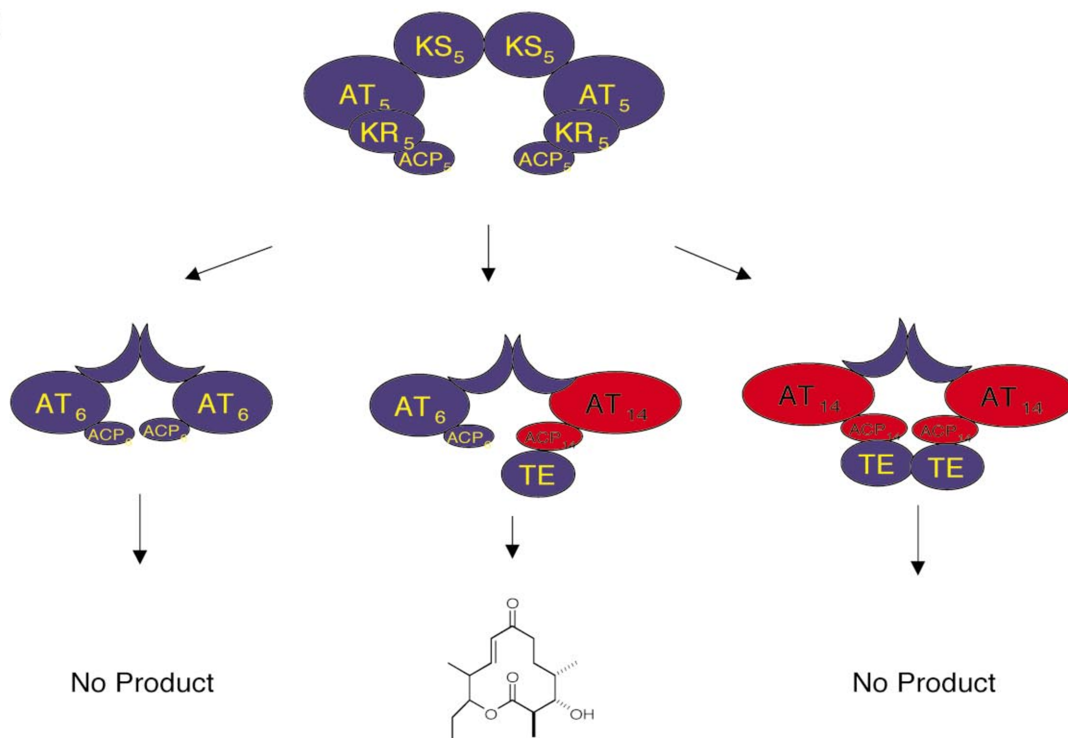
Plasmid-based (pSC43) expression of the hybrid PikAIV (KS_6[-AT_{14}-ACP_{14}-]$_{rap}$TE) in the TE-deleted AX912 strain did, however, produce narbonolide. No narbonolide was observed in a control when this plasmid (pSC43) was expressed in SC017 (expressing the same hybrid PikAIV) (Table 1). The level of production of this aglycone was 10-fold less than seen for plasmid-based (pDHS707) expression of native PikAIV. Production of narbonolide rather than 2-desmethylnarbonolide and the dependence on the presence of TE-deleted PikAIV is consistent with the elongated polyketide chain being generated using the AT_6 domain on this C-terminal truncated PikAIV and the TE module on the hybrid PKS (Figure 3A). Thus, it seems likely that a heterodimeric polypeptide must form consisting of this hybrid PikAIV and the TE-deleted PikAIV (Figure 3A). The decrease in narbonolide production relative to a control experiment using pDHS707 is consistent with the proposal that only the heterodimeric PikAIV complexes (which consists of one, rather than two complete sets of catalytic activities) but neither of the homodimeric complexes, can produce narbonolide (Figure 3A).

Evidence for formation of heterodimeric PKSs have been previously reported for the erythromycin and pikromycin PKSs [8,12]. In these cases the two polypeptides either differed in mutations in active site residues of specific domains, or contained specific domain deletions. Studies with the erythromycin heterodimers have clearly demonstrated that the polyketide chain is transferred both within and between the two polypeptide chains at different stages as it is processed through the PKS [8,10]. These studies have also shown that the AT domain can catalyze acylation of the ACP domain on either subunit [9]. It is therefore possible that the AT_6 within the proposed PikAIV heterodimer might function on both ACP_6 and $_{rap}ACP_{14}$ (Figure 3A). It remains to be determined which of these ACP domains carries the fully elongated polyketide chain that is cleaved by the TE.

Dimerization of KS_6 domains is essential for formation of a functional heterodimer between an inactive hybrid PikAIV and a TE-truncated form of PikAIV

In the current study the proposed heterodimeric complex





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forms between significantly different polypeptides containing AT and ACP domains from different PKSs (Figure 3A). Both polypeptides contain the PikAIV KS_6 domain and the importance of this in formation of the proposed heterodimeric PikAIV complex was probed through a series of complementation experiments.

First, the KS_6 domain from the hybrid PKS was removed. A plasmid (pSC73) capable of expressing a polypeptide ($[-AT_{14}-ACP_{14}-]_{rap}TE$) composed of the RapC AT_{14}-ACP_{14} and the PikAIV TE domain was constructed. When this plasmid was introduced into the AX912 strain, neither 10-deoxymethynolide nor narbonolide were detected, supporting the hypothesis that the proposed heterodimer cannot form in the absence of KS_6 (Table 1). In contrast, both of these products have previously been observed with plasmid-based expression of AT_6-ACP_6-TE (pDHS708) in the same AX912 strain [12], presumably because of a dimerization of the AT_6-ACP_6 domains. In the latter case it is noted that the dimer contains only one KS domain which must, therefore, be responsible for catalyzing the final elongation step. This domain is located on the TE-deleted form of PikAIV, while the TE responsible for catalyzing narbolide formation is contained on the AT_6-ACP_6-TE polypeptide chain.

Second, a plasmid (pSC51) capable of expressing a hybrid PikAIV containing only the N-terminal region of PikAIV (including the first 118 amino acids of KS_6) and the TE and a KS-AT-DH-ACP domain from a module of the putative *Streptomyces collinus* naphthomycin (*nap*) PKS [16] was constructed. A second plasmid (pSC63) capable of expressing a hybrid PikAIV containing just the N-terminal region of PikAIV (including the first 81 amino acids of KS_6) and the KS_7-AT_7-DH_7-ACP_7-TE domain of the module 7 of the tylosin (*tyl*) PKS was prepared. Expression of either pSC63 or pSC51 in the AX912 mutant failed to produce detectable levels of narbonolide, 10-deoxymethynolide, or any other aglycone products under standard fermentation conditions (Table 1). These observations support the hypothesis that formation of a heterodimer capable of generating 10- or 12-membered ring macrolactone products requires at least one domain such as KS_6 to be present on both polypeptide chains. Such a finding supports the proposed structural models for the erythromycin PKS where the core structure is achieved by strong association of KS domains [10]. We propose that the apparent inability of different KS domains to form a functional dimer may ensure that only homodimeric complexes are formed in unmodified PKSs, thus maximizing the production of a single polyketide product.

A TE-deleted PikAIV is required for a hybrid, functionally inactive PikAIV containing a TE domain to release 10-deoxymethynolide products from PikAIII

Plasmid-based expression (pSC43) of the hybrid PikAIV in AX912 gave approximately an eight-fold increase in the levels of 10-deoxymethynolide as compared to plasmid-based expression (pDHS707) of the wild-type PikAIV. The TE attached to the hybrid PikAIV must terminate polyketide chain extension at PikAIII. In contrast a PikAIV chromosomal mutant (SC1017) containing only this hybrid PikAIV was unable to generate 10-deoxymethynolide. Similarly no 10-deoxymethynolide was generated when SC1017 was transformed with pSC43. These observations suggest that formation of a heterodimeric PikAIV complex is also necessary for TE to act upon PikAIII. Only the latter half of the KS_6 can be used to form heterodimers because the N-terminal region is not generated in the truncated form of PikAIV that produces 10-deoxymethynolide [12] (Figure 3B).

The observation that AX912/pSC43 strain can produce significant levels of 10-deoxymethynolide is surprising given that the PikAIV homodimers generated in this system are predicted to be inactive (Figure 3A,B). The SGGP media allows production of both 10-deoxymethynolide and narbonolide products and thus AX912/pSC43 presumably produces the hybrid PikAIV and wild-type PikAIV in both the complete and N-terminally truncated form allowing six different PikAIV dimeric complexes to be present (Figure 3A,B). If the N-terminally truncated PikAIV and full length PikAIV polypeptides can dimerize then as many as 10 dimeric forms of PikAIV may be present, of which only heterodimeric forms may be active. A static model for the pikromycin PKS in which these different PikAIV polypeptides associate with PikAIII in vivo and do not readily dissociate would give rise predominantly to inactive PKSs not containing a TE domain, and significantly decreased yields of *both* narbonolide and 10-deoxymethynolide. A dynamic model in which the various forms of PikAIV rapidly temporarily associate and dissociate can also be envisioned. In this model, PikAIII would associate with PikAIV heterodimers capable of forming both narbonolide and 10-deoxymethynolide. In such a model a dramatic decrease in narbonolide production due to the presence of

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Figure 3. Proposed interaction of PikAIII with a heterodimer comprised of a TE-deleted PikAIV and a hybrid PikAIV. The AT_{14} and ACP_{14} domains of RapsC are shown in red, while the natural domains of the pikromycin PKS are shown in blue. This heterodimer expressed with the complete (**A**) and truncated KS_6 domain (**B**) is proposed to be responsible for generation of narbonolide and 10-deoxymethynolide, respectively, in the AX912 strain carrying pSC43. Homodimers comprised solely of either the hybrid PikAIV, or the TE-deleted PikAIV, are unable to generate either product (Table 1).

mutant PikAIV polypeptides unable to elongate, could produce a concomitant increase in 10-deoxymethynolide. This increase would occur so long as the heterodimers containing a truncated PikAIV polyketide are able to catalyze chain termination at PikAIII. The overall suppression of aglycone production in the AX912/pSC43 (33%) and AX912/pDHS707 (50%), compared to the SC1022 control, presumably reflects a slowing of polyketide chain extension due to the temporary association of PikAIII with inactive forms of PikAIV.

The PikAIV AT_6 domain is essential for generating both 12- and 14-membered ring macrolactones

A series of different chromosomal mutants in the *pikAIV* locus were constructed to investigate the reason why a hybrid PikAIV, in which AT_6-ACP_6 had been replaced with AT_{14}-ACP_{14} of RapC, cannot produce either 10-deoxymethynolide, narbonolide or any other product. Strain SC1017 that produced this hybrid PikAIV using a fusion point that allows 35 amino acids of a unique linker region upstream of AT_{14} of RapC [15] was generated (the same fusion point was used for pSC43 plasmid-based expression of this hybrid PikAIV). This KS_{14}-AT_{14} linker of RapC, based on published sequence pile-up data [14], was used in place of normal PikAIV KS_6-AT_6 linker. Strain SC1020 produced a hybrid PikAIV with the $AT_6/_{rap}AT_{14}$ fusion point 22 amino acids into the conserved domain of AT_6. A similar fusion point using the $_{rap}AT_{14}$ (three amino acids into a conserved AT domain) has previously been reported in the successful generation of a hybrid erythromycin PKS capable of producing a desmethyl 6-deoxyerythronolide B analog [17]. Strain SC1021 contained a hybrid AT having the N-terminal region of PikAIV AT_6 and the C-terminal region of $_{rap}AT_{14}$. The fusion point used to generate this chimeric AT was in a highly conserved region that comes 25–35 amino acids after the proposed AT binding motifs which specify malonyl CoA or methylmalonyl CoA specificity [18]. The fusion juncture was, however, before the recently described hypervariable region of the AT domain, suggested to determine substrate specificity [19].

None of these mutants (SC1017, SC1020, SC1021) were observed to generate detectable levels of either 10-deoxymethynolide or narbonolide (Figure 4). Only a mutant (SC1016) expressing a hybrid PikAIV containing the entire conserved AT_6 domain (the fusion point in this mutant was

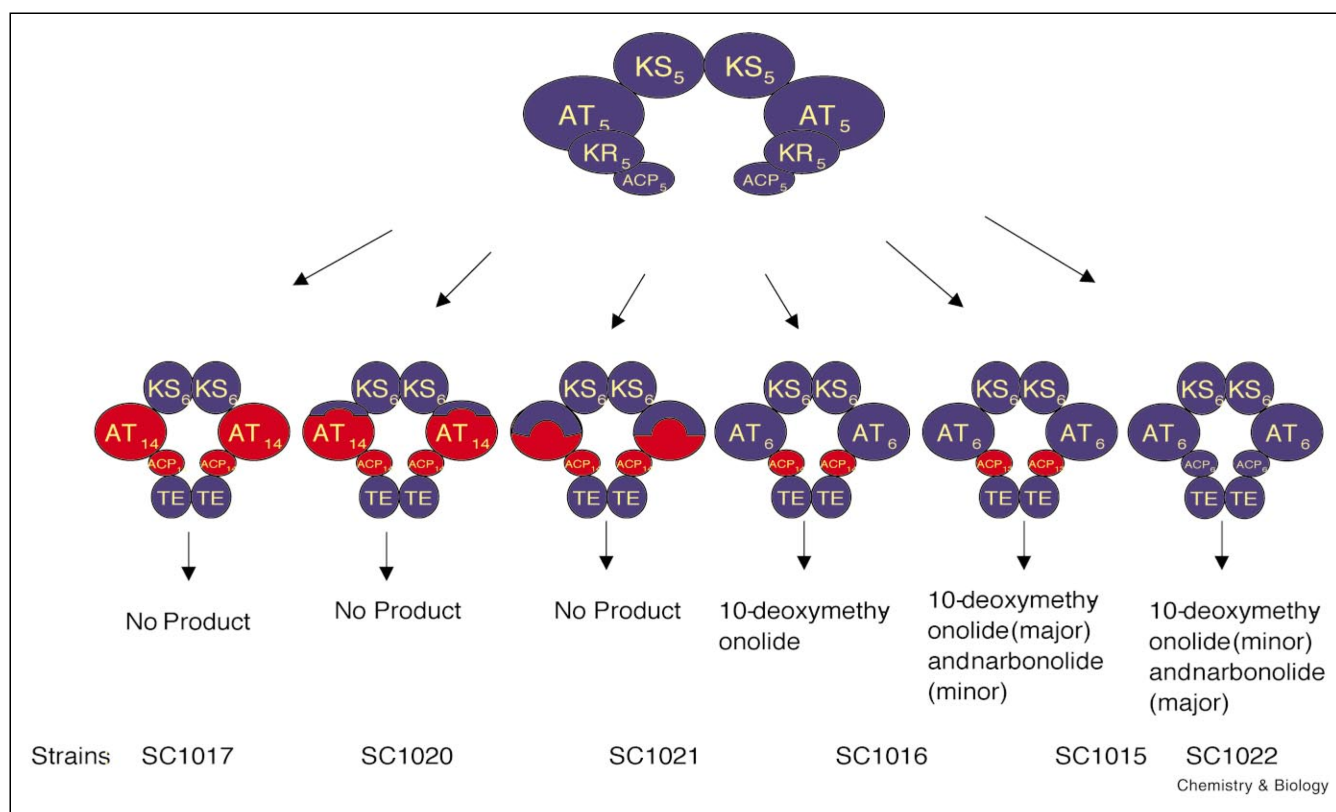

Figure 4. Production of narbonolide and 10-deoxymethynolide using pikromycin PKS in five *S. venezuelae* strains which produce different hybrid PikAIV polypeptides. The rapamycin PKS domains from module 14 and 13 are shown in red. As all strains were grown in SGGP media, the full length PikAIV products would be accompanied by N-terminal truncated PikAIV products (not depicted).

made at the beginning of the linker region between AT_6 and ACP_6) produced 10-deoxymethynolide (Table 1 and Figure 4). Even in this mutant no detectable levels of narbonolide were generated. In terms of 10-deoxymethynolide production, these results indicate that the N-terminal truncated hybrid PikAIV is unable to bring TE into the appropriate position at the end of PikAIII, unless AT_6 is present (an interaction is either facilitated by AT_6, or blocked by the presence of alternative AT domains). This hypothesis was supported by the observation that plasmid-based (pDHS704) expression of the TE domain in SC1020 did not result in the production of 10-deoxymethynolide, even though significant levels (12 mg/l) were observed when this complementation experiment was carried out using the AX912 strain (Table 1). In the complementation studies when plasmid-based expression of a hybrid PikAIV with AT_{14} does produce 10-deoxymethynolide (AX912/pSC43), the AT_6 is presumably provided by dimerization with the TE-deleted PikAIV. Thus, in all experiments carried out in the absence of AT_6, a TE domain whether attached to a hybrid PikAIV, or expressed as a separate polypeptide, cannot cleave polyketide products from PikAIII.

Recent studies using the erythromycin PKS have suggested that the N-terminal region upstream of a KS domain of an N-terminal module contains an 'interpolypeptide linker' which plays an important role in facilitating transfer of an extended polyketide chain from the ACP domain of the preceding polypeptide [20]. In the case where the N-terminal region of PikAIV is deleted, such a linker would be absent and thus one of the roles of AT_6 in the PikAIV may be to facilitate interaction with PikAIII. This interaction may explain the surprising efficiency of 10-deoxymethynolide production when TE is provided in *trans* (pDHS704) to the AX912 strain (Table 1).

The lack of detectable narbonolide or 2-desmethylnarbonolide production with mutants expressing PikAIV hybrids with alterations in the AT and ACP domains is most likely indicative of a catalytically inactive or inefficient module. The fact that SC1016 expressing a hybrid PikAIV with only ACP_6 substitution can produce 10-deoxymethynolide but not narbonolide, suggests that at least in this case an association of the PikAIII and hybrid PikAIV polypeptides is possible. It is now well established that using gene fusion approaches to create hybrid PKSs with domain substitutions often leads to decreased in vivo productivity [5,21]. Suggested reasons for the lower productivity include suboptimal catalysis within the altered PKS, structural instability of the hybrid protein, or inefficient processing of the nonnatural polyketide intermediate by downstream modules [20]. Some of the nonproductive changes in PikAIV (SC1016) should not in principle affect the final product structure, and the TE domains in many of the PikAIV hybrids can release products from either PikAIII, or PikAIV in the proposed heterodimers. Presumably then, most hybrid PikAIV homodimers are unable to produce significant levels of the predicted 14-membered ring macrolactone due to inefficient catalysis.

Hybrid PikAIV polypeptides containing different ACPs produce different ratios of 12- and 14-membered ring macrolactones

Only one hybrid PikAIV, in which the ACP_6 was replaced by the ${}_{rap}ACP_{13}$ was able to generate significant levels of the 14-membered ring narbonolide product (SC1015). The reason why a hybrid PikAIV containing the ${}_{rap}ACP_{13}$ (SC1015) and not the ${}_{rap}ACP_{14}$ (SC1016) can catalyze elongation is unclear, but may reflect the difference in linker regions flanking the two ACPs. The ${}_{rap}ACP_{14}$ switch included the 15 amino acid linker region between ${}_{rap}AT_{14}$ and ${}_{rap}ACP_{14}$ of RapsC, while the ${}_{rap}ACP_{13}$ switch included the 35 amino acid linker region between ${}_{rap}KR_{13}$ and ${}_{rap}ACP_{13}$ of RapsC [15]. The ACP_{13} switch was linked to TE using the amino acid region between ${}_{rap}ACP_{13}$ and ${}_{rap}KS_{14}$, while a similar fusion using ACP_{14} involved the unusual C-terminal region of RapsC (a region that might be involved in forming an interaction with RapP, the protein involved in termination of rapamycin polyketide chain extension).

Even though strain SC1015 produces narbonolide, in vivo production is only approximately 30% of that observed for the control strain (SC1022) expressing the wild-type PikAIV. Thus, the hybrid PikAIV with the ${}_{rap}ACP_{13}$ is still less efficient at elongation. This strain, grown in SGGP media, however, produced the highest levels of 10-deoxymethynolide (four-fold higher than the SC1022 control) out of all experiments performed. Interpreting this data from a static model for polyketide production in which the full length and N-terminal truncated forms of PikAIV are used to form distinct pikromycin PKSs (Figure 2) would require that (a) the hybrid PikAIV is less efficient at elongation yet more efficient at chain termination at PikAIII, and (b) that chain termination in both forms is rate-limiting. As described above, a dynamic model in which these two forms rapidly associate and dissociate from PikAIII must be considered. In such a model a hybrid PikAIV less efficient at chain elongation would decrease narbonolide production and provide greater opportunities for the truncated PikAIV to terminate elongation at PikAIII, thus increasing 10-deoxymethynolide production.

Significance

Modular PKSs are large multifunctional enzymes containing discrete modules, responsible for elongating small carboxylic acid precursors into complex polyketide products via a sequential process. The individual polypeptide components of bacterial type I PKS systems contain one or more dimerized modules. The interactions between the dimers, and the manner in which the different polypeptide

components interact are important factors that allow the molecular assembly line process to typically generate one aglycone product. The generation of novel polyketides through rational bioengineering of these PKS systems will be aided by understanding these processes and how they can be altered.

The pikromycin PKS produces ketolide antibiotics, a class of natural products that have recently attracted significant interest because of their activity against a number of macrolide-resistant microorganisms [22]. The last polypeptide (PikAIV) of this PKS generates the ketolide functionality in 14-membered ring macrolactone products, and also plays a role in producing 12-membered ring macrolactones by terminating chain extension at the C-terminus of PikAIII. We have provided strong evidence that the AT_6 domain within PikAIV plays an important role in facilitating TE-catalyzed chain termination at ACP_5 of PikAIII. In addition, these studies show for the first time that in vivo PKS heterodimer complexes can be formed using modules that share only a KS domain in common. In such a heterodimer, the TE domain responsible for terminating polyketide chain extension and the AT domain responsible for loading the methylmalonyl CoA extender unit are apparently located on separate polypeptides. Finally, we have shown that the pikromycin PKS can tolerate the presence of multiple forms (active and inactive) of PikAIV, the polypeptide catalyzing the final elongation step in pikromycin. This observation suggests that there is a dynamic interaction between PikAIV and the PikAIII polypeptide responsible for catalyzing the preceding chain elongation step. These findings may be useful in various combinatorial biosynthetic applications, such as the generation of a range of polyketide products from a single recombinant microorganism.

Materials and methods

Bacterial strains and culture conditions

The mutant AX912 was previously created by targeted gene replacement with wild-type *S. venezuelae* ATCC 15439 [12]. All the subsequent mutants substituted at AT_6 and/or ACP_6 domains on chromosome were obtained from AX912 by standard homologous recombination procedures. AX912 was also used as the host for the production of polyketides from the engineered plasmids derived from pAX702. *Escherichia coli* TG1 (Stratagene) was used throughout this study as a cloning host, growing in standard culture conditions. *S. venezuelae* strains were grown on R2YE agar plates after transformation and then transferred to sporulation agar for production of spores. SGGP liquid medium was used for propagation of *S. venezuelae* mycelia and antibiotic production [23].

Manipulation of DNA and organisms

The manipulation and transformation of DNA in *E. coli* was performed by general molecular cloning procedures. Polymerase chain reaction (PCR) was performed with *rTth* enzyme (Perkin-Elmer) under conditions recommended by the manufacturer. *S. venezuelae* were transformed by standard procedure and transformants were selected on agar plates using thiostrepton and/or apramycin (50 μg/ml).

PCR amplification of DNA fragments

DNA fragments used for constructing the various recombination and expression plasmids were PCR amplified from plasmids or cosmids containing *pikAIV* (pDHS707, cosLZ51) [12], *rapsC* (cos2, cos8, cos9, provided by Leonard Katz, Abbott Laboratories), *tylG* (pOJ566, a gift from Eli Lilly Co.) and the gene encoding a module from the putative naphthomycin PKS (cos7G11) [16]. The following oligonucleotides with designed restriction sites (italics) for ligation were used (all shown as 5′→3′): A 960 bp *Nde*I–*Bam*HI fragment containing 3′ region of *pikAIV* (encoding the TE domain), forward GGCGGAGGAG*CATATG*GAAGAAGATCCGGC and reverse GCGCC*GGATCC*ACAGTCCGCTGTCCAC; a 1.32 kb *Eco*RI–*Nde*I/*Xba*I fragment containing the internal region of *pikAIV* encoding the AT_6-ACP_6 (for pSC992 construction), forward CCC*GAATTC*GCCGCCGCCATGGCCGAAT (*Eco*RI) (primer 1) and reverse GACGGC*TCTAGA*CGGCC*CATATG*CGCTCGGCC (*Nde*I/*Xba*I); a 1.75 kb *Xba*I fragment containing *pikAV* and *desVIII* region and the 3′ region of *pikAIV* (for pSC992 construction), forward GGC*TCTAGA*TCGATGGCATCGAGGGGGCGGGCAAGTGACCGACA and reverse GGG*TCTAGA*GCTGCACCGGCGGGTCGTAGCGGA; a 1 kb *Eco*RI–*Xho*I fragment containing a region of *pikAIV* encoding AT_6 (for pSC1002 and pSC1003 construction), primer 1 (forward) and reverse GGCGACGGC*CTCGAG*CCCGGAAGCGGTG; a 420 bp *Xho*I–*Nde*I fragment (fragment A) containing a region of *rapsC* encoding ACP_{13} (for pSC1015 construction), forward TGCGCGGA*CTCGAG*ACGGTGAAGCGTCC and reverse GGCGATCGG*CATATG*CGCCGACGGCTCA; a 480 bp *Xho*I–*Nde*I fragment (fragment B) containing a region of *rapsC* encoding ACP_{14} (for pSC1016 construction), forward CTACTGG*CTCGAG*CCGGCCGCCCCGGAT and reverse CGTGTGACACC*CATATG*TTCGTTCCTGGTCC (primer 2); a 1.4 kb *Eco*RI–*Nde*I fragment encoding the region of *rapsC* encoding AT_{14}-ACP_{14} (for pSC1020 construction), pSC1020 forward AGCTACCCC*GAATTC*GCACGTGCCTGGGAG (*Eco*RI), and primer 2 (reverse); a 1.06 kb *Hin*dIII–*Nde*I fragment of *rapsC* encoding the rapAT_{14}-ACP_{14} region (for pSC1019 construction), forward GGTGAGG*AAGCTT*TGAGGGAGTTCCTCAGC (*Hin*dIII) and primer 2 (reverse); a 330 bp *Eco*RI–*Hin*dIII fragment of *pikAIV* encoding the AT_6-ACP_6 region (for pSC1019 construction), primer 1 (forward), and reverse GCTGCCGGG*AAGCTT*CCTCGCTGAGGGC (*Hin*dIII); a 1.6 kb *Bam*H1–*Nde*I fragment (fragment C) of *rapC* encoding the AT_{14}-ACP_{14} region (for pSC1017 and pSC43 construction), forward TCGCGGTG*GGATCC*GGGCCGGGAGGACT and primer 2 (reverse); a 1.6 kb *Bam*HI–*Nde*I/*Bam*HI fragment containing the same region of *rapC* as fragment C, using the same forward primer and reverse CGCGGATCCATATGCCTCTCCATTCGTTCCTGGTCCTCCATTTT; a *Bam*HI–*Nde*I fragment (fragment D) of the same region of *rapsC* encoding AT_{14}-ACP_{14} (for pSC73 construction), forward TCGCGGTG*GGATCC*GCCGGGAGGACT and primer 2 (reverse); A 410 bp *Eco*RI–*Nco*I fragment of *pikAIV* encoding the RBS site and first 120 amino acids of KS_6 (for pSC51 construction), forward AGTGAGTCCGAG*GAATTC*ATCGAGGGG (primer 3) and reverse AACAGGATCCGCTGCTGCGGGT*CCATGG*CCACCGCCTC; a 320 bp *Eco*RI–*Bam*HI fragment of *pikAIV* encoding the RBS site and first 80 amino acids of KS_6 (for pSC63 construction), primer 3 (forward), reverse TTGCGCCCG*GGATCC*GGGTCGTAGAGC; a 5.1 kb *Nco*I–*Nde*I fragment from cos7G11 encoding KS-DH-AT-ACP domains of a putative naphthomycin PKS module from *S. collinus* (for pSC51 construction), forward AGGCGGTGG*CCATGG*ACCCGCAGCAG and reverse GATGGGATCCATTTC*CATATG*CACGGCCACCCCGGAGAGAC.

Construction of homologous recombination plasmids

pSC992 was obtained by first ligating the *Eco*RI–*Nde*I/*Xba*I PCR fragment into pUC119. A *Xba*I fragment was then cloned into the *Xba*I site. Two *Eco*RI–*Nde*I fragments were generated by ligating a *Eco*RI–*Xho*I PCR fragment and the two *Xho*I–*Nde*I PCR fragments (fragments B and C) and used to replace the corresponding region of pSC922 to generate pSC1002 and pSC1003, respectively. The *Nde*I–*Bam*HI PCR fragment was then cloned into pSC1002 and pSC1003 that had been digested

with *Nde*I–*Bgl*II. Subsequently, the *Eco*RI–*Xba*I fragments from these pSC1002 and pSC1003 derivatives were cloned into pKC1139 and made pSC1015 and pSC1016. A coligation of a natural 2.4 kb *Eco*RI–*Bam*HI fragment of *pikAIV* (encoding AT_6-ACP_6-TE, and the first 13 amino acids of TEII), with a 1 kb *Bgl*II–*Xba*I fragment of pSC992 produced an *Eco*RI–*Xba*I fragment that was cloned into pKC1139 to give pSC1022. A 1.6 kb *Eco*RI–*Bam*HI fragment (containing the region of *pikAIV* encoding KS_6) from pDHS707 was ligated with the *Bam*HI–*Nde*I PCR fragment (fragment C) and subsequently used to replace the corresponding *Eco*RI–*Nde*I fragment in pSC1015, to generate pSC1017. A 1.8 kb *Eco*RI fragment from pSC43 (see below) was ligated with the *Eco*RI–*Nde*I PCR fragment and subsequently used to replace the corresponding *Eco*RI–*Nde*I fragment in pSC1015, to generate pSC1020. The PCR *Hin*dIII–*Nde*I fragment was ligated with the *Eco*RI–*Hin*dIII fragment and subsequently used to replace the corresponding *Eco*RI–*Nde*I fragment in pSC1016 to generate pSC1019. The 1.8 kb *Eco*RI fragment from pSC43 was then cloned into the corresponding site in pSC1019 to generate pSC1021. The plasmids pSC1015–1017 and pSC1020–1022 were used to generate the corresponding *S. venezuelae* mutant, as described above.

Construction of expression plasmids

All the expression plasmids were derivatives of pDHS702 [12] a shuttle vector, with an *E. coli* colE origin and a low copy *Streptomyces* origin (based on SCP2*). Antibiotic selection for pDHS702 is provided by thiostrepton in *Streptomyces* and ampicillin in *E. coli*. The expressed genes are under the control of *pikAI* promotor, P*pikAI*. Plasmid pDHS707 was digested with *Nde*I and religated after end-filling to obtain pSC39. The 1.6 kb PCR *Bam*HI–*Nde*I/*Bam*HI PCR fragment was cloned into the *Bam*HI site of pSC39 to give pSC40. The *Nde*I–*Bam*HI PCR fragment was used to replace the *Nde*I–*Bgl*II fragment of pSC40 to give pSC43. pDHS702 [12] was digested with *Nde*I and religated after end-filling to obtain pSC38. The *Bam*HI–*Nde*I PCR fragment (fragment D) was ligated with the *Nde*I–*Bam*HI PCR fragment and subsequently used to replace the *Bam*HI–*Bgl*II fragment of pSC38 to generate pSC73. The *Eco*RI–*Nco*I PCR fragment was ligated with the *Nco*I–*Nde*I PCR fragment and then used to replace the *Eco*RI–*Nde*I fragment of pSC43 to create pSC51. The *Eco*RI–*Bam*HI PCR fragment was ligated with a natural 8.07 kb *Bam*HI–*Nsi*I fragment from pOJ566 (encoding almost the entire tylosin module 7) and then cloned into the corresponding sites of pSC38 to generate pSC63.

Purification and characterization of polyketide

Innoculated SGGP liquid media were incubated in a 30°C shaker for 3 days and 10-deoxymethynolide, narbonolide and related compounds were extracted following published procedures [24]. Thin layer chromatography ($CHCl_3$:MeOH, 9:1) was routinely used to detect the antibiotics. Further purification was conducted using flash column chromatography ($CHCl_3$:MeOH, 9:1) and HPLC (MeCN:H_2O, 50:50). Crude extracts and purified compounds were analyzed by LC-MS.

Quantification of polyketide production

HPLC-purified 10-deoxymethynolide and narbonolide were dissolved in MeOH and the absorbancy measured at UV 226 and 228.5 nm, respectively. The concentration of the solutions was then calculated using the known narbonolide (ε, 8200) and 10-deoxymethynolide (ε, 9100) molar extinction coefficients [24,25]. Samples were then dried and serially diluted with acetonitrile (MeCN). The dilutions were injected onto a C18 reverse phase HPLC analytical column and eluted with 50:50 MeCN:H_2O and the aglycones were detected by absorbance at 230 nm. Peak areas were integrated and plotted against the injected amount of pure aglycone to obtain a standard curve. This was used subsequently to evaluate aglycone production in *S. venezuelae*. Antibiotic production was averaged from at least three separate fermentations with two selected colonies. In plasmid-based complementation, fresh transformed colonies were used for inoculating a production culture.

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