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Generation of Multiple Bioactive Macrolides by Hybrid Modular Polyketide Synthases in *Streptomyces venezuelae*

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Summary

The plasmid-based replacement of the multifunctional protein subunits of the pikromycin PKS in *S. venezuelae* by the corresponding subunits from heterologous modular PKSs resulted in recombinant strains that produce both 12- and 14-membered ring macrolactones with predicted structural alterations. In all cases, novel macrolactones were produced and further modified by the DesVII glycosyltransferase and PikC hydroxylase, leading to biologically active macrolide structures. These results demonstrate that hybrid PKSs in *S. venezuelae* can produce a multiplicity of new macrolactones that are modified further by the highly flexible DesVII glycosyltransferase and PikC hydroxylase tailoring enzymes. This work demonstrates the unique capacity of the *S. venezuelae* pikromycin pathway to expand the toolbox of combinatorial biosynthesis and to accelerate the creation of novel biologically active natural products.

Introduction

Modular polyketide synthases (PKSs) are large multifunctional enzymes that are responsible for the biosynthesis of macrolides and other macrocyclic polyketides whose members have diverse structural and pharmacological properties [1]. These modular PKS assemblies are formed by giant multifunctional enzymes harboring one to many modules that catalyze serial condensations of activated coenzyme-A thioester monomers derived from small organic acids such as acetate, propionate, and butyrate [2–4]. Each module contains distinctive active site domains required for one cycle of polyketide elongation. Active sites required for condensation include an acyltransferase (AT), an acyl carrier protein (ACP), and a β-ketoacyl synthase (KS). Each condensation cycle results in a β-keto group that can undergo additional processing steps. Catalytic domains that perform these reactions include a keto reductase (KR), a dehydratase (DH), and an enoyl reductase (ER). The absence of any β-keto-processing domain results in the presence of a ketone, a KR alone gives rise to a hydroxyl, and a KR and DH generate an alkene, whereas a KR, DH, and ER combination leads to complete reduction to an alkane. After assembly of the polyketide chain, the molecule typically undergoes cyclization by a thioesterase (TE) domain at the C terminus of the final module. The linearity between the catalytic domains present and the structure of its polyketide products makes modular PKSs attractive systems for combinatorial biosynthesis [5, 6]. Hybrid PKSs have been generated via a number of genetic-engineering strategies, including (1) inactivation, deletion, insertion, and substitution of one or more catalytic domains, (2) deletion or exchange of complete modules, and (3) combining complete subunits from heterologous PKS clusters [1, 5, 7, 8].

The pikromycin (Pik) PKS of *S. venezuelae* has several remarkable features that make it a powerful system for combinatorial biosynthesis. Such features include the ability to produce both 12- and 14-membered ring macrolides [9] (Figure 1A). Recent work has shown that alternative expression of the Pik PKS results in the generation of two macrolactone structures [10]. Expression of full-length PikAIV (the last module required for heptaketide chain elongation) generates the 14-membered ring macrolactone narbonolide, whereas expression of an N-terminally truncated form of PikAIV (with the alternative translation start codon 600 amino acids downstream of the normal *pikAIV* start codon) results in skipping of the final condensation cycle to generate the 12-membered ring macrolactone 10-deoxymethynolide. The unusual nature of the *S. venezuelae* system provides a potentially useful tool for generating multiple products from a single hybrid modular PKS by combinatorial biosynthesis [11, 12].

Another strength of the *S. venezuelae* system is the presence of two tailoring enzymes that have unusual substrate flexibility (they recognize both 12- and 14-membered ring macrolactones). Specifically, there is one DesVII glycosyltransferase that can accept both 12- and 14-membered ring aglycones [13]. PikC (a P450 hydroxylase) also accepts both macrolide substrates and is active at two positions on the macrolactone system [14]. Because structural modifications are often critical for biological activity, a current challenge for combinatorial biosynthesis is to develop approaches that lead not only to novel macrolactones but also to ones that provide fully elaborated structures.

Here, using a set of hybrid modular systems based on the Pik, tylosin (Tyl) and erythromycin (DEBS) PKSs (Figures 1A, 1B, and 1C), we report novel combinatorial biosynthetic approaches in *S. venezuelae*. The three systems were chosen based on biochemical architecture (e.g., the presence of bimodular PikAI, DEBS1, and TylGI; two final monomodular PKSs in PikAIII/PikAIV and TylGIV/TylGV) as well as the structural similarity of their

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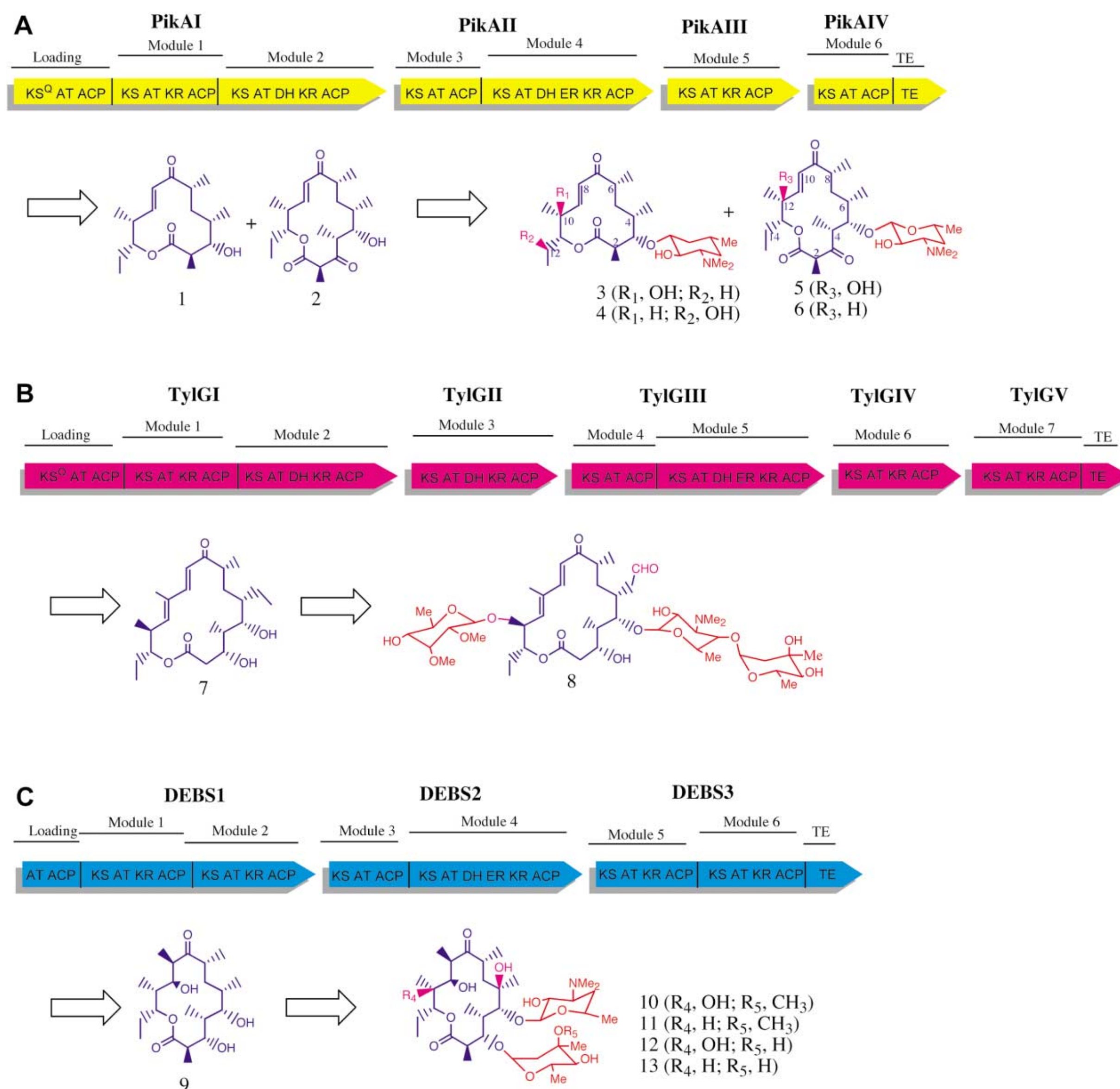

Figure 1. Modular Organization and the Products of Pik PKS, Tyl PKS, and Erythromycin PKS

Modular organization and the products of (A) Pik PKS, (B) Tyl PKS, and (C) erythromycin (DEBS) PKS (6-deoxyerythronolide B synthase). Each protein subunit is shown as a broad arrow, with the domains indicated by symbols explained in the text. Each module catalyzes one cycle of chain elongation and associated β-keto processing for the biosynthesis of corresponding macrolactones. Pik PKS produces 10-deoxymethynolide (1) and narbonolide (2). Final products are methymycin (3), neomethymycin (4), pikromycin (5), and narbomycin (6). Tyl PKS generates tylactone (7), which is modified further to tylosin (8). DEBS PKS produces 6-deoxyerythronolide B, which is converted to erythromycin A, B, C, and D (10, 11, 12, and 13, respectively). Post-polyketide modifications are highlighted in different colors: hydroxylation, pink; glycosylation, red.

product profiles. These attributes provided a framework from which to address new questions about molecular recognition between heterologous monomodular PKSs as well as to probe the flexibility of the DesVII glycosyltransferase and PikC hydroxylase toward novel substrates.

Results and Discussion

In a recent report, we described an initial investigation of the mechanisms of molecular recognition in the PikAIV (module 6) of the Pik PKS. One aspect of this analysis involved creation of heterodimeric forms of the multifunctional protein in which native PikAIV interacted with a hybrid bearing AT and ACP domains from the Rap PKS [12]. The results provided new insights into molecular recognition of the Pik PKS, but they also demonstrated that some homodimeric forms of the hybrid PikAIV proteins are unable to produce detectable levels of polyketide because of inefficient catalysis. Previous work has shown that protein subunits from heterologous modular PKSs can be functionally assembled in ways that improve the yield of product formation relative to engi-

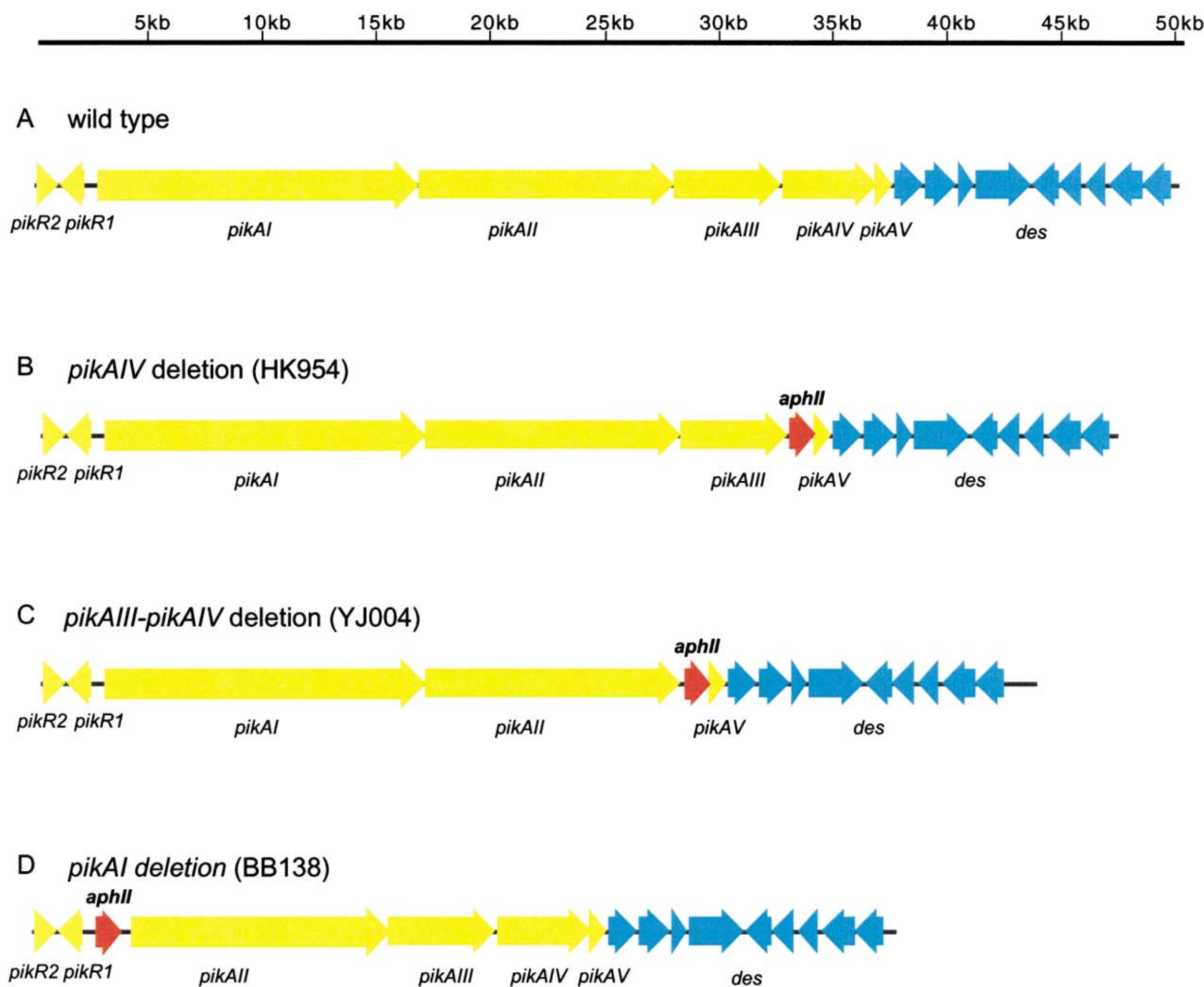

Figure 2. Organization of the *pik* Cluster in Wild-Type *S. venezuelae* and Pik PKS Deletion Mutants

Organization of the *pik* cluster in (A) wild-type *S. venezuelae*, (B) *pikAIV* deletion mutant HK954, (C) *pikAIII-pikAIV* deletion mutant YJ004, and (D) *pikAI* deletion mutant BB138. Each arrow represents an ORF. The direction of transcription and relative sizes of the ORFs are indicated. *aphII* and *pikAV* encode the kanamycin resistance gene [25] and type II thioesterase [13], respectively. After PKS gene deletion, *pikAV* and other downstream genes remain intact and can still be expressed.

neered domain replacements [7]. We therefore were motivated to design a more efficient system for the creation of hybrid Pik PKSs by subunit replacements in a genetic background lacking expression of the corresponding native Pik modules.

Complementation of the *S. venezuelae pikAIV* Deletion Mutant by *pikAIV* and *tylGV*

To construct hybrid PKSs in the final module of the Pik PKS, we generated an *S. venezuelae* mutant strain (HK954) bearing a deletion of *pikAIV* (Figure 2B) and performed a series of complementation experiments. Complementation was carried out with a low-copy plasmid of SCP2* origin (pDHS4162) [10, 11] under control of the native *pikAI* promoter. Expression of PikAIV in the HK954/pDHS4162 strain (Figure 3A) restored production of 12- and 14-membered ring macrolides to wild-type levels (our unpublished data). This initial experiment demonstrated that subunit complementation of plasmid-expressed PikAIV restores function of the Pik PKS for the production of both 12- and 14-membered ring macrolides at levels similar to those of wild-type *S. venezuelae*.

Upon successful complementation of the *S. venezuelae pikAIV* deletion mutant with pDHS4162, we proceeded to test the ability of a related heterologous monomodular PKS to functionally replace the mutant Pik PKS system. A plasmid expressing the Tyl PKS module 7 multifunctional protein (TylGV, [pDHS3007]) was constructed and transformed into *S. venezuelae* HK954. Analysis of the complementation results provides information on several key issues. First, the AT domain in Tyl module 7 is specific for malonyl-CoA extension as opposed to PikAIV, which specifies chain extension by methylmalonyl-CoA. Secondly, Tyl module 7 includes a KR domain that is absent from Pik module 6. Thus, the 14-membered ring macrolactone expected from the resulting Pik-Tyl hybrid PKS is 2-desmethyl-3-dihydro-narbonolide (**14**). Moreover, the expected macrolides from glycosylation/hydroxylation are 2-desmethyl-3-dihydro-pikromycin (**15**) and 2-desmethyl-3-dihydro-narbomycin

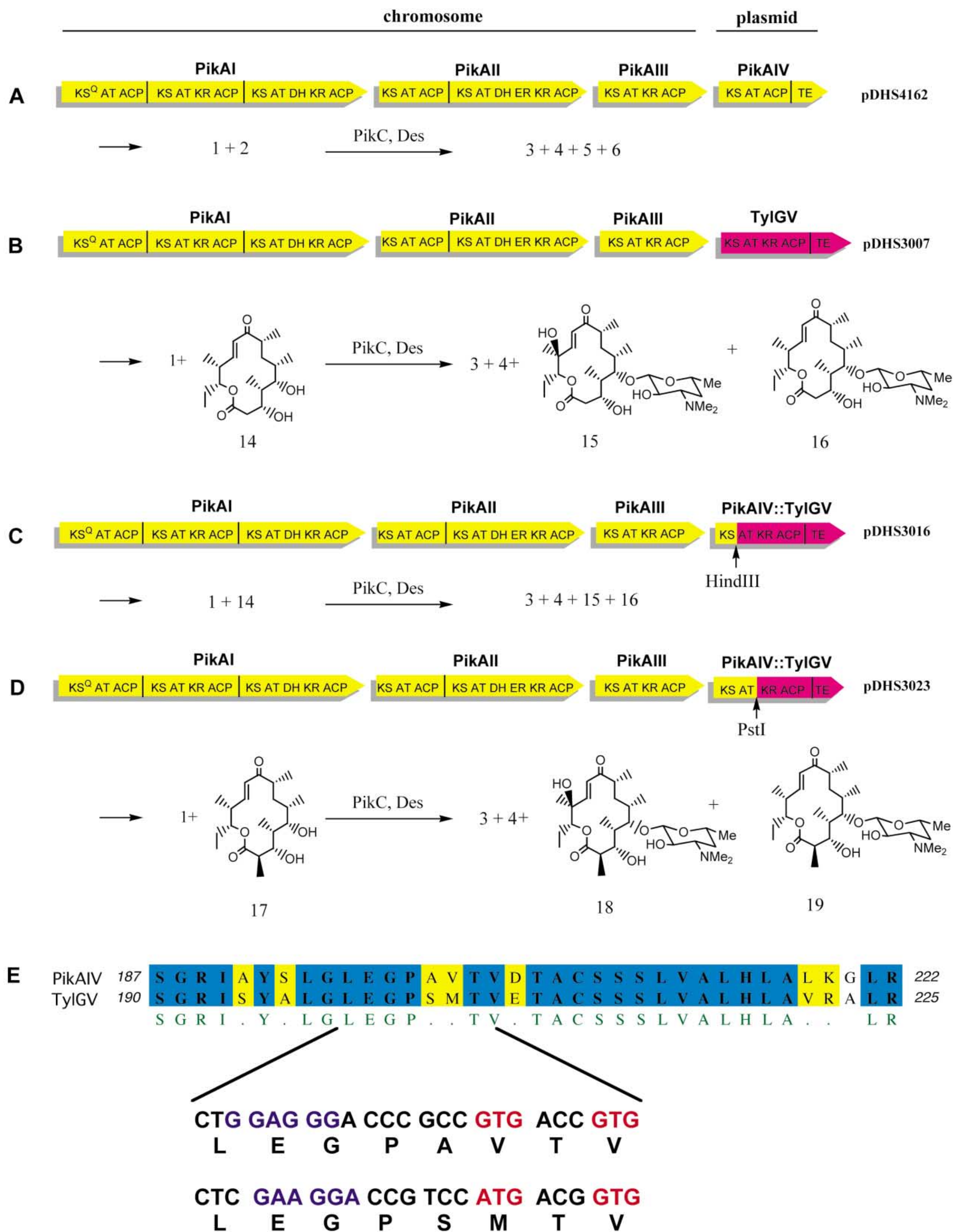

Figure 3. Polyketides Produced by Functional Replacement of PikAIV and Partial Sequence Alignment of PikAIV and TylGV

Polyketides produced by complementation of PikAIV with native and hybrid TylGV (B–D) and partial sequence alignment of PikAIV and TylGV. Alternative ribosome binding sites and start codons are shown in blue and red text, respectively (E). Domains from Pik PKS are marked in yellow, and those from Tyl PKS (A–D) are in red. Des represents all eight enzymes for desosamine biosynthesis and transfer [13], and PikC is the cytochrome P450 monooxygenase responsible for hydroxylation [14]. Alternative ribosome binding sites and start codons are shown in blue and red text, respectively.

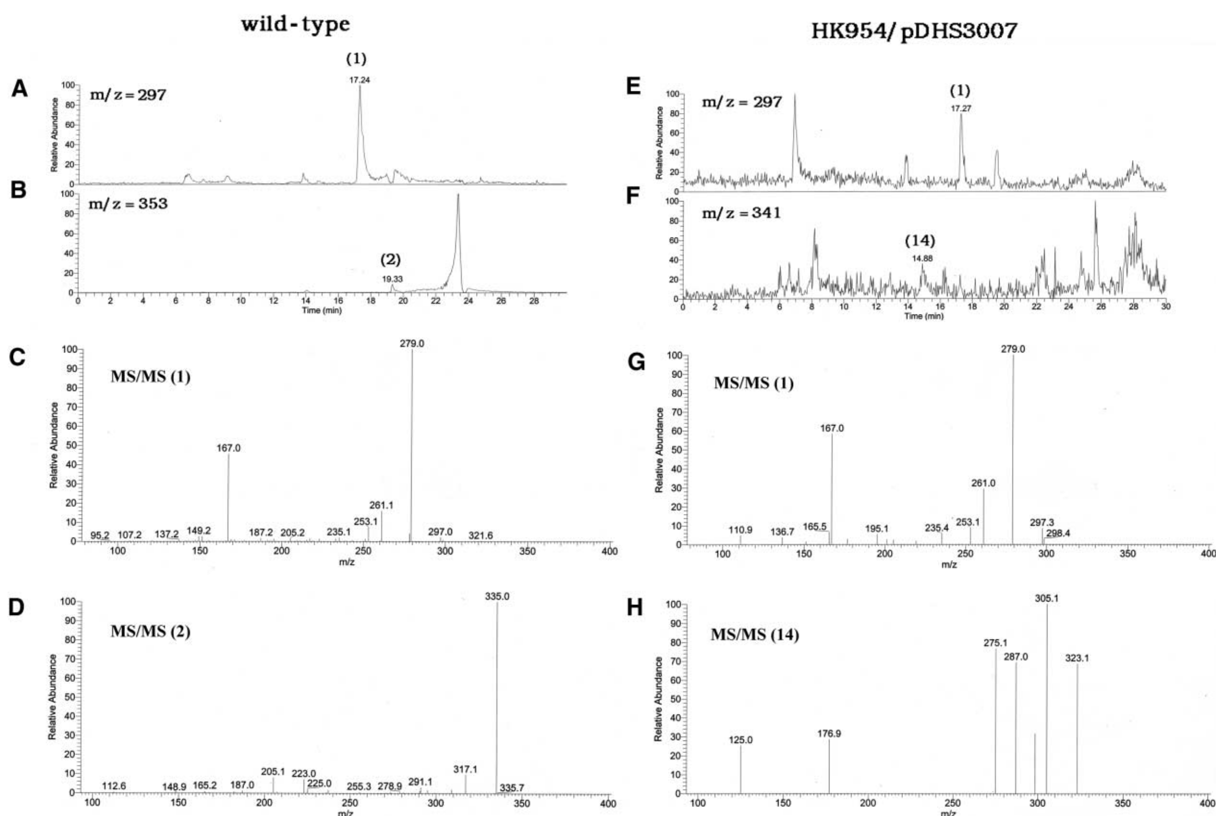

Figure 4. LC/MS and MS/MS Analyses of Aglycones Produced from *S. venezuelae* Wild-Type and HK954/pDHS3007 Strains

Comparison of LC/MS and MS/MS analyses of aglycones produced from *S. venezuelae* wild-type (A–D) and HK954/pDHS3007 (E–H) strains.

(A) LC/MS selected for amu = 297, corresponding to compound **1** produced from the *S. venezuelae* wild-type strain.

(B) LC/MS selected for amu = 353, corresponding to compound **2** produced from the *S. venezuelae* wild-type strain.

(C) MS/MS spectrum corresponding to compound **1** produced from *S. venezuelae* wild-type strain.

(D) MS/MS spectrum corresponding to compound **2** produced from *S. venezuelae* wild-type strain.

(E) LC/MS selected for amu = 297, corresponding to compound **1** produced from HK954/pDHS3007 strain.

(F) LC/MS selected for amu = 341, corresponding to compound **14** produced from HK954/pDHS3007 strain.

(G) MS/MS spectrum corresponding to compound **1** produced from HK954/pDHS3007 strain.

(H) MS/MS spectrum corresponding to compound **14** produced from HK954/pDHS3007 strain (see text for a detailed description of the MS/MS signals).

(**16**). Clear evidence that the recombinant strain (HK954/pDHS3007) produces the predicted macrolactone (**14**) was obtained by the combination of liquid chromatography/mass spectrometry (LC/MS) and mass/mass spectrometry (MS/MS) (Figure 3B). We detected the parent ion ($M + H^+$) of the expected compound (**14**) at 341 amu. At 323 and 305 amu, we detected two characteristic ions for the corresponding dehydration products, which are produced by the loss of two hydroxyl groups at C-3 and C-5 of the new hybrid aglycone (**14**) under the ionization conditions used (Figure 4). Moreover, LC/MS and MS/MS demonstrated production of the predicted macrolide antibiotics. The parent ions (514 amu and 498 amu, respectively) of the new macolides **15** and **16** were detected along with the characteristic desosamine moiety ion (158 amu) as well as patterns of the corresponding dehydration products. Thus, the hybrid 14-membered ring aglycone (**14**) was elaborated further by DesVII (glycosyltransferase) and PikC (P450 hydroxylase) to form novel glycosylated and hydroxylated compounds (structures **15** and **16,** Figure 3B). Previous work has shown that DesVII can accept a range of novel sugar substrates generated in vivo by genetic manipulation of the *des* biosynthetic system in *S. venezuelae* [15–17]. The current study takes this a step further by showing that novel macrolactone substrates generated in vivo through combinatorial biosynthesis can serve as substrates for both DesVII and PikC.

Surprisingly, the 12-membered ring macrolactones 10-deoxymethynolide and the corresponding macrolides, methymycin and neomethymycin, were detected from culture extracts of the HK954/pDHS3007 recombinant strain (Figures 3B and 4). The unique ability of the Pik PKS of *S. venezuelae* to produce both 12- and 14-membered ring macrolactones was shown previously to involve an N-terminally truncated form of PikAIV that is unable to catalyze the final chain elongation step [10]. Nucleotide sequence alignment of the 5′ regions of *pikAIV* and *tylGV* indicated a high degree of homology. However, a minor difference was identified in the predicted ribosome binding site and in one of two possible start codons (Figure 3E). A recent report suggested that *Streptomyces narbo-*

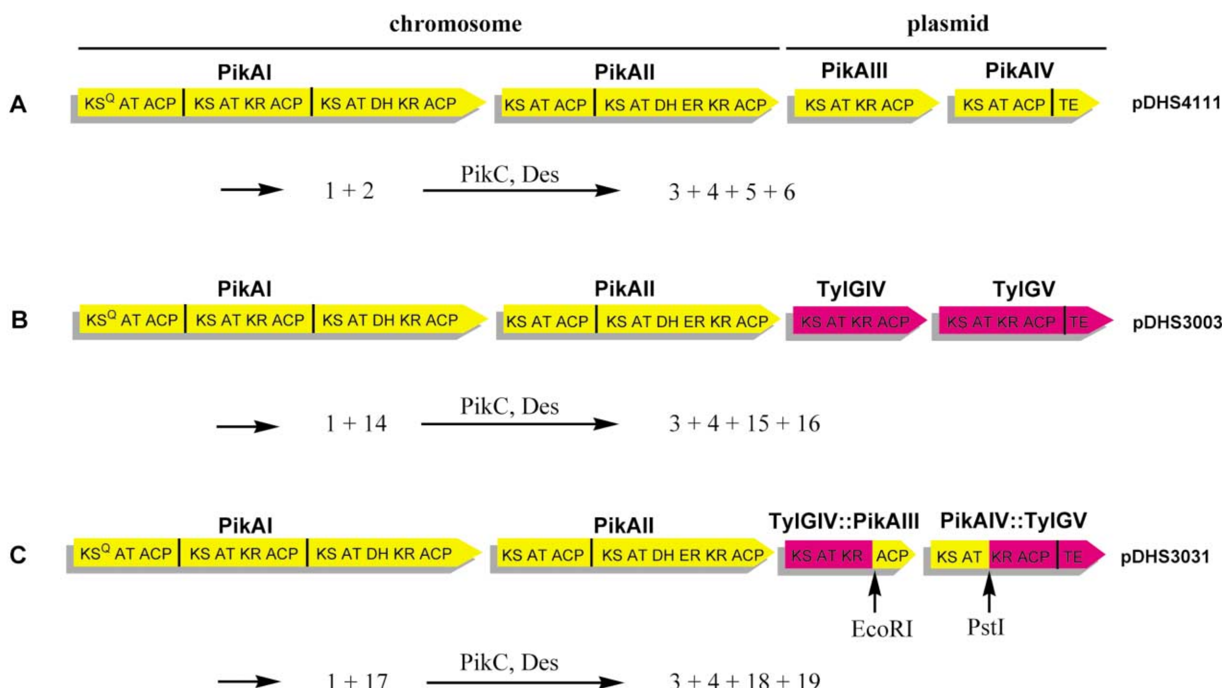

Figure 5. Polyketides Produced by Functional Replacement of PikAI

Domains from Pik PKS are marked in yellow, and those from Tyl PKS are in red. Des represents all eight enzymes for desosamine biosynthesis and transfer [13], and PikC is the cytochrome P450 monooxygenase responsible for hydroxylation [14].

nensis, which produces only 14-membered ring macrolides, is unable to generate a truncated form of PikAIV$_{nrb}$, compared to PikAIV, due to a nucleotide sequence variation in the downstream ribosome binding site [9]. It remains unclear whether differential expression of TylGV is the basis for production of 10-deoxymethynolide in this hybrid system, or whether other mechanism(s) enable these hybrid PKSs to produce two different macrolactones [18]. However, these results demonstrate clearly that hybrid PKSs involving Tyl module 7 in *S. venezuelae* are capable of producing two macrolactone ring systems from a single hybrid PKS.

One of the key challenges of combinatorial biosynthesis involving modular PKSs is the significantly reduced productivity of the hybrid systems [7, 19]. The yield of new macrolides produced from the hybrid PKSs in *S. venezuelae* (see below) requires sensitive analytical methods such as LC/MS for detection. Moreover, the yields of all the new compounds in this study (see below) were approximately one hundred-fold less (approximately 50–100 μg/liter) than typical *S. venezuelae* wild-type production levels, which complicates the quantitative comparisons between mutant strains based on LC/MS analysis.

To enhance productivity of the HK954/pDHS3007 strain, we cloned the PikD transcriptional activator [13, 20] in a second compatible vector (which compared to pDHS3007 has the same origin of replication and *pikAI* promoter, but a unique resistance marker) and transformed it into *S. venezuelae* HK954/pDHS3007. LC/MS analysis revealed that the resulting recombinant strain provided a greater-than-2-fold increase in the production of both 12- and 14-membered ring macrolides (data not shown). This result demonstrates that metabolic engineering of the host strain can improve production levels of hybrid biosynthetic systems in *S. venezuelae* and ease the challenge of product identification.

Complementation of the *S. venezuelae pikAIV* Deletion Mutant by a *tylGV::pikAIV* Hybrid Module

Previous work in the DEBS system has shown that productive transfer of a polyketide chain elongation intermediate in a modular PKS is facilitated by preserving the native intermolecular contacts between an ACP and the following KS domain [21]. The production levels of macrolides in the HK954/pDHS3007 strain was lower as compared to wild-type *S. venezuelae*. We therefore sought to increase the production efficiency by maintaining the native interaction between an ACP and its cognate KS domain. We also wished to investigate the effect of native KS and AT domains in Pik module 6 on production of 12-membered ring macrolides and to access novel structures. Two plasmids were constructed and introduced into HK954 for expression of hybrid PikAIV modules. One comprises the KS domain of PikAIV and the AT-KR-ACP-TE domains from TylGV (PikAIV KS_6-$[AT_7$-KR_7-ACP_7-$TE]_{TylGV}$), and the other comprises the KS-AT domains of PikAIV and the KR-ACP-TE domains from TylGV, respectively (PikAIV KS_6-AT_6-$[KR_7$-ACP_7-$TE]_{TylGV}$).

Transformation of the *pikAIV*-deleted HK954 strain by pDHS3016 expressing PikAIV KS_6-$[AT_7$-KR_7-ACP_7-$TE]_{TylGV}$ resulted in a strain (HK954/pDHS3016) capable of producing the same hybrid macrolides as *S. venezuelae* HK954/pDHS3007 (Figure 3C). The amount of both 12-

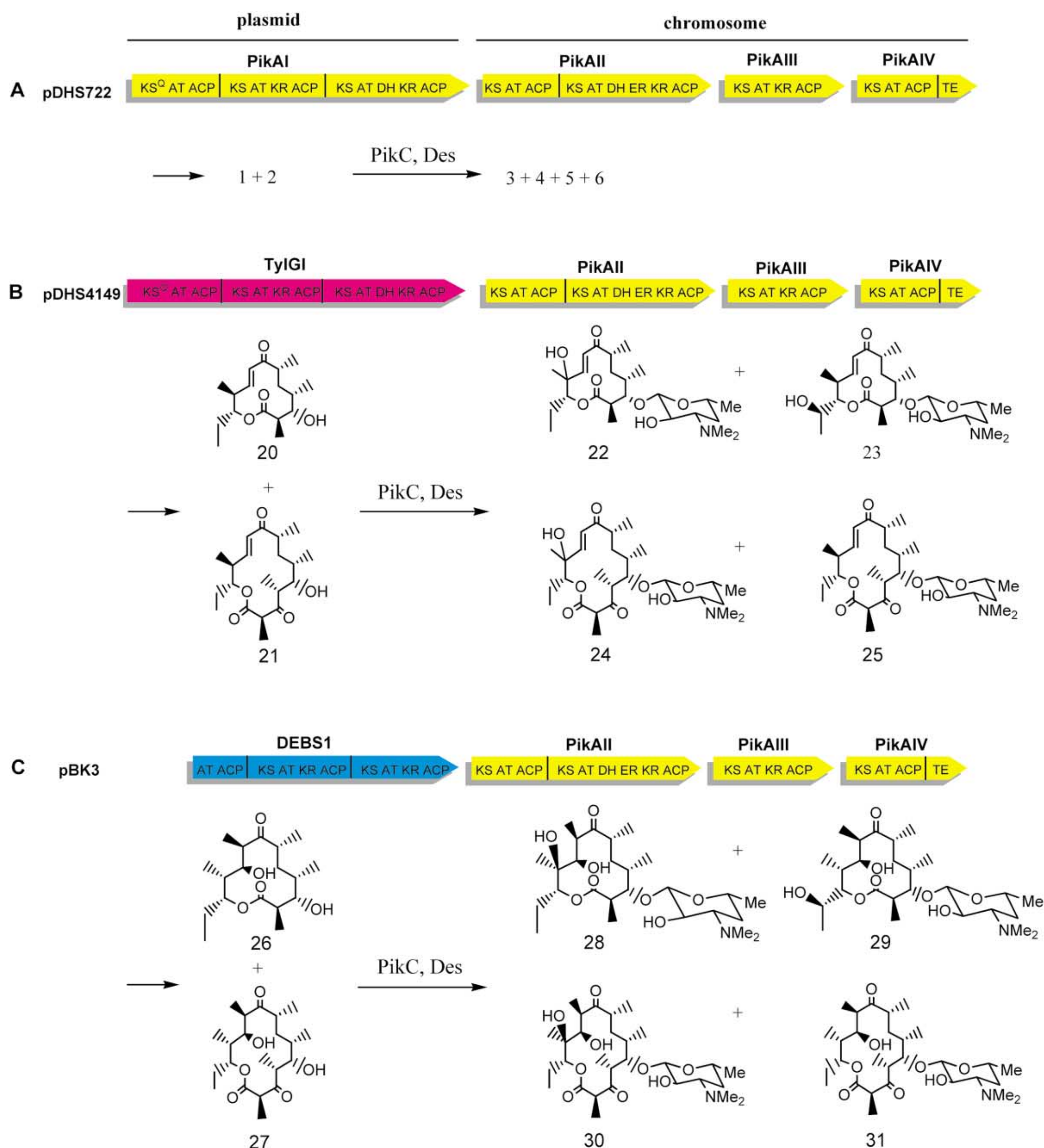

Figure 6. Polyketides Produced by Functional Replacement of PikAI

Domains from Pik PKS are marked in yellow, those from Tyl PKS are in red, and those from DEBS PKS are in blue. Des represents all eight enzymes for desosamine biosynthesis and transfer [13], and PikC is the cytochrome P450 monooxygenase responsible for hydroxylation [14].

and 14-membered ring macrolides produced from HK954/pDHS3016 was similar to levels produced when the complete TylGV module was used (HK954/pDHS3007). Compared to expression in HK954/pDHS3007 and HK954/pDHS3016, plasmid-based (pDHS3023) expression of the PikAIV KS_6-AT_6-$[KR_7$-ACP_7-$TE]_{TylGV}$ hybrid polypeptide comprising KS and AT domains of PikAIV and KR-ACP-TE domains from TylGV in the HK954 strain also produced equivalent levels of 14-membered ring macrolactones, 3-dihydro-narbonolide (**17**), 3-dihydro-pikromycin (**18**), and 3-dihydro-narbomycin (**19**), and 12-membered ring macrolactones as determined by LC/MS and MS/MS (Figure 3D). The parent ions (355, 528, and 512 amu, respectively) of the new macrolactone **17**, and macrolides **18** and **19** were detected along with the characteristic patterns of the corresponding dehydration products, as well as the desosamine moiety ion (158 amu) for the macrolide compounds. These results indicate that simply preserving the native Pik ACP_5/KS_6 domains in a hybrid Pik-Tyl PKS construct is not sufficient to restore molecular recognition for efficient polyketide assembly.

Interestingly, introduction of engineered restriction sites used for domain fusion does not adversely affect

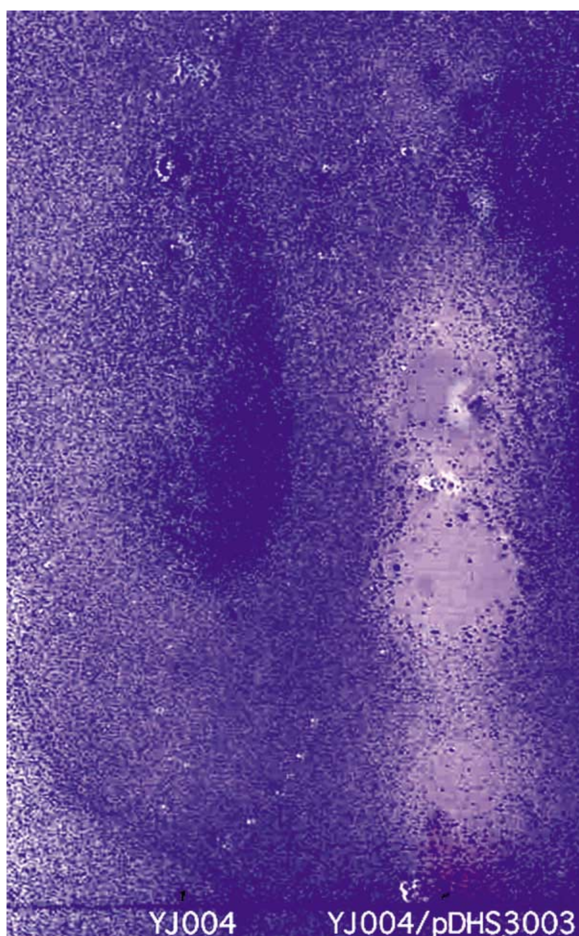

Figure 7. Biological Activity of Fractionated Organic Solvent Extracts from Strains Containing Hybrid PKSs

The text below each TLC spot indicates the strain name from which the extract was prepared. The multiple zones of growth inhibition demonstrate the generation of multiple macrolides by a single engineered biosynthetic pathway.

production levels of the native macrolactone systems. The HindIII restriction site introduced in pDHS3016 (Figure 3C), located at the inter-domain linker between KS_7-AT_7 domains in TylGV altered one amino acid residue (Ala449 to Ser). Successful fusions between DEBS modules using this junction have been described previously [21]. Moreover, when the same amino acid change was introduced into the protein product of pDHS3007 and transformed in to HK954 strain, the level of antibiotic production of the resulting mutant was indistinguishable from that of the HK954/pDHS3007 strain, indicating that this engineered fusion site has no adverse effect on PKS function or molecular recognition. The PstI restriction site introduced in pDHS3023 (Figure 3D), located at the inter-domain linker between AT-ACP domains in TylGV also changed one amino acid residue (Ala935 to Gln). As reported previously for fusions of DEBS modules using an identical engineered junction site [19, 22], there was no change in the level of macrolides produced when the Ala935Gln alteration was introduced into the product of pDHS3007.

Complementation of the *S. venezuelae pikAIII-pikAIV* Deletion Mutant by *tylGIV-tylGV*

It is conceivable that the native genetic architecture of *pikAIII-pikAIV* plays a significant role in the regulation of macrolactone ring size formation in *S. venezuelae*. Specifically, transcriptional and/or translational control of domain skipping may occur through cis-related elements (e.g., unique transcriptional start point) inherent to *pikAIII-pikAIV* on the *S. venezuelae* chromosome rather than by trans-activated mechanisms. To address this question and to extend the investigation of generating hybrid PKS systems, we deleted the *pikAIII* and *pikAIV* genes from the *S. venezuelae* (YJ004) chromosome by exchanging them with *aphII* (Figure 2C). This was followed by functional replacement using plasmids expressing TylGIV-TylGV and hybrid TylGIV-TylGV modules containing the ACP domain of PikAIII and KS-AT domains from PikAIV (Figures 5B and 5C). Plasmid-based (pDHS4111) expression of PikAIII and PikAIV in the YJ004 strain (Figure 5A) restored antibiotic production to wild-type levels (data not shown), demonstrating that complementation of two protein subunits from a plasmid in *S. venezuelae* generates a fully functional PKS.

Transformation of the YJ004 strain by the pDHS3003 expressing a complete TylGIV and TylGV generated a strain (YJ004/pDHS3003) that produces the same 12- and 14-membered ring macrolides as HK954/pDHS3007 and HK954/pDHS3016 strains at the same levels (Figure 5B). Because the catalytic domain organization of TylGIV and PikAIII is identical (e.g., KS-AT-KR-ACP), the predicted structure of the 14-membered ring macrolides produced by YJ004/pDHS3003 strain is the same as the structure of those produced by HK954/pDHS3007.

A plasmid (pDHS3031) expressing the hybrid polypeptide TylGIV KS_6-AT_6-KR_6-$[ACP_5]_{PikAIII}$–PikAIV KS_6-AT_6-$[KR_7$-ACP_7-$TE]_{TylGV}$ was constructed and introduced into the YJ004 strain. The resulting recombinant (YJ004/pDHS3031) generated the same 12- and 14-membered ring macrolides, at approximately the same production levels as HK954/pDHS3023 (Figure 5C).

Production of 12-membered ring macrolides from the YJ004/pDHS3003 and YJ004/pDHS3031 strains suggests that domain skipping occurs with similar efficiency from TylGV or the PikAIV-TylGV (pDHS3031) hybrid module. Thus, the requirement for a native PikAIII and PikAIV interaction is not a necessary condition for alternative expression of PikIV (module 6). It is also worthy of note that the engineered EcoRI restriction site located at the intermodular linker between KR_6-ACP_6 domains in TylGIV (resulting in an Ala1385-to-Gly amino acid change in TylGIV; tested in pDHS3003) as mentioned above has no effect on the phenotype of the mutant (Figure 5C).

These results demonstrate that *S. venezuelae* is particularly amenable as a host for combinatorial biosynthesis. First, the hybrid PKSs generated by subunit complementation produce multiple macrolides when native or heterologous KS domains are used. It is possible that the heterologous subunits (TylGIV and TylGV) used in this study have an exceptionally high evolutionary relationship with the Pik PKS and thus share the mechanism for production of two macrolactone ring systems. Interestingly, constraints on ring cyclization due to the double

Table 1. Strains and Plasmids Used for PKS Expression

Strain/Plasmid	Description	Reference
Strain		
S. venezuelae ATCC 15439	Wild-type	
S. venezuelae HK954	*pikAIV* deletion mutant	This study
S. venezuelae YJ004	*pikAIII-pikAIV* deletion mutant	This study
S. venezuelae BB138	*pikAI* deletion mutant	This study
Plasmid		
pDHS618	*E. coli-Streptomyces* shuttle plasmid vector	[11]
pDHS702	*E. coli-Streptomyces* shuttle plasmid vector	[11]
pSE34	*E. coli-Streptomyces* shuttle plasmid vector	This study
pDHS722	*pikAI*	This study
pDHS4111	*pikAIII* and *pikAIV*	This study
pDHS4149	*tylGI*	This study
pDHS4162	*pikAIV*	This study
pBK3	*eryAI*	This study
pDHS3003	*tylGIV-tylGV*	This study
pDHS3007	*tylGV*	This study
pDHS3016	PikAIV KS_6-$[AT_7$-KR_7-ACP_7-$TE]_{TylGV}$	This study
pDHS3023	PikAIV KS_6-AT_6-$[KR_7$-ACP_7-$TE]_{TylGV}$	This study
pDHS3031	TylGIV KS_6-AT_6-KR_6-$[ACP_5]_{PikAIII}$-PikAIV KS_6- AT_6-$[KR_7$-ACP_7-$TE]_{TylGV}$	This study

bonds in the penultimate tylactone chain elongation intermediate would preclude formation of a 14-membered ring macrolactone in *S. fradiae*. Second, the post-polyketide tailoring enzymes in *S. venezuelae* are flexible enough to accept a broad range of hybrid aglycones to create biologically active molecules (see below).

Complementation of the *S. venezuelae pikAI* Deletion Mutant by *tylGI* and *eryAI*

To expand the applicability of hybrid PKS systems in *S. venezuelae* and to investigate the behavior of a hybrid Pik PKS in which the first protein subunit is engineered, we deleted from the *S. venezuelae* (BB138) chromosome the *pikAI* gene encoding modules L, 1, and 2 (Figure 2D) and complemented it with the plasmids expressing TylGI and DEBS1, respectively (Figure 6). Complementation of the *pikAI* deletion mutant (BB138) by *pikAI* expressed on a plasmid (pDHS722) restored antibiotic production to wild-type levels (data not shown).

Transformation of BB138 by the plasmid (pDHS4149) expressing TylGI resulted in a strain that produces macrolactones of molecular weight identical to those from wild-type *S. venezuelae* (Figure 6B). The compounds' MS/MS fragmentation patterns, including the signature dehydration pattern as well as the daughter ion of the desosamine moiety, were identical to those obtained from the antibiotic products of wild-type *S. venezuelae*. The predicted structures of the aglycones produced from the resulting hybrid PKS are 10-*epi*-10-deoxymethynolide (**20**) and 12-*epi*-narbonolide (**21**) because TylGI module 1 catalyzes ketoreduction to give the opposite stereochemistry as compared to that of the corresponding module in PikAI (Figure 1). The expected structures of the macrolides that are modified further from aglycones **20** and **21** are 10-*epi*-methymycin (**22**), 10-*epi*-neomethymycin (**23**), 12-*epi*-pikromycin (**24**), and 12-*epi*-narbomycin (**25**). Although we cannot distinguish by LC/MS and MS/MS the stereochemistry at C-10 of compounds **22** and **23** and at C-12 of compounds **24** and **25**, it is likely that the stereochemistry at C-10 of the 12-membered ring macrolactone and at C-12 of the 14-membered ring macrolactone was introduced according to the deduced catalytic specificity of the TylGI KR domain. This is evident based on the slight difference between the relative retention times of LC/MS of the predicted new *epi*-isomers and those of wild-type products.

The plasmid (pBK3) expressing DEBS1 under control of the *ermE** promoter [23] was constructed and introduced into the *pikAI*-deletion strain. Since the AT domain in module 2 of DEBS1 is specific for methylmalonyl-CoA instead of malonyl-CoA (the extender unit of module 2 of the Pik PKS) and DEBS1 module 2 lacks a DH domain (present in module 2 of Pik PKS [Figure 1]), the expected structures of the 12- and 14-membered ring macrolactones are 8,9-dihydro-8-methyl-9-hydroxy-10-deoxymethynolide (**26**) and 10,11-dihydro-10-methyl-11-hydroxynarbonolide (**27**), respectively (Figure 6C). Indeed, these two novel aglycones were detected from the culture extract of the resulting strain (BB138/pBK3) and were modified further by DesVII and PikC, leading to 8,9-dihydro-8-methyl-9-hydroxymethymycin (**28**), 8,9-dihydro-8-methyl-9-hydroxyneomethymycin (**29**), 10,11-dihydro-10-methyl-11-hydroxypikromycin (**30**), and 10,11-dihydro-10-methyl-11-hydroxy-narbomycin (**31**) (Figure 6C). LC/MS analysis provided clear evidence for the presence of compounds **28, 29, 30,** and **31** based on predicted molecular weights and fragmentation patterns, although the proposed stereochemistry of compounds **26–31** remains to be established.

Biological Activity of Hybrid Macrolides

An important strength of the *S. venezuelae* expression system is the ability to engineer direct in vivo production

of multiple bioactive macrolides from one recombinant strain. Ultimately, this allows the production of novel macrolides whose biological activity can be assayed by TLC separation of the culture extract. As an example, the organic solvent extract from the YJ004/pDHS3003 recombinant strain displayed multiple zones of growth inhibition when separated by TLC (Figure 7). The result shows that there are at least two new clearing zones in the extract of the YJ004/pDHS3003 strain compared to the *pikAIII-IV* deletion mutant (YJ004) strain, suggesting the existence of multiple bioactive macrolides. The zones of target cell growth inhibition in the middle and top of the TLC plate are due to methymycin/neomethymycin and the hybrid 14-membered ring macrolides (**15**, **16**), respectively. The culture extracts of HK954/pDHS3007, HK954/pDHS3023, and YJ004/pDHS3031 strains displayed very similar zones of growth inhibition (data not shown).

Significance

The hybrid PikA, TylG, and DEBS modular PKS systems engineered at the beginning or end of the Pik PKS complex in *S. venezuelae* resulted in the production of multiple macrolides that differ in size of the macrolactone core and are modified diversely at multiple positions by glycosylation and hydroxylation. Taken together, these results demonstrate the unique advantages of the *S. venezuelae* system for combinatorial biosynthetic creation of natural-product structural diversity. A continuing challenge of this approach is the relatively low productivity of these hybrid systems. However, an understanding of the regulation of the Pik biosynthetic pathway has already provided a strategy for improving the level of production of hybrid macrolide compounds. Current efforts are focused on enhancing the metabolic efficiency of these hybrid systems in the *S. venezuelae* host strain.

Experimental Procedures

Bacterial Strains and Culture Conditions

S. venezuelae ATCC 15439 was used for the construction of Pik PKS deletion mutants. *S. venezuelae* transformants were selected on R2YE agar plates [23] by overlaying with 1 ml of an appropriate antibiotic, apramycin (0.5 mg/ml), kanamycin (1 mg/ml), or thiostrepton (0.5 mg/ml). SGGP liquid medium [24] was used for propagation of *S. venezuelae*. *S. venezuelae* containing plasmids were grown on solid SPA medium (1 g of yeast extract; 1 g of beef extract; 2 g of tryptose; 10 g of glucose; trace amount of ferrous sulfate; 15 g of agar/liter) with an appropriate antibiotic or combination of antibiotics (25 mg/liter of apramycin; 50 mg/liter of kanamycin; 25 mg/liter of thiostrepton) for the production and analysis of polyketides. *Escherichia coli* DH5α was used as a host for DNA manipulation.

Manipulation of DNA and Organisms

The routine manipulation of DNA in *E. coli* was performed with standard procedures in LITMUS28 (New England Biolabs). The polymerase chain reaction (PCR) was performed with *Pfu* Turbo polymerase (Stratagene) under the recommended conditions. *S. venezuelae* transformation was performed by the standard protoplast procedure [23].

Construction of Deletion Mutants

A replicative plasmid-mediated homologous recombination approach was developed previously to conduct gene deletion/replacement in *S. venezuelae* [13]. Plasmids for gene deletion were constructed with a kanamycin resistance gene (*aphII*) [25] replacing the complete targeted gene and DNA fragments flanking both upstream and downstream of the targeted gene. Constructs for gene deletion/replacement were generated with pKC1139 [26], which is a shuttle plasmid for *E. coli* and *Streptomyces*, and they were introduced into *S. venezuelae* via protoplast transformation. Spores from individual transformants were cultured on nonselective agar plates, and the cycle was typically repeated up to three times to enhance the probability for recombination. Double crossovers yielding targeted gene replacement with *aphII* were selected and screened for the kanamycin-resistant, apramycin-sensitive phenotype, and the mutant genotype was confirmed by Southern blot hybridization of genomic DNA. The *pikAIII-pikAIV*-flanking DNA fragments that were used for constructing the *pikAIII-pikAIV* deletion plasmid (pDHS3004) were PCR amplified from cosmid-containing *pikAII-AV* and *desVIII-desR* genes (pLZ71) [13]; the following nucleotides with engineered restriction sites (in italics) were used for cloning: a HindIII-XbaI fragment containing the 3′-region of *pikAII*, forward 5′-AAA*AAGCCT*CGAGGACGGGGTGCTCCGGCACCT-3′ and reverse AAA*TCTAGA*GGTGCCGGTGGTGTCGGGCAGGTC-3′; and a KpnI-EcoRI fragment containing *pikAV* and part of *desVIII*, forward 5′-AAA*GGTACC*GAGGGGGCGGGCAAGTGACCGACAGA-3′ and reverse 5′-AAA*GAATTC*GGACAGCGGCCGGAGCCGCTCCA-3′. The *pikAIV*-flanking DNA fragments that were used for the *pikAIV* deletion plasmid (pDHS954) were PCR amplified from the same cosmid pLZ71 by the use of the following nucleotides: a HindIII-XbaI fragment containing the 3′-region of *pikAIII*, forward 5′-AAA*AAGCCT*GTGGTCCGTCACCCAGGGCGCGGT-3′ and reverse 5′-AAA*GAATTC*GTGGGGCAGGACCGCGGTCGGGTC-3′; and a fragment containing *pikAV* and part of *desVIII* as described above.

The *pikAI*-flanking DNA fragments that were used for constructing the *pikAI* deletion plasmid (pDHS4138) were PCR amplified from cosmid pLZ51 [13]; the following primers with engineered restriction sites (in italics) were used for cloning: a HindIII-XbaI fragment containing the promoter region of *pikAI*, forward 5′-AAA*AAGCTT*GGGGATGTGGCGCCGGAGGATC-3′ and reverse 5′-TTT*TCTAGA*CATCCGGCTCCGTCTCCGGAAGCC-3′; and a KpnI-EcoRI fragment containing the 5′-end of *pikAII*, forward 5′-AAA*GGTACC*CCGTCCCGGGCACCTCGACTC-3′ and reverse 5′-TTT*GAATTC*GCCGACACCAGCCACGGCACC-3′. The strains used in this study are summarized in Table 1.

Construction of Plasmids for Hybrid PKS Expression

All the expression plasmids were derivatives of pDHS618, pDHS702 [11], and pSE34 (derived from pWHM3 [27]), which are shuttle vectors with an *E. coli colE* origin of replication and either a low-copy *Streptomyces* SCP2* origin [28] or a high-copy pIJ101 origin [28]. Plasmids were maintained in *S. venezuelae* by using the appropriate antibiotic at a concentration of 25–50 mg/liter. The expression of genes cloned in plasmids pDHS618 and pDHS702 is driven by the *pikAI* promoter, whereas gene expression in pSE34 derivatives is dependent on the *ermE** promoter.

The *tylGI* gene was subcloned from pSET506 (a gift from Eli Lilly Co., GenBank accession number SFU78289) as a MunI-NsiI fragment into pDHS702 through a series of PCR and subcloning steps and designated pDHS4149.

The *eryAI* gene was placed into pSE34 as an XbaI-HindIII fragment through a series of PCR and subcloning steps, yielding plasmid pBK3.

The *tylGV* gene was first subcloned from pOJ463 and pOJ566 (a gift from Eli Lilly Co.) as an EcoRI-XbaI fragment into LITMUS28 (pDHS3006) through a series of PCR and subcloning steps and placed into the expression vector pDHS618, yielding plasmid pDHS3007.

Plasmid pDHS3012 is a pDHS3006 derivative in which a HindIII site has been engineered immediately downstream of the KS domain in TylGV (by mutating the sequence CCGGCCCCGGCGGACCGGGGTGTCCGCCTT into CCGGCCCCGGCGGACCGGGGTGTCAAGCTT) and the introduced restriction site results in the alteration of a single amino acid residue. This fusion junction was engineered by standard PCR mutagenesis procedures.

Plasmid pDHS3015 was constructed by replacement of the KS domain of TylGV in pDHS3012 with the KS domain of PikAIV as an EcoRI-HindIII fragment, which was PCR amplified from cosmid pLZ71 by the use of the following oligonucleotide (cloning sites in

italics): forward 5′-AAA*GAATTC*TGACCCGACCGCGGTCCTGCC CCA-3′ and reverse 5′-AAA*AAGCTT*GAGACGCCCGCCCGGCGGAG CCGG-3′. The resulting hybrid PKS gene was placed into pDHS618 as an EcoRI-XbaI fragment, generating the expression plasmid pDHS3016.

Plasmid pDHS3020 is a pDHS3006 derivative in which a PstI site has been engineered immediately downstream of the AT domain in TylGV (by mutation of the sequence GCCTCCCGGCACCGCCGCTA CTCCCTCGCC into GCCTCCCGGCACCGCCGCTACTCCCTGCAG) and the introduced restriction site results in the alteration of a single amino acid residue. This fusion junction was engineered by standard PCR mutagenesis procedures.

Plasmid pDHS3021 was constructed by replacement of the KS-AT domains of TylGV in pDHS3020 with the KS-AT domains of PikAIV as an EcoRI-PstI fragment, which was PCR amplified from cosmid pLZ71 with the following oligonucleotide (cloning sites in italics): forward 5′-AAA*GAATTC*TGACCCGACCGCGGTCCTGCCCCA-3′ and reverse 5′-AAA*CTGCAG*CCAGTACGAGCGGTGCTGGAAGGC-3′. The resulting hybrid PKS gene was placed into pDHS618 as an EcoRI-XbaI fragment, generating the expression plasmid pDHS3023.

The *tylGIV* and *tylGV* genes were first subcloned as a BglII-XbaI fragment into LITMUS28 (pDHS3002) through a series of subcloning steps and placed into pDHS618, generating pDHS3003

Plasmid pDHS3027 is a pDHS3002 derivative in which the same PstI site has been engineered immediately downstream of the AT domain in TylGV as in pDHS3020, and an EcoRI site has been engineered at the interdomain region of KR and ACP domains in TylGIV (by mutation of the sequence GTCGACTGGGAGCGGTTCGCCCCG GCCTTC into GTCGACTGGGAGCGGTTCGCCCCGGAATTC). Both restriction sites result in the alteration of a single amino acid residue. These fusion junctions were engineered by standard PCR mutagenesis procedures.

Plasmid pDHS3030 was constructed by replacement of the ACP domain of TylGIV and the KS-AT domains of TylGV in pDHS3027 with the ACP domain of PikAIII and the KS-AT domains of PikAIV as an EcoRI-PstI fragment, which was PCR amplified from cosmid pLZ71 with the following oligonucleotide (cloning sites in italics): forward 5′-AAA*GAATTC*ACGGTGTCCCGTCCCAGCCTTCTG-3′ and reverse 5′-AAA*CTGCAG*CCAGTACGAGCGGTGCTGGA AGGC-3′. The resulting hybrid PKS gene was placed into pDHS618 as a BglII-XbaI fragment, generating the expression plasmid pDHS3031.

Standard PCR amplification and molecular-biology techniques were used to amplify the *pikAIV* and *pikAIII-IV* genes from cosmid pLZ71 and cloned into pDHS618 and pDHS702, respectively. The *pikAIV* expression vector was designated pDHS4162, and the *pikAIII-IV* expression vector was designated pDHS4111. The *pikAI* gene was placed into pDHS702 as an EcoRI-NsiI fragment from cosmid pLZ51 through a series of PCR and subcloning steps, yielding the expression plasmid pDHS722. The plasmids used for PKS expression in this study are summarized in Table 1.

Production and Analysis of Polyketide Analogs

The *S. venezuelae* wild-type strain and mutants were cultured on SPA solid medium at 30°C for 3 days under appropriate antibiotic selection. Agar medium production gives more reproducible results than liquid fermentation, although the liquid fermentation typically supports the same level of antibiotic production. The agar-grown culture was diced and extracted with two volumes of methanol. The extract was washed with water, extracted again with one volume of chloroform, and concentrated. A 50 μl aliquot of the concentrated solvent extract was analyzed on a reverse-phase C_{18} column with 0%–100% acetonitrile in water gradient by the combination of LC/MS and MS/MS. The hybrid polyketides were identified by mass spectral fragmentation pattern corresponding either to the predicted products or to known standards. Under the ionization conditions used, aglycones and their analogs generate signature dehydration patterns, and the macrolides and their analogs produced the characteristic desosamine moiety ions as well as patterns of the dehydration products. The relative amount of each compound produced was compared by the peak intensity in the LC/MS.

Biological Activity Assays

The biological activity of extracts prepared as above was assayed against *Bacillus subtilis* by overlaying a TLC plate that had been developed with each extract with soft agar containing freshly grown *B. subtilis*. The solvent system for TLC was chloroform:methanol:25% ammonium hydroxide (90:10:1). The TLC plate spotted with 5–10 μl of each of the extracts was developed, dried, placed on a water agar plate and overlaid with soft agar containing 50 μl of culture of *B. subtilis* grown overnight. After overnight incubation at 37°C, the TLC plate was stained with Coomassie blue solution and washed with water for visualization of zones of target cell growth inhibition.

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