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Functional Dissection of a Multimodular Polypeptide of the Pikromycin Polyketide Synthase into Monomodules by Using a Matched Pair of Heterologous Docking Domains

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The pikromyin polyketide synthase (PKS) in *Streptomyces venezulae* is comprised of a loading module and six extension modules, which generate the corresponding 14-membered macrolactone product. PikAI is a multimodular component of this PKS and houses both the loading domain and the first two extension modules, joined by short intraprotein linkers. We have shown that PikAI can be separated into two proteins at either of these linkers, only when matched pairs of docking domains (DDs) from a heterologous modular phoslactomycin PKS are used in place of the intraprotein linker. In both cases the yields of pikromycin produced by the *S. venezuelae* mutant were 50% of that of a *S. venezuelae* strain expressing the native trimodular PikAI. This observation provides the first demonstration that such separations do not dramatically impact the efficiency of the entire in vivo biosynthetic process. Expression of module 2 as a monomodular protein fused to a heterologous N-terminal docking domain was also observed to give almost a tenfold improvement in the in vivo generation of pikromycin from a synthetic diketide intermediate. These results demonstrate the utility of DDs to manipulate biosynthetic processes catalyzed by modular PKSs and the quest to generate novel polyketide products.

Introduction

A large array of structurally diverse, and medically important, bacterially-derived natural products are biosynthesized by modular polyketide synthases (PKSs).[1–6] These are multifunctional enzymes containing any number of distinct modules responsible for each successive round of coenzyme A thioester monomer (typically malonyl or methylmalonyl CoA) decarboxylative condensations into the final polyketide core structure. At minimum each module contains a ketosynthase (KS), acyltransferase (AT) catalytic domains as well as an acyl carrier protein (ACP), to which the polyketide chain is attached. Additionally, modules can contain a variety of tailoring domains responsible for catalyzing keto and enoyl group reduction as well as dehydration activities. The pikromycin (Pik) PKS from *Streptomyces venezuelae* ATCC 15439 contains six such distinct modules, which together with a loading module and a termination domain, are responsible for the formation of the 14-membered ring macrolide product, narbonolide (early termination without use of module 6 provides the 12-membered ring macrolide, 10-deoxymethynolide;[7] Figure 1). These two structures are then modified by glycosylation and hydroxylation to their corresponding antibiotics pikromycin and methymycin.[8]

In many PKSs, multiple modules (typically 2–6) can be housed on a single polypeptide. There are also many PKSs in which several or even all of the modules are contained on separate polypeptides. The six extension modules of the pikromycin PKS are housed on a combination of multimodular (PikAI and PikAII) and monomodular (PikAIII and PikAIV) polypeptides (Figure 1). In contrast the six extension modules of the 6-deoxyerythronolide B synthase (DEBS) are all housed on three bimodular polypeptides.[9]

A short intraprotein linker, typically about 20–30 amino acids, is responsible for linking modules within multimodular polypeptides.[10,11] Much longer docking domains (DDs, ~100 amino acids) are found at the C- and N terminus of both monomodular and multimodular type I PKS polypeptides. The proper DD interactions have been shown to be essential in promoting the appropriate protein–protein and thus module–module interactions required for the biosynthetic processes.[12–17] Perhaps the most vital aspect of DD interactions is the selective binding that discrete DD pairs exhibit for one another.

The binding affinities for matched DD pairs from both the DEBS and Pik PKS systems have been determined by surface plasmon resonance (SPR), and have shown that these docking appendages selectively bind to their cognate partners while mismatched pairs showed no binding.[18] While other protein–protein interactions between the ACP and KS domains could play a role in the binding and association of sequential modules, the specific amino acids involved have not yet been determined.[19,20]

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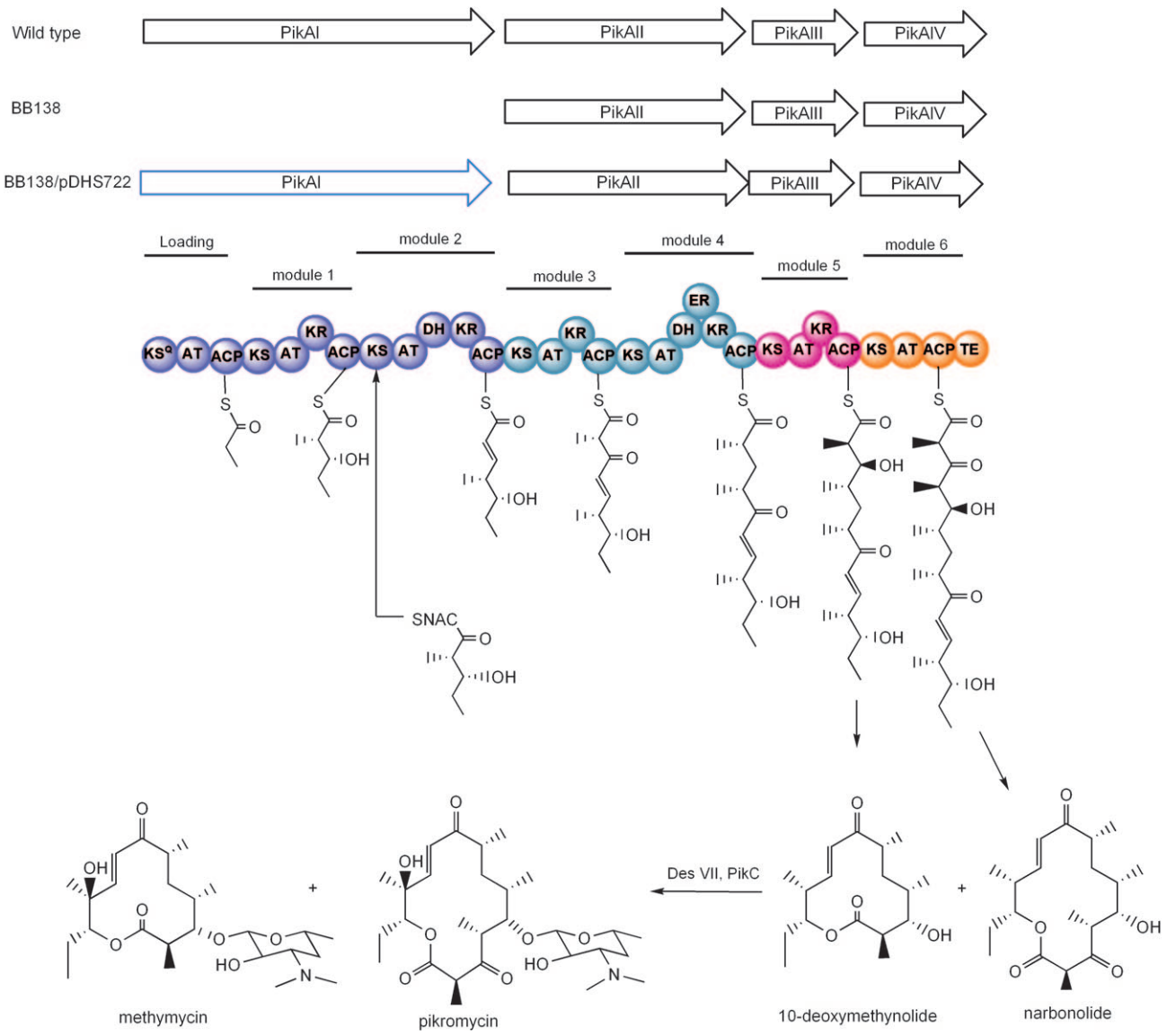

Figure 1. Two routes to prime the pikromycin PKS and provide 12- and 14-membered macrocyclic products. The natural process occurs with the generation of the enzyme-bound propionate starter unit of the loading domain from methylmalonyl CoA. An alternate less efficient route, which can occur in the absence of the loading domain and module 1 or mutational inactivation of module 1, is the transfer of the diketide intermediate, (2*S*)-methyl-(3*R*)-hydroxypentanoic acid (activated as a NAC thioester) onto the KS domain of module 1.

Both NMR spectroscopy and X-ray crystallographic structural models of the DD interactions for DEBS2/DEBS3[15] and PikAIII/PikAIV[18] junctions, respectively, have been reported. Both of these models depict the DD interactions as being primarily determined by a set of α helices formed by the DD peptide regions. These interactions play a critical role in both the stabilization of the PKS homodimer and the docking interactions of the C-terminal ACP bound domain with the N-terminal DD of the subsequent KS.[15,18] Due to the fact that these DD sequences tend to be unconserved in nature, classification of these interactions has been limited. However, recently Thattai et al. have reported an in-depth computational analysis based on the previously mentioned NMR structural model of the DEBS DD pair. This work has classified and organized PKS DD pairs into distinct subclasses, depending on key amino acid residues making direct interactions.[21]

While the length and structure of intraprotein linkers and DD is different,[11,16] their functional role is equivalent; they mediate transfer of a growing polyketide chain from the C-terminal ACP of one module to the N-terminal KS of the next module. This functional equivalency raises the intriguing question of whether they can be exchanged without significantly reducing overall production levels.

Replacement of docking domains with an intraprotein linker would permit an increase in the number of modules on a polypeptide. An increase from dimodular to tetramodular polypeptides in the DEBS system has been accomplished by a different approach of fusing the N- and C-terminal docking domains.[22]

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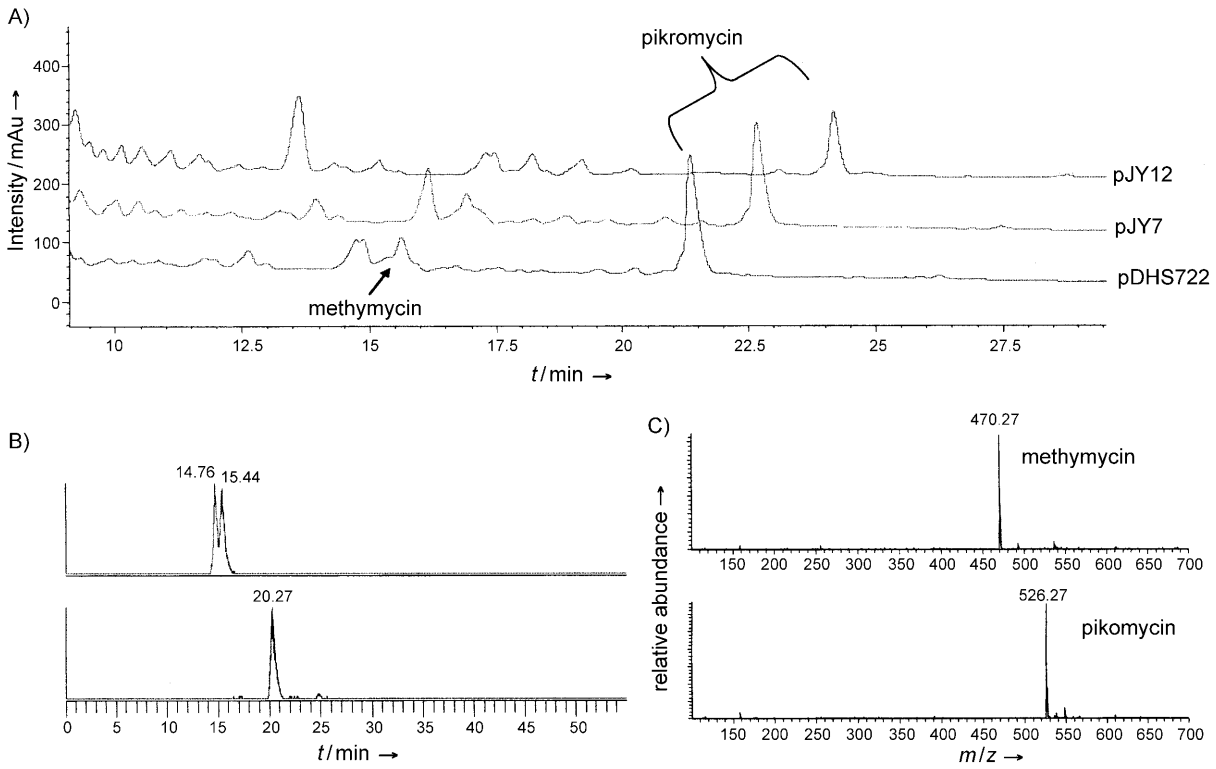

Figure 2. HPLC and LC-MS analysis of methylmycin and pikromycin production. A) Overlaid HPLC trace for fermentations of BB138 carrying the indicated complementation plasmids. B) Selected ion monitoring for the expected macrolide products methymycin and pikromcyin, which eluted at approximately 15 and 21 min, respectively. C) Mass spectra of the products methymycin and pikromycin at *m/z* 470 and 526 in the positive mode, respectively.

Other examples of functional PKS polypeptide fusions have been reported in the aureothin,[23] borrelidin,[24] and rapamycin[25] systems. The fusion of two of the three polypeptides (DEBS1+2 or DEBS 2+3) together yielded comparable production levels, but fusion of all three polypeptides resulted in barely detectable levels when compared to the wild-type system.[22] In the aureothin and borrelidin examples, functional fusion of AurA and BorA5 monomodules with their respective adjacent PKS polypeptides AurB and BorA4 and or BorA6 provided evidence of their iterative functionality. In the case of rapamycin, the second module of this PKS was fused between the first and second module of DEBS to produce a small amount (5%) of the expected tetraketide product.[25] With the exception of the DEBS polypeptide fusions these examples yielded a significant drop in production levels compared to control experiments with native modules.

Conversely, replacement of intraprotein linkers with a matched pair of docking domains from a heterologous system should permit separation of a multimodular polypeptide into monomodules. Such an experiment has been reported for both the multimodular components of the rifamycin[26] and epothilone[27] PKS proteins when these complex pathways were reconstructed in *E. coli*. For the epothilone case, EpoD, which contains four modules, was split into two modules by using a matched DD pair from the stigmatellin PKS.[27] For the rifamycin case the tetramodular RifA was split into two proteins by using a matched DD from the erythromycin PKS.[26] In both cases the yields of the fully extended polyketide products were very low (10 $\mu g\,L^{-1}$ for epothilone C). The multimodular separation was also needed to address problems with expression of soluble multimodular PKS component. Therefore, the effect of multimodular separations on polyketide production in hosts in which the multimodular PKS is expressed as a soluble protein is unknown.

We have investigated this question in the pikromycin producing *Streptomyces venezuelae* host using a heterologous DD pair from the phoslactomycin (Plm) PKS[28] to replace the two PikAI intraprotein regions that link the loading domain and first two extension modules. The results show that such separations lead to only modest decreases in the overall pikromycin yields.

Results and Discussion

We employed an experimental approach that was built upon an established plasmid-based complementation system in which pDHS722 drives expression of PikAI in BB138—a PikAI deletion strain of *S. venezuelae*.[29] Three pDHS722 derivatives were constructed pJY12, pJY7 and pJY9. In pJY7, the DNA sequence encoding the intraprotein linker between modules 1 and 2 of PikAI was replaced with a fragment that spans the region encoding the C-terminal docking domain of Plm1 to that encoding the N-terminal docking domain of Plm2-3. This plasmid was designed to express PikAI in which the two extension modules are now contained on separate polypeptides. A similar replacement of the DNA sequence encoding the load-

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ing domain–module 1 intraprotein linker in pDHS722 generated pJY12, which was designed to determine if docking domains can permit the loading domain to function as a separate polypeptide. In the case of pJY9, the DNA sequence for the intraprotein linker between modules 1 and 2 was replaced with a stop codon, and the start codon (and associated ribosome binding site) for Plm2-3 expression. This plasmid was designed to test the effect of separating modules 1 and 2 without using DDs. In all cases fusion points were based on multiple alignments of the KS and ACP domains from multiple PKS systems.

Triplicate liquid fermentations of each *S. venezuelae* BB138 carrying pDHS722, pJY12, pJY7 or pJY9 were carried out in order to determine if these three complementation variants produced the expected macrolide products. Detection and quantification of methymycin and pikromycin products was performed by using HPLC analysis combined with UV and MS (*m/z* 470 and 526 in positive mode) and confirmed with authentic standards (Figure 2). Production of methymycin and pikromycin was observed in both BB138/pJY12 and BB138/pJY7, and the yields were approximately 50% of that observed for BB138/pDHS722 (Figure 3). However, in the case of BB138/pJY9, for which no linker or DD pair was present between modules 1 and 2, only trace amounts of the polyketide products were detected: $<0.1\%$ compared to intact PikA1 (pDHS722; Figure 3). This dramatic decrease in pikromycin yields likely indicates that in the separated modules without DDs, protein recognition between the ACP of module 1 and the KS of module 2 is not sufficient to permit transfer from modules 1 to 2. Alternatively, the expression of a soluble separate module 2 might be lower without an N-terminal DD present. Indeed, work by Khosla et al. on in vitro expression of an individual PKS monomodule[13,30–32] and specifically Pik module 2[33] has shown that an N-terminal stretch of amino acids (linker or DD) is required for the expression and catalytic activity of these proteins (the trace levels of pikromycin by BB138/pJY9 demonstrate some level of expression of this protein).

There are numerous examples for which it has been demonstrated that *N*-acetylcysteamine (NAC) thioesters of polyketide intermediates can be loaded and processed by KS domains to a final polyketide product.[34,35] However, this process is inefficient relative to priming from the cognate ACP of a previous module. The successful separation of module 2 of PikAI as a distinct polypeptide by using the Plm DD pair results in this KS domain being present at the N terminus rather than the center of the polypeptide; this permitted us to assess if this change leads to an increased efficiency in processing of the NAC thioester of the natural diketide intermediate. For this work three plasmids, pBK51, pBK51* and pJY7* were constructed (Figure 3). Plasmid pBK51 is a derivative of pDHS722 and expresses PikAI in which module 1 is catalytically inactive as a result of the Cys to Ala mutation in the KS active site.[36] Plasmid pBK51* expresses the same mutated PikAI but as two separate polypeptides; this plasmid was generated from pBK51 in a manner analogous to pJY7 from pDHS722. The final plasmid, pJY7*, expressed only module 2 of PikAI with the N-terminal docking domain of Plm2-3 and was created by deletion of the appropriate DNA region in pJY7.

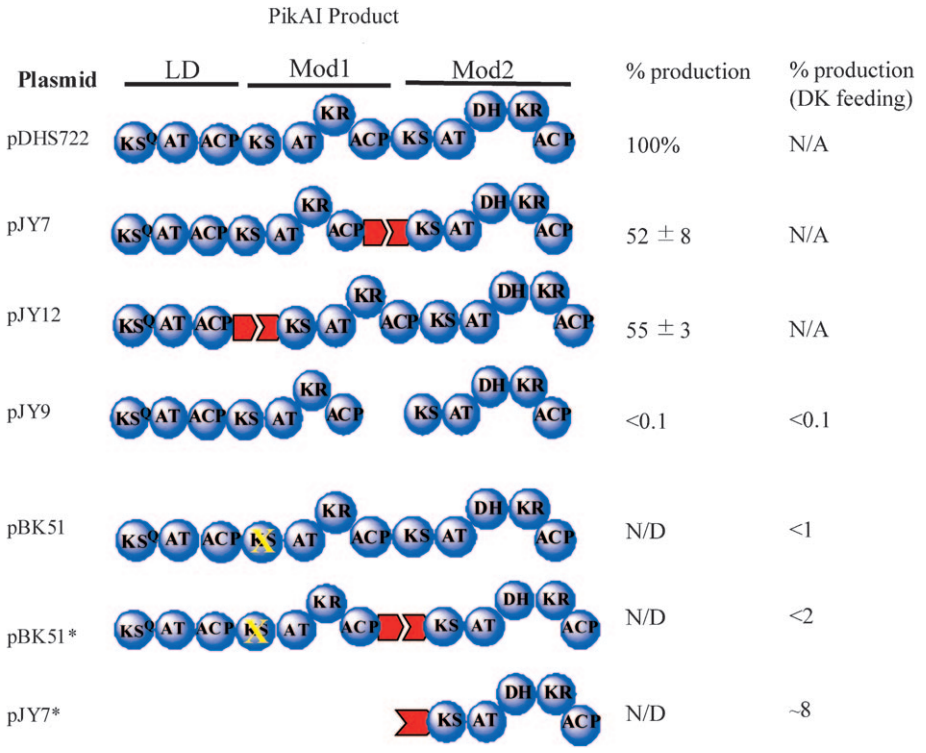

Figure 3. Levels of pikromycin products made in the BB138 PikAI deletion strain of *S. venezuelae* with the respective complementation plasmids. Red blocks indicate the Plm1–Plm2-3 docking domain pair from the phoslactomycin system. Diketide feeding experiments were carried out only with BB138 carrying constructs expressing PikAI in which module 1 was either inactive (through mutational inactivation of the KS domain, indicated by an X), absent (pJY7*) or missing the C-terminal DD (pJY9). N/A: not applicable; N/D: not detectable.

Fermentations of BB138 carrying pBK51, pBK51* or pJY7* did not produce any detectable levels of methymycin or pikromycin; this is consistent with either loss of the loading domain and module 1 of PikAI, or mutational inactivation of module 1. These fermentations were also performed in the presence of the NAC thioester of (2*S*)-methyl-(3*R*)-hydroxypentanoic acid, the natural diketide intermediate. Fermentations were conducted three times; product yields and standard deviations were calculated relative to those obtained with BB138/pDHS722 (Figure 2). Levels of pikromycin production in the case of BB138/pBK51 were extremely low, much less than 1% of that observed in the control

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culture. This result is in contrast to experiments that have demonstrated that for the DEBS PKS, the natural diketide intermediate is able to load onto the second module of a dimodular polypeptide.[37] This observation with DEBS has been reported in a number of hosts, and we have used BB138/pBK3,[38] which produces the truncated DEBS PKS triketide lactone product, to show that it also functions similarly in *S. venezuelae*. We have also shown that feeding the natural triketide intermediate to either BB138 or BB138/pBK51 gives pikromycin yields of 10% of that observed for the control.[36] The low levels of pikromycin production increased to about 2% of the control with BB138/pBK51*; this indicates that a modest improvement in diketide utilization can result from expressing module 2 as a monomodular polypeptide. Notably, however, a significant increase was seen with BB138/pJY7* in which the pikromycin yields were about 8% (Figure S1a–c in the Supporting Information) of the control and comparable to that observed with feeding the triketide intermediates. Thus, removal of the preceding module rather than separation of the two modules is more effective at improving utilization of the diketide intermediate (improvements in utilization of diketides for erythromycin biosynthesis have also been observed upon removal of module 1 of DEBS).[39] This result could indicate that even as separate polypeptides the loading domain and module 1 might negatively impact loading of diketide onto module 2. Alternatively, the process could simply be inefficient regardless of the presence of the preceding module, and the improvements might reflect better expression of the new monomodular module 2. The efficient utilization of the natural diketide by a separated module 2 was only observed when there was an N-terminal docking domain. There was no improvement in the trace levels of pikromycin made by BB138/pJY9 when grown in the presence the diketide intermediate; this suggests that that the separate module 2 without a N-terminal DD is either catalytically impaired or poorly expressed.

Conclusions

In summary, these experiments have provided an additional in vivo example that a multimodular PKS can be separated into individual modules by replacing the intrapolypeptide linker with a heterologous DD pair; this demonstrates that the separation does not work in the absence of the DDs. Furthermore, the observations demonstrate for the first time that multimodular separation can lead to only a modest decrease in the overall production of the final polyketide production. Such dissected systems make multimodular PKS more amenable for creation of hybrid PKSs, and potentially, the creation of new polyketide products. However, challenges associated with: 1) the appropriate molecular recognition between the ACP and KS domains, and 2) the substrate specificity of the catalytic domains, remain. An alternative approach to generating novel polyketide products is feeding NAC thioesters of analogues of polyketide intermediates. This approach also faces the challenge associated with substrate specificity of the catalytic domains and inefficiencies of loading onto the KS of the appropriate module. In the case of PikAI we have shown that the in vivo utilization of a diketide intermediate by a multimodular protein is very inefficient, but can be improved significantly by removal of the preceding modules and expression of the appropriate loading module with an N-terminal docking domain.

Experimental Section

Plasmids, strains and culture conditions: All constructed plasmids were derivatives of pDHS722, a PikA1 complementation plasmid. All plasmid manipulations were completed in *E. coli* hosts. Transformants were cultivated on LB–agar media and plasmids were isolated from LB liquid media, both of which were supplemented with the appropriate antibiotics.

Variants generated from pDHS722 were transformed into BB138 by using established protocols,[40] and were cultured on R2YE plates. Transformants were subsequently grown on SPA agar plates (50 mg L^{-1} thiostrepton). SCM liquid media was used for fermentation and propagation of *S. venezuelae* strains.

DNA manipulation: Variants of pDHS722 were prepared by a series of PCR reactions and subsequent PCR targeting recombination crossovers in *E. coli*, before final transformation into *S. venezuelae*. DNA sequences encoding the docking domains between modules 1 and 2 of phoslactomycin (PLM) PKS was PCR amplified from cosmid 10B4[28] with primers PLMDDF (5′-CTG GGG CAC CTC GAT TTC GG-3′) + PLMDDR (5′-GTG GTG CTT CGT CTC GGC GT-3′). The PCR product was gel purified with Qiagen gel extraction kit and cloned into TOPO TA cloning vector (Invitrogen). The inserts were verified by sequencing with manufacture supplied M13F and M13R primers, and construct pJY1 was obtained. The *aac(3)IV* resistance marker was amplified from pIJ773[41] by using primers PlmDD-ApraF (5′-CTG ATG CAG TTC ATC GAC ACC GAG TTG GGG GAC ATC TGA GTA *TAC ***GGT TCA TGT GCA GCT CCA TC***-3′) + PlmDD-Apra-R (5′-GAA CAG GGC GCG ACG GGA ACA GGA GTG GTG GCA GAG GAT GTA *TAC ***TGT AGG CTG GAG CTG CTT C***-3′); bold, italicized sequences represent annealing sites for the antibiotic marker and * indicates the location of a unique Bstz17I restriction sites introduced for later removal of this marker. The resulting PCR product was recombined with pJY1 by using established procedures.[41] The resulting recombinant plasmid, pJY2, was isolated with GeneJET Plasmid Miniprep Kit (Fermentas) and confirmed by sequencing. Construct pJY2 was used as a template to generate PCR products with the needed 39 nucleotide region complementary to the desired point of recombination between the linked ACP and KS domains of PikA1. PCR products for the exchange of DNA sequences encoding the intramodular linkers between modules 1–2 and LD–module 1 for that coding for the Plm docking domain pair, were amplified with primer pairs PikA1F (5′-GTC TTC GAC CAC CCG ACG CCG CTG GCC CTC GTG TCG CTG ***CTG GGG CAC CTC GAT TTC GG***-3′) + PikA1R (5′-GGG GTA GCG GCA GCT CAT CGC GAC GAT CGC GAT CGG ATC ***GTG GTG CTT CGT CTC GGC GT***-3′), and LDSepF (5′-TTC GAC TTC CCC ACC CCC GAG GCT CTC GCG GAG CAG CTG ***CTG GGG CAC CTC GAT TTC GG***-3′) + LDsepR (5′-GGG CAG GCG GCA GGC CAT GCC GAC GAT CGC CAC CGG CTC ***GTG GTG CTT CGT CTC GGC GT***-3′), respectively; primer sequences in bold italicized font represent annealing sites on the template pJY2, and the remaining 5′ region represents the complementary 39 nucleotide region of PikAI used for recombination. PCR products were introduced into pDHS722 by homologous recombination by using established protocols.[41] Recombinant pDHS722 plasmids, were isolated as before, treated with Bstz71I to remove the *apra* marker, ligated, overnight, at 12 °C, transformed into *E. coli* and single colonies screened for both (+)Amp and (−)Apra; this yield-

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ed pJY7 and pJY12, respectively. Plasmid pJY9, which had the region encoding the intrapolypeptide linker between modules 1 and 2 deleted, was also generated by homologous recombination by using established protocols.[41] The *aac(3)IV* resistance marker amplified from pIJ773 was used to delete the linker-encoding region with primers PiklinkΔF (5′-GTC TTC GAC CAC CCG ACG CCG CTG GCC CTC GTG TCG CTG **TGA** GTA* TAC ***GGT TCA TGT GCA GCT CCA TC***-3′) + PiklinkΔR (5′-GGG GTA GCG GCA GCT CAT CGC GAC GAT CGC GAT CGG ATC **CAC** CTA TCG GCT TCG CGG CTG TCC GCC CGG TA*T AC***T GTA GGC TGG AGC TGC TTC***-3′). Bold and italicized sequences represent annealing regions for the resistance marker, and * indicates the location of the unique the Bstz17I restriction sites introduced for later removal of this marker. A TGA stop codon (underlined and bold) was introduced at the 3′ end of the module 1 ACP-encoding region in the forward primer. The reverse primer contained the complementary sequence to the putative ribosome binding site (underlined) and start codon (CAC) of the N-terminal Plm DD (underlined and bold) fused to the beginning of the KS module 2 encoding region. This PCR product was again recombined into pDHS722 by using established protocols,[41] the *aac(3)IV* resistance marker was removed by restriction enzyme digestion as before, and religated to yield the final pJY9 plasmid. PBK51* was generated in the exact same manner as pJY7, but by using pBK51, which encodes PikAI with a Cys to Ala mutation on KS1, as the final recombination plasmid. Plasmid pJY7*, which expresses only module 2 of PikAI, was generated from pJY7 by using the PCR-targeted *Streptomyces* gene replacement method.[41] For this, the DNA encoding the polypeptide containing just the loading domain and module 1 in pJY7 was replaced with the kanamycin resistance marker. This marker was amplified from pKD13[42] with primers PikA1scarF (5′-GTT GGG TGA AAG CGC GGC TTC CGG AGA CGG AGC CGG ATG ***ATT CCG GGG ATC CGT CGA CC***-3′) + PikAIscarR (5′-GCG ACG GGA ACA GGG GTG GTG GCA GAG GAT GTA TAC TCA ***TGT AGG CTG GAG CTG CTT C***-3′); bold sequences represent annealing sites for the antibiotic marker, and the remaining 5′ regions represent recombination crossover sites. Successful replacement in pJY7 was followed by FLP-mediated excision of the marker region to generate pJY7*, which encodes PikAI module 2 with the C-terminal docking domain of Plm2-3 at the N terminus.

Polyketide production analysis: SCM media fermentations (50 mg L^{-1} thiostrepton) were carried out at 30 °C with agitation (220 rpm) for 72 h. After fermentation the medium was separated by centrifugation at 11000 *g* for 10 min. The fermentation media was extracted 3× with ethyl acetate (10 mL), dried and resuspended in MeOH (1 mL). Extracts of BB138/(pJY7, pJY12, pJY9, pBK51, pBK51*, pJY7*) were analyzed by reverse phase HPLC by using an Agilent 1100 series system with a 5 μm Discovery HS C18 reversed-phase column (25×4.6 cm, Supelco), a linear gradient system from solvent A (20:80 acetonitrile/water and 10 mM ammonium acetate) to solvent B (80:20 acetonitrile/water and 10 mM ammonium acetate) over 50 min at a flow rate of 1 mL min^{-1}, and detection at 220 nm. Relative amounts were determined by integrating and averaging the peak areas for pikromycin produced in three separate extracts and comparison to extracts of BB138/pDHS722 (Figure 2A). Extracts were also analyzed by LC-MS to correlate the observed retention times with the appropriate masses for both methymycin and pikromycin (Figure 2B). LC-MS analysis was carried out with extracts (20 μL) under the same solvent system conditions at a rate of 0.3 mL min^{-1} with a Surevor HPLC system (ThermoFinnigan) connected to a diode array detector equipped with a 2.1 μm Discovery HS C18 reversed-phase column (25× 4.6 cm Supleco). Mass spectra were collected by using an LCQ quadrupole ion trap (ThermoFinnigan) mass spectrometer equipped with an electrospray ion source in positive mode.

Diketide feeding experiments were completed by using an overnight seed culture to seed fresh SCM (10 mL) fermentations. After agitation for 10 h under the above-mentioned conditions, the diketide was added from a stock in MeOH to a final concentration of 1 mM—MeOH was used a negative control—and the fermentation process was permitted to continue for approximately 60 h. Analyses were carried out as described above.

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Keywords: biosynthesis · natural products · polyketides

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