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Functional dissection of a multimodular polypeptide of the pikromycin polyketide synthase into monomodules using a matched pair of heterologous docking domains

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Abstract

The pikromycin polyketide synthase (PKS) in *Streptomyces venezuelae* is comprised of a loading module and 6 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 an *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 10-fold 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.

Keywords

biosynthesis; natural products; polyketides

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

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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 may 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 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 two-six) can be housed on a single polypeptide. There are also many PKSs where 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- terminal 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 has been determined via surface plasmon resonance (SPR), showing that these docking appendages selectively bind to its cognate partner while mismatched pairs showed no binding.^[18] While, other protein-protein interactions between the ACP and KS domains may play a role in binding and the association of sequential modules, the specific amino acids involved have not yet been determined.^[19, 20]

Both NMR 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 alpha 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 un-conserved 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 via a different approach of fusing the N- and C-terminal docking domains.^[22] 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, producing 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 reconstructing these complex pathways in *E. coli*. For the epothilone case, EpoD which contains 4 modules was split into two modules using matched DD pair from the stigmatellin PKS.^[27] For the rifamycin case the tetramodular RifA was split into two proteins using a matched DD from the erythromycin PKS.^[26] In both cases the yields of the fully extended polyketide products were very low (10 µg/L 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 where 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 (Figure 2). 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 encoding the intraprotein linker between module 1 and module 2 of PikAI, was replaced with a DNA 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 encoding the loading domain-module 1 intraprotein linker in pDHS722 generated pJY12 and was designed to determine if docking domains would permit the loading domain to function as a separate polypeptide. In the case of pJY9 the intraprotein linker between module 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 see the effect of separating module 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 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 were observed in both BB138/pJY12 and BB138/pJY7, and were approximately 50% of that observed for BB138/pDHS722 (Figure 3). However, in the case of BB138/pJY9 where no linker or DD pair is 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 module 1 to 2. Alternatively the expression of a soluble separate module 2 may be lower without an N-terminal DD present. Indeed, work by Khosla et al on *in vitro* expression of 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 where it has been demonstrated that 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 using the Plm DD pair results in this KS domain being present at the N-terminus rather than the center of the polypeptide, permitting 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 the module 1 is catalytically inactive as a result of mutation of the KS active site Cys to Ala.^[36] The 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 appropriate DNA region in pJY7.

Fermentations of BB138 carrying pBK51, pBK51* or pJY7* did not produce any detectable levels of methymycin or pikromycin, 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 deviation were calculated relative to those obtained with BB138/pDHS722 (Figure 2). Levels of pikromycin production in the cases of BB138/pBK51 were extremely low, much less than 1% of that observed in the control culture. This result contrasts experiments which 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 control.^[36] The low levels of pikromycin production were increased to about 2% of the control with BB138/pBK51*, indicating a modest improvement in diketide utilization may 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) 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 may indicate that even as separate polypeptide the loading domain and module 1 may negatively impact loading of diketide onto module 2. Alternatively, the process may simply be inefficient regardless of the presence of the preceding module and the improvements may 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 (suggesting that that the separate module 2 without a N-terminal DD is either catalytically impaired or poorly expressed).

Conclusion

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, demonstrating that the separation does not work in the absence of the DDs. Furthermore, the observations demonstrate for the first time that the 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 a) the appropriate molecular recognition between the ACP and KS domains, and b) the substrate specificity of the

catalytic domains remains. An alternative approach to generating novel polyketide products is feeding NAC thioesters of analogs 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 plasmid 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 isolated from LB liquid media, both supplemented with appropriate antibiotics.

Generated variants of the pDHS722 were transformed into BB138 by established protocols^[40] onto R2YE plates. Transformants were subsequently grown on SPA agar plates (50mg mg/L 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 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 plasmid sequencing with manufacture supplied M13F and M13R primers, yielding construct pJY1. The *aac* (3)IV resistance marker was amplified from pIJ773^[41], using primers PlmDD-*Apra*F (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 location of unique Bstz17I restriction sites introduced for later removal of this marker). The resulting PCR product was recombined with pJY1 via 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 39nt 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 remaining 5' region represent the complementary 39nt of the PikAI region for recombination). PCR products were introduced to pDHS722 by homologous recombination via 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, yielding JY7 and JY12 respectively. The plasmid pJY9, which has had the region encoding the intrapolypeptide linker between modules 1 and 2 deleted was also generated by homologous recombination via established protocols^[41]. The *aac* (3)IV resistance marker amplified from pIJ773 was used for deletion of the linker encoding region with primer Pik link F (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') + Pik link R (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 ACT ***GTA GGC TGG AGC TGC TTC*** - 3'). Bold and italicized regions represent annealing regions for the resistance marker and * indicates location of unique the Bstz17I restriction sites introduced for later removal of this marker. A TGA stop codon (underlined and bold text) 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 presumed RBS (underlined) and start codon (CAC) of the N-terminal Plm DD (underlined and bold text) fused to the beginning of the KS module 2 encoding region. This PCR product was again recombined into pDHS722 via established protocols^[41] and the *aac* (3)IV resistance marker removed by restriction enzyme digestion as before and relegated to yield the final pJY9 plasmid. PBK51* was generated in the exact manner as JY7 but using pBK51 (encoding PikAI with a Cys to Ala mutation on KS1) as the final recombination plasmid. The plasmid pJY7*, which expresses only module 2 of PikAI was generated from PJY7 by 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 PCR. 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') + Pika1scarR (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 with remaining 5' regions representing recombination crossover sites). Successful replacement in pJY7 was followed by FLP mediated excision of the marker region generating pJY7*, which encodes for the expression of 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 thiostrepton) were carried out at 30°C with agitation (220 rpm) for 72 hours. After fermentation the medium was separated by centrifugation at 10,000 rpm for 10 min. The fermentation media was extracted 3× with (10 mL) ethyl

acetate, dried and re-suspended in (1mL) MeOH. Extracts of BB138/(pJY7, pJY12, pJY9, pBK51, pBK51* and pJY7*) were analyzed by reverse phase HPLC on a Agilent 1100 series system with a 5 μ m Discovery HS C18 reverse phase column (25 $\times$ 4.6 cm, Supelco), a linear gradient system from solvent A (20:80 acetonitrile: water -10mM ammonium acetate) to solvent B (80:20 acetonitrile: water -10mM ammonium acetate) over 50 min at a flow rate of 1 mL/min, and detection at 220nm. Relative amounts were determined by integrating and averaging the peak areas for pikromycin produced in 3 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 (1) and pikromycin (2) (Figure 2B). LC-MS analysis was carried out with (20 μ L) extracts under the same solvent system conditions at a rate of 0.3 mL/min with a Surevor HPLC system (ThermoFinnigan) connected to a diode array detector equipped with a 2.1 μ m Discovery HS C18 reverse phase column (25 $\times$ 4.6 cm Supelco). Mass spectra were collected on 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 (10 mL) SCM fermentations. After agitation for 10 hours at the above conditions, the diketide was added from a stock in MeOH to a final concentration of 1mM, (MeOH was used a negative control), and the fermentation process permitted to continue for approximately additional 60 hours. Analyses were carried out as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 generation of 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 transfer of the diketide intermediate, 2(*S*)-methyl-3(*R*)-hydroxypentanoic acid (activated as a NAC thioester) onto the KS domain of module 1.

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 methylmycin and pikromycin eluting at approximately 15 and 21 min respectively. C) Mass spectra of the products methylmycin and pikromycin ($m/z = 470$ and 526) in positive mode respectively

Figure 3. Levels of pikromycin products made in the BB138 PikAI deletion strain of *S. venezuelae* with respective complementation plasmids

Red block shapes indicate the Plm1- Plm2-3 docking domain pair from the phoslactomycin system. Diketide feeding experiments were carried out only with BB138 carrying constructs with PikAI in which the module 1 was either inactive (through mutational inactivation of the KS domain, indicated by an X), absent, as well as in the case of pJY9 where no docking domains were present. N/A (not applicable) and N/D (not detectable).