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Evolution of Efficient Modular Polyketide Synthases by Homologous Recombination

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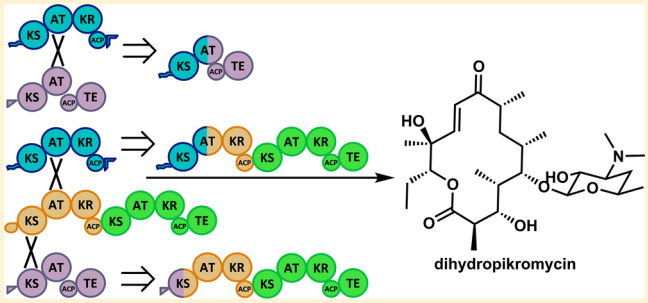
Supporting Information

ABSTRACT: The structural scaffolds of many complex natural products are produced by multifunctional type I polyketide synthase (PKS) enzymes that operate as biosynthetic assembly lines. The modular nature of these megaenzymes presents an opportunity to construct custom biocatalysts built in a lego-like fashion by inserting, deleting, or exchanging native or foreign domains to produce targeted variants of natural polyketides. However, previously engineered PKS enzymes are often impaired resulting in limited production compared to native systems. Here, we show a versatile method for generating and identifying functional chimeric PKS enzymes for synthesizing custom macrolactones and macrolides. PKS genes from the pikromycin and erythromycin pathways were hybridized in *Saccharomyces cerevisiae* to generate hybrid libraries. We used a 96-well plate format for plasmid purification, transformations, sequencing, protein expression, in vitro reactions and analysis of metabolite formation. Active chimeric enzymes were identified with new functionality. *Streptomyces venezuelae* strains that expressed these PKS chimeras were capable of producing engineered macrolactones. Furthermore, a macrolactone generated from selected PKS chimeras was fully functionalized into a novel macrolide analogue. This method permits the engineering of PKS pathways as modular building blocks for the production of new antibiotic-like molecules.



INTRODUCTION

Microbes efficiently assemble a plethora of polyketides with high medicinal value, including macrolide antibiotics, by gene clusters that encode type I polyketide synthase (PKS) enzymes. These multifunctional enzymes catalyze the initiation, elongation, and elaboration steps of the polyketide scaffold (see Figure 1a). Almost all PKS genes encode a minimal set of domains (ketosynthase [KS], acyl transferase [AT], and acyl carrier protein [ACP]), and are often accompanied by additional domains (e.g., ketoreductase [KR], dehydratase [DH], enoylreductase [ER], denoted as X and Y in Figure 1a) with the final module terminating with a thioesterase domain (TE). Tailoring enzymes (e.g., P450, glycosyltransferase, epoxidase, *O*-methyl transferase, halogenase and others) provide unique structural modifications of macrolactones and linear polyketide chains necessary for bioactivity. Bioinformatic tools with increasing power and automation are able to predict the domain architecture and catalytic reactions of typical PKS enzymes based on DNA sequence information. Conceptually, de novo design of a PKS pathway is possible by assembling a specific sequence of domains and modules with predicted/known catalytic functions (termed "lego-ization") for targeted small molecule biosynthesis.[1] This general approach is also applicable to the core enzymes of nonribosomal polypeptide synthetase (NRPS) and hybrid PKS-NRPS systems.[2−4]

Natural products often serve as the lead compound for improving pharmaceutical properties by medicinal chemistry, but their complexity often presents challenges for drug development.[5,6] The ability to introduce or eliminate reactive groups may be required to generate various types of drug conjugates, or improve bioavailability, pharmacokinetic and other parameters. The structural diversity of polyketides arises from their extensive repertoire of catalytic domains that yield a unique architecture and three-dimensional structure. A specific structural alteration to the polyketide is achievable by editing the architecture of the PKS enzymes. However, the current dearth of comprehensive structural data continues to limit the use of rational structure-guided approaches to generate highly functional engineered PKSs. Thus, we were motivated to explore a new strategy for rapid generation and testing of libraries of chimera PKSs for synthesizing complex polyketides in vitro and in a *Streptomyces* host.

Homologous recombination is a method that Nature employs to increase genetic diversity yielding higher overall

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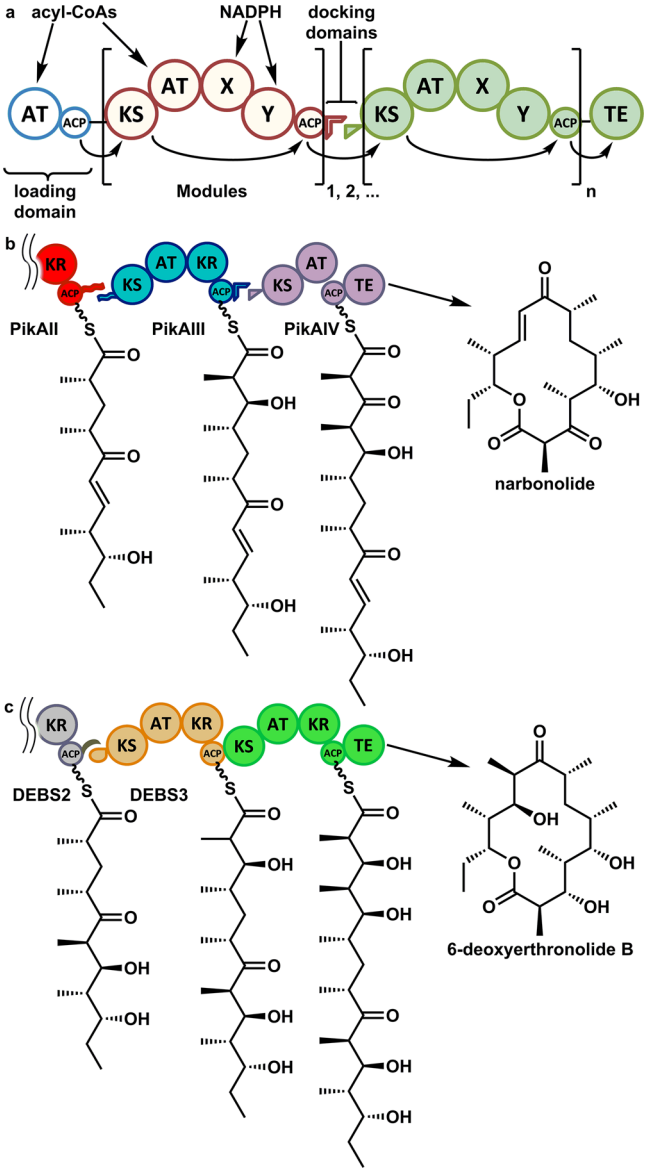

Figure 1. PKS assembly and products. (a) Typical PKS modular architecture with domain substrates and pathway of the elongating polyketide chain. (b) Pikromycin structures and abridged biosynthesis of narbonolide. (c) Erythromycin structures and abridged biosynthesis of 6-deoxyerythronolide B.

fitness of the chimeras relative to random mutations.[7] Hybridization can occur between similar genes with short stretches of identical DNA sequences resulting in hybrids with significant variation relative to either parental gene. In canonical PKS pathways, there is a limited scope of catalytic domains and substrates responsible for their vast structural diversity. Evolutionary pressure preserves secondary and tertiary protein structures and key catalytic residues to maintain catalytic activity,[8−10] which is reflected in regions of highly conserved DNA sequences among PKS and NRPS genes, respectively. PKS and NRPS pathways, therefore, may evolve by hybridization between two separate genes or gene clusters.[11] Moreover, *Streptomyces* species contain both linear genomes and large plasmids, and are particularly well-suited for exchanging genetic material.[12] Horizontal gene transfer permits the migration of gene clusters on plasmids or other mobilizable elements as indicated by certain pathways appearing in variant *Streptomyces* species.[12,13] A comparison of sequenced and annotated gene clusters revealed that recombination has indeed occurred between separate gene clusters and gene conversions appear to occur frequently within a gene cluster.[14] Thus, we envisioned homologous recombination as an attractive approach due to the successes revealed by Nature for the creation and diversification of PKS/NRPS pathways.

We selected genes from the pikromycin and erythromycin pathways (see Figure 1b,c) for developing a proof of principle homologous recombination system implemented in *Saccharomyces cerevisiae*. This approach was applied to first construct libraries of hybrid PKS genes, followed by expression in *Escherichia coli* and screening of chimera enzymes for production of specified macrolactones in vitro. Upon screening, a significant fraction of the libraries were catalytically active many with gained or lost functionality compared to the parental enzymes. Activity was demonstrated by a diverse library of chimeras with sequences not limited to splicing two PKS genes at the boundaries of annotated catalytic domains.[15] Through this approach, we identified a chimera PKS enzyme that yielded dihydronarbonolide in vitro, which was subsequently converted into dihydropikromycin, a new antibiotic-like macrolide via mutasynthesis. Engineered *Streptomyces venezuelae* strains expressing selected PKS chimeras were capable of producing pikromycin pathway analogues in vivo. Thus, homologous recombination is a general strategy to create functional PKS chimeras for producing custom-designed small molecules.

RESULTS

As a first step toward establishing an experimental homologous recombination approach, we chose well characterized PKS genes from the pikromycin and erythromycin pathways to generate hybrid genes in yeast. The hybrid library plasmids were assembled in *S. cerevisiae* by transforming with a linearized plasmid and a second DNA fragment (see Figure 2). The

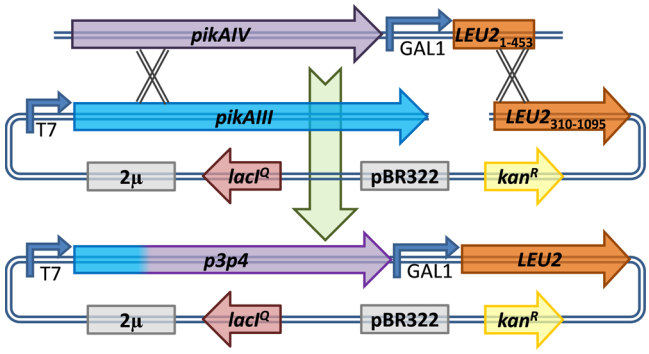

Figure 2. Generation of plasmids bearing a library of hybrid PKS genes through homologous recombination in yeast.

linearized plasmid contained the first PKS gene, either monomodular *pikAIII* or *pikAIV*, to establish the N-terminal docking domain and a 3′ end of a partial sequence of the auxotroph marker, *LEU2*. The second DNA fragment contained the second PKS gene, either *pikAIV* or bimodular *eryAIII*, to establish the origin of the TE domain and the 5′ end of partial sequence of *LEU2* downstream of the GAL1 promoter. Circular plasmids that assembled by hybridization of the *LEU2* gene fragments and the two PKS genes enabled growth of colonies on defined dropout media without leucine. Selected clones were grown in 96-well format and named based

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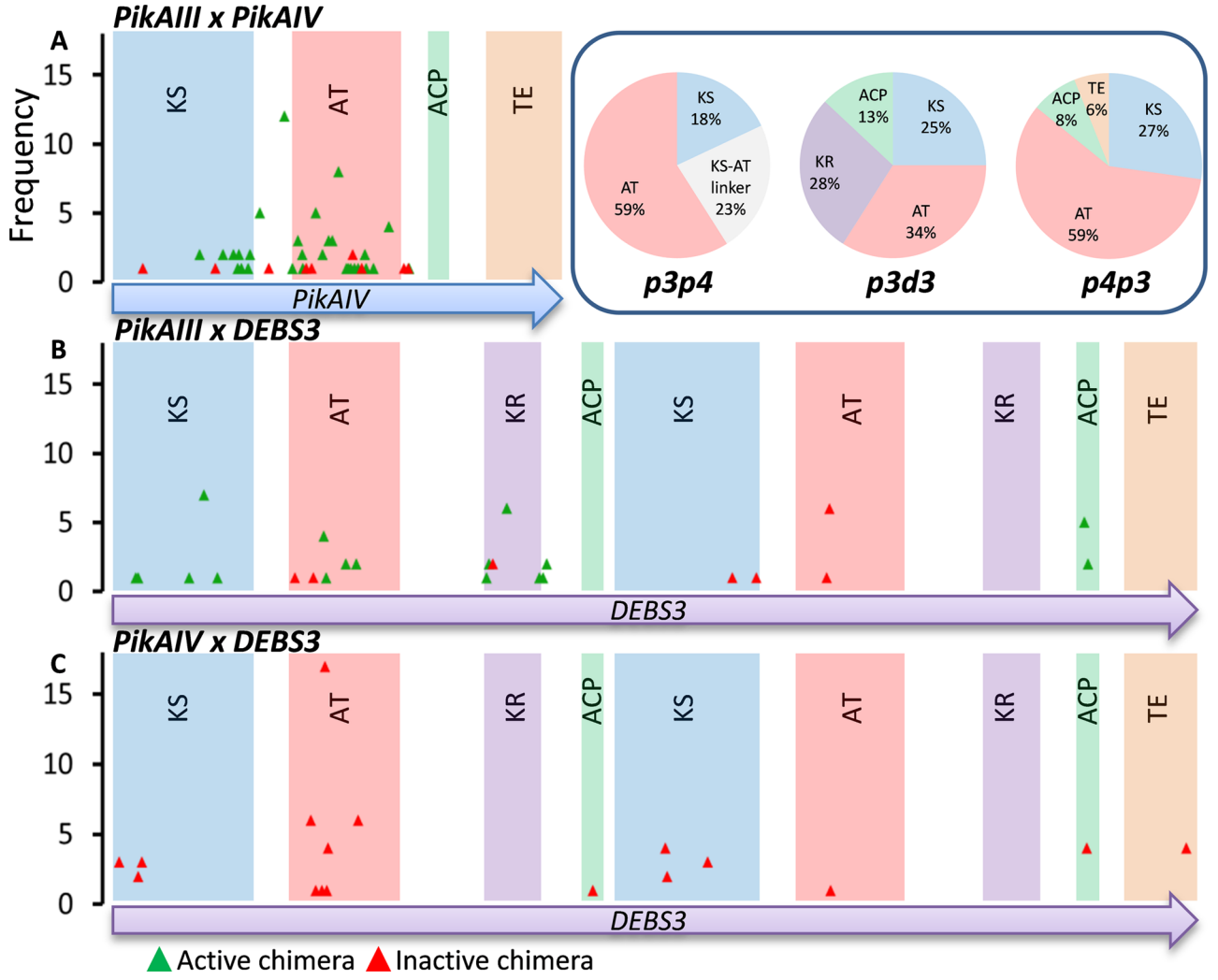

Figure 3. Frequency of occurrence of a hybrid sequence, location of the recombination event (scaled relative to gene sequence), and in vitro activity of the chimera. The boxed inset shows frequency of recombination within the respective catalytic domains.

on their well position (A1 to H12) and parental genes (*pikAIII* × *pikAIV* [*p3p4*], *pikAIII* × *eryAIII* [*p3d3*] and *pikAIV* × *eryAIII* [*p4d3*]).

Sequence Analysis. Hybrid sequences were aligned to parental gene sequences to identify the location of the recombination relative to the PKS gene with the terminal sequence (see Figure 3). Approximately 90% of all sequences were in-frame hybrid genes, 5% of which had stretches of only five identical nucleotides at the crossover location (see Supporting Information). Plasmids apparently assembled by illegitimate/nonhomologous recombination events represented 0%, 16%, and 13% of all the *p3p4*, *p3d3*, and *p4d3* hybrid libraries, respectively.

For the *p3p4* hybrid library, 82 clones were sequenced to identify the location of the crossover event, of which 38 were unique hybrids. The entire library contained hybrids with the same domain architecture of PikAIV predicted to generate 3-dehydro-10-deoxymethynolide (**1**) (see Figures 3 and 4). The locations of the crossovers clustered predominantly within the AT domain and the adjacent linker region between the KS and AT domains. The *p3d3* hybrid library contained 52 in-frame clones of which 24 were unique sequences, consisting of a mix of hybrids, both mono- and bimodules with either the architecture of Ery6 or DEBS3 predicted to yield 10-deoxymethynolide (10-dml, **2**) and dihydronarbonolide (**3**) (see Figures 3 and 4). The *p4d3* hybrid library contained 65 in-frame clones of which 18 were unique sequences consisting of both mono- and bimodules with a mix of four distinct architectures leading to four possible structures (see Figures 3 and 4). For both *p3d3* and *p4d3* hybrid libraries, about 70% of the in-frame clones were bimodular genes.

In Vitro Screening. Clones from the *p3p4*, *p3d3*, and *p4d3* hybrid library were transformed into *E. coli* (BAP1 pRARE-CDF strain) for protein expression. To minimize the number of handling steps, recombinant proteins were prepared as clarified crude cell lysates and hybrid PKS enzymes were assayed with the PikAIII thiophenol-pentaketide,[16] with detection of macrolactone production by LCMS (see Figure 4). The thiophenol-pentaketide is the native substrate for the KS domain of PikAIII with a thiophenol leaving group instead of the pantetheine group attached to the upstream ACP domain of PikAII module 4 (see Figure 1b).

The P3P4 chimeras were expected to contain enzymes with the same architecture of PikAIV and yield **1**. Of the 38 unique clones, 28 of them had detectable levels of macrolactone production. The P3D3 library contained a mix of mono- and bimodular hybrids predicted to produce **2** and **3**, respectively. Of the 24 unique hybrids screened, 15 had detectable

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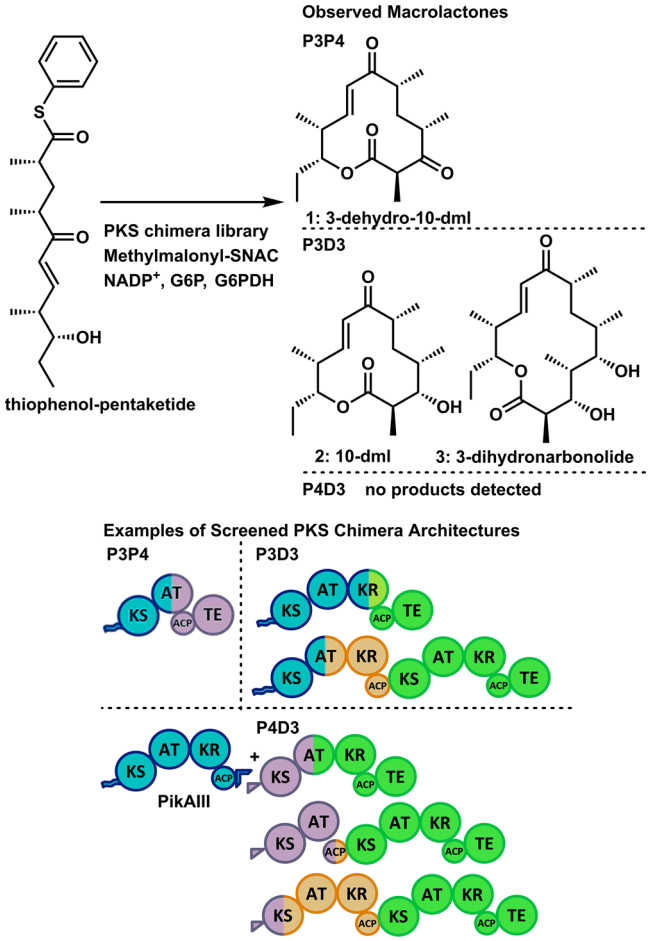

Figure 4. In vitro screening reactions of chimera libraries and macrolactones detected using LCMS.

macrolactone formation. The P4D3 reactions were screened with PikAIII for initial extension of the Pik pentaketide followed by hexaketide transfer to the P4D3 chimera. Following extension by PikAIII, single and double extension by hybrid P4D3 enzymes was predicted to generate specific 14- and 16-member rings. However, in this case, no macrolactone formation was observed.

Enzyme Kinetics. The most active PKS chimeras, based on product formation assessed in the LCMS screening assay, were overexpressed and purified by nickel affinity and size exclusion chromatography. Their apparent Michaelis–Menten kinetic parameters were determined (see Supporting Information Figure S4 and S5). The results are summarized in Table 1.

Table 1. Summary of Enzyme Kinetic Constants

enzyme	k_{cat} (min^{-1})	K_m (μM)	product
PikAIII-TE[a]	nd[a]	nd	1
P3P4_A3	2.36 ± 0.14	444 ± 61	1
P3P4_F4	2.21 ± 0.18	127 ± 23	1
P3P4_G3	nd[a]	nd	1
DEBS3	1.44 ± 0.095	465 ± 67	3
P3D3_A3	nd[a]	nd	3
P3D3_G9	2.13 ± 0.15	314 ± 57	3

[a]Kinetic data could not be fitted properly to the nonlinear curve and kinetic constants were not determined (nd).

Biotransformation of Dihydronarbonolide (3) into Dihydropikromycin (4). The last steps toward generating a biologically active macrolide analog required further modification by installing D-desosamine and the final C–H functionalization reaction using PikC P450-mediated hydroxylation (see Figure 5). In vitro reactions containing purified P3D3 chimeras

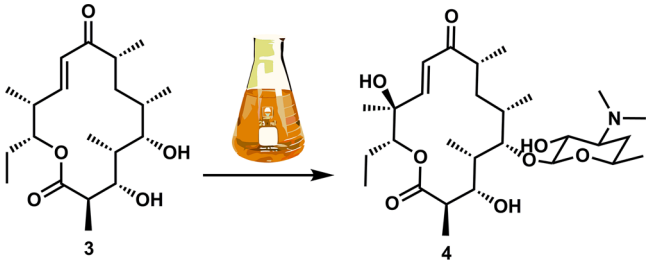

Figure 5. Dihydronarbolide (3) is converted stepwise into dihydropikromycin (4).

were used to synthesize **3** from the Pik thiophenol-pentaketide. The engineered variant of *S. venezuelae* strain DHS2001 (ATCC 15439 Δ*pikAI-IV*::*hph*)[17] was cultured for introduction of substrate **3**, which was converted into dihydronarbomycin and **4** (see Supporting Information). Levels of dihydronarbomycin were transient and **4** was recovered from the culture media after 24 h.

Biosynthesis of 3-Dehydro-10-deoxymethynolide (1). Although scalable chemoenzymatic synthesis of macrolactones using modular PKS proteins can be highly effective,[16] we were interested in extending our work by expressing engineered hybrid PKS genes in *S. venezuelae*. Thus, the engineered strain YJ004 (ATCC 15439 Δ*pikAIII-IV*::*aphII*)[18] was transformed with the expression plasmid, pDHS618 bearing the *p3p4_a3* hybrid gene under the control of the *pikAI* promoter. This strain was expected to yield 3-dehydro-10-dml (**1**); however, no products were detected initially from liquid culture. To test whether expression had been disabled by an unexpected polar effect, the plasmid was also transformed into strains HK954 (ATCC 15439 Δ*PikAIV*::*aphII*)[18] and the wild type *S. venezuelae* ATCC 15439. Only cultures of the transformed wild type produced **1** along with pikromycin. We deduced that the production of **1** depended on induction of gene expression using narbonolide or acetyl-narbonolide.[16] Thus, addition of acetyl-narbonolide to the culture with YJ004 *p*DHS-*p3p4_a3* provided **1** with maximum titers achieved (25 ± 8 mg/L) within 24 h of the fermentation.

Biosynthesis of Dihydropikromycin (4) in *S. venezuelae*. In an effort to test additional engineered modules for expression, YJ004 was transformed with selected hybrid PKS genes from the *p3d3* hybrid library including hybrids *p3d3_a3*, *a11*, *c7*, and *g9*. Despite in vitro production of macrolactones from modules generated through homologous recombination, it was evident that considerable protein instability was leading to low level, or no production of expected metabolites in *S. venezuelae*. Some improvement was observed when fermentation conditions were modified by lowering the culture temperature to 18 °C in an attempt to stabilize the chimeric modular PKS enzymes. The YJ004 strain expressing *p3d3_a3* provided **4** only during the first 24 h (see Supporting Information), further demonstrating the ability to create PKS modules with unique functions and engineered microbial

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strains that generate new macrolides through direct fermentation.

DISCUSSION

Over the past 20 years, numerous efforts have been made to re-engineer PKS enzymes to produce natural product analogs with moderate success.[15,18−24] Almost all of these relied solely on the approximate boundary demarcations of catalytic domains for creating fusions of different genes. Fusions usually have attenuated catalytic activity, which correlates with low levels of metabolite formation when expressed in a heterologous host. Prior efforts also include dissection of a bimodule into interacting monomodules,[25] reconstitution of enzymatic function after separation of catalytic domains,[26] docking domain engineering[27] and domain exchanges.[28] One notable success was the engineering of the rapamycin pathway through site-directed gene conversion to produce rapalogs with only a moderate decrease in titers compared to the native pathway.[29] In this case, a methylmalonyl-CoA specific AT domain was replaced with a malonyl-CoA specific domain by identifying junctures of high homology among the AT domains within the rapamycin system. Despite early promise,[23] efforts to engineer highly efficient PKS modules have been hindered by the lack of structural information on the entire enzyme. Generating this information from NMR or protein crystallography has been limited to partial structures containing only one or two PKS catalytic domains.[27,30−33] Recently, using cryo-electron microscopy, Whicher, Dutta et al. solved structures at 7 to 8 Å resolution for the complete PikAIII modular PKS from the pikromycin pathway.[34,35] This relatively comprehensive structural information provides important new insights that should facilitate engineering of efficient PKS and NRPS pathways by rational design.

Nature evolves natural product biosynthetic systems in many ways, including homologous recombination between two different PKS pathways. The preservation of PKS catalytic function provides a selective pressure to maintain overall structure, stability and key catalytic residues. Sequence analysis has revealed that the maintenance of regions of high identity provides sites for homologous recombination to occur between different PKS gene clusters.[14,36] The exact nature of the selection pressure(s) that maintains functional secondary metabolic gene clusters remains unknown.

In this study, we designed a strategy to take advantage of homologous recombination to generate hybrid PKS libraries. We first selected *S. cerevisiae* as a host to perform the homologous recombination due to its exceptional DNA repair mechanisms[37] for creating hybrid libraries by hybridizing genes that share some sequence homology. Indeed, sequencing of the hybrid libraries revealed that about 5% of all in-frame hybrids had local homologous sequences as short as five nucleotides. This is consistent with strain invasion during DNA repair requiring as few as two base pairs of homology in *S. cerevisiae*.[38] A minimum of five nucleotides needed for hybridization sets an estimate for an upper limit to the size of the possible number of unique hybrids in a library. DNA sequence alignments between of *pikAIII*, *pikAIV*, and *eryAIII* revealed there are between 100 and 200 locations with at least five nucleotides of identity.

The PKS enzymes, PikAIII, PikAIV, and DEBS3, were selected because each can be expressed exceptionally well as an active, soluble enzyme in *E. coli*. In addition, DEBS3 contains an extra ketoreductase domain relative to PikAIII and PikAIV to provide additional functionality. Furthermore, in this case, hybrids could be assessed in vitro by monitoring macrolactone formation by using the natural pentaketide chain elongation intermediate previously designed for PikAIII.[16]

Previous work had shown that PikAIV had a marginal ability to convert the Pik pentaketide into 3-dehydro-10-dml (**1**), likely due to substrate selectivity by PikAIV KS_6.[39] Therefore, the activity of the P3P4 chimeras was presumed to be due to the inherent specificity of the PikAIII KS domain. The wild type DEBS3 demonstrated substrate flexibility in its KS_5 domain by its ability to accept Pik pentaketide, catalyze double extension and cyclize the resulting heptaketide macrolactone.[39] Thus, both PikAIII and DEBS3 possessed the ability to process the Pik pentaketide substrate, which was reflected in the P3D3 library having a significant fraction of functional chimeras. In the case of the P4D3 library, the chimeras predominately contained the PikAIV KS_6 domain and therefore were not predicted to process the Pik pentaketide on its own. As expected, the production of 14-membered macrolactones failed to occur with the P4D3 chimeras. The addition of PikAIII would be required to present a hexaketide to the P4D3 chimeras. Still, no active chimeras in the screened library were identified with the addition of PikAIII. PikAIV and DEBS3 evolved to process specific 14-membered macrolactones with the TE domains likely serving as strict structural gatekeepers.[40] The incorporation of a TE domain that naturally catalyzes formation of 16-membered ring macrolactones such as tylactone, protomycinolide IV and platenolide may be required to accommodate an octaketide chain elongation intermediate.[41−43]

A functional PKS network requires a number of proper inter- and intramolecular protein−protein interactions to ensure efficient biocatalysis and optimal metabolic flux. For instance, the cognate docking domain pairs, attached to the ACP_n and KS_{n+1} of two interacting modules, are crucial for proper association between two specific PKS enzymes, especially when multiple PKS pathways are functioning within a single microbial cell.[44,45] In the case of the P3P4_A3 chimera, the docking domain and KS domain were properly matched with PikAII ACP_4. Following expression in a mutant *S. venezuelae* strain, production of significant amounts of **1** was achieved. Similarly, a P3D3 chimera provided **4**, albeit only when cultured at a reduced temperature. With only the first 100 residues originating from PikAIII, this chimera contained the proper docking domain from PikAIII, but interactions between PikAII ACP_4 and the DEBS3 KS_5 were mismatched and likely contribute to the reduced titers. Attenuated pathway productivity is expected when an ACP is mismatched with non-native KS partners.[46,47] As a control, a plasmid containing wild type DEBS3 transformed into *S. venezuelae* was tested for production. The expected product **4** was not detected during typical and reduced temperature fermentation conditions further illustrating the need for proper interactions between cognate docking domains.[45]

Moreover, the production of **1** from an engineered *S. venezuelae* strain YJ004 pDHS618-*p3p4_a3* occurred only following addition of the modified pikromycin pathway intermediate, acetyl-narbonolide, an inducer of *pik* gene expression.[16] Previously, we demonstrated that the addition of narbonolide or acetyl-narbonolide was required to induce the conversion of **2** into methymycin by presumed up-regulation of *des* and *pikC* via the bidirectional *pikC* promoter.[16,48] In the current study, the *p3p4* hybrid PKS gene was under the control

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of the *pikAI* promoter, which suggests that it is also regulated by narbonolide in a positive feedback loop.[49]

Many of the chimeras tested in vitro from the *p3d3* library yielded macrolactones, yet selected chimeras did not yield products when expressed in *S. venezuelae* under typical fermentation conditions. These chimeras, expressed in *E. coli*, purified as mixtures of truncated and full-length polypeptides, suggesting that the chimeras are unstable. The issue of unstable PKS chimeras was observed previously when DEBS3 AT_6 was replaced with rapamycin AT_2 domain.[50] Improper interactions between domains and between domains and linker regions of a PKS chimera may further contribute to attenuated functionality.[51] These observations further underscore the need for the PKS enzyme to be thermally stable to be functional and avoid proteolysis in vivo. The instability and proteolysis of a chimera protein was more likely the primary explanation for absent or decreased macrolide production from the chimeras, while ineffective inter- or intradomain interactions certainly also play a role.

Fortunately, there are approaches available to stabilize chimera proteins. Random mutagenesis is frequently used as part of a directed evolution approach. Relative to unmutated chimeras, titers of NRPS metabolites were improved in two examples after the non-native adenylation domains within chimeras underwent iterative rounds of random mutagenesis and selection.[52] The beneficial mutations were distributed throughout the adenylation domains. The incorporation of emerging structural information obtained from cryo-EM, protein NMR, and X-ray crystallography will enable rational approaches to improve the stability of chimera PKSs. For instance, the interfaces between the ACP_n and KS_{n+1} of PikAIII were identified above a side entrance of the KS active site.[34,35] Furthermore, algorithms like SCHEMA have been used to identify specific residue pairs with broken contacts within a chimera compared to a parental structure.[53] Mutating sequences to re-establish residue contacts was employed successfully to improve thermal stability and catalytic activity.[54] We expect that increasing success in engineering efficient PKS and NRPS chimeras will occur as more comprehensive structural data becomes available.

CONCLUSION

We have presented a straightforward and versatile method for rapid generation of chimeric forms of large multifunctional PKS enzymes. These efforts revealed facile creation of biosynthetic pathways to produce designer macrolide-type antibiotics. Following homologous recombination between annotated PKS genes, hybrid libraries yielded a significant fraction of catalytically active proteins with high sequence diversity. Library design can be implemented based solely on domain annotation, relying on evolutionarily conserved sequences to obtain enzymes with both improved activity and desired functionality. This approach is highly amenable to further improvement by incorporating additional directed evolution approaches as information regarding the dynamic structure and catalytic domain selectivity of full size PKS and NRPS proteins becomes increasingly available.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b04842.

Hybrid library sequence analysis (XLS)
Full experimental details, spectroscopic data (PDF)

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Notes

The authors declare no competing financial interest.

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