Modular polyketide biosynthesis can be harnessed to generate rationally designed complex natural products through bioengineering. A detailed understanding of the features that govern transfer and processing of polyketide biosynthetic intermediates is crucial to successfully engineer new polyketide pathways. Previous studies have shown that substrate stereochemistry and protein-protein interactions between polyketide synthase modules are both important factors in this process. Here we investigated the substrate tolerance of different polyketide modules and assessed the relative importance of inter-module chain transfer versus chain elongation activity of some of these modules. By constructing a variety of hybrid modular polyketide synthase systems and assaying their ability to generate polyketide products, it was determined that the substrate tolerance of each individual ketosynthase domain is an important parameter for the successful recombination of polyketide synthase modules. Surprisingly, however, failure by a module to process a candidate substrate was not due to its inability to bind to it. Rather, it appeared to result from a blockage in carbon-carbon bond formation, suggesting that proper orientation of the initially formed acyl thioester in the ketosynthase active site was important for the enzyme-catalyzed decarboxylative condensation reaction.

Polyketides are a large family of structurally diverse and complex natural products produced by bacteria and fungi. Many of these compounds, such as the important clinical agents erythromycin (1, 2), rifamycin (3, 4), and epothilone (5, 6), are generated by multifunctional proteins called modular polyketide synthases (PKS). These proteins catalyze the repetitive condensation of acetate or propionate units, similar to fatty acid biosynthesis, to produce these diverse and complex molecules (7, 8).

Experimental Procedures

Chemicals, Strains, and DNA Manipulation—[1-14C]Propionic acid (56.7 mCi/mmol), (±)-[2-14C]methylmalonyl-CoA (60.0 mCi/mmol), and [2-14C]malonyl-CoA (55.0 mCi/mmol) were obtained from
Hybrid Polyketide Synthases

**Fig. 1.** A PKS module contains the catalytic domains responsible for acyl chain elongation. Each module contains three core catalytic domains, ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP). The CoA-activated extender unit (malonyl, R = H or methyl malonyl, R = CH3) is loaded onto the module by the acyltransferase domain (A) and then transferred to the acyl carrier protein domain (B). The KS domain is loaded with the substrate from the transferase domain (A) and then transferred to the acyl carrier protein shown) and is transferred to the next module (C). P-pant, 4'-phosphopantetheinyln.

ARC, Inc. (2S,3R)-[1-14C]2-Methyl-3-hydroxypentanoic acid N-acetyl-cysteaminethioester ([14C]-labeled product 4, 5.5 mCi/mmol) was obtained by custom synthesis from Amersham Biosciences. All other chemicals were purchased from Sigma. DNA manipulations were performed in Escherichia coli XLI Blue (Strategene) using standard procedures (22). Restriction enzymes were from New England Biolabs. PCRs were carried out using Pfu polymerase (Stratagene) as recommended by the manufacturer.

**Engineering of Module Cassettes and Module Nomenclature—**By using module boundaries as defined earlier (13), modular (typically BsaBI-SpeI) cassettes were engineered for a selected set of modules including modules 2, 3, 5, and 6 of DEBS (hereafter referred to as eryM2, eryM3, eryM5, and eryM6, respectively), modules 2, 3, 5, and 6 of the pikromycin synthetase (hereafter referred to as picM3 and picM6, respectively), and modules 2, 3, 5, and 6 of the rifampicin synthetase (hereafter referred to as rifM2, rifM3, rifM5, and rifM6, respectively). The 5' end of each cassette could be fused to either the N-terminal linker segment of eryM3 (M3N) or eryM5 (M5N), or an additional domain (ketoreductase domain (KR), dehydratase domain (DH), and enoylreductase (ER) domains; not shown) and is transferred to the next module (E). P-pant, 4'-phosphopantetheinyln.

**Detection and Characterization of Polyketide Products—**Polyketides were considered to be produced if [14C]-labeling experiments produced a detectable spot at the correct Rf on a radio-TLC assay. The lower limit of sensitivity for this assay was 0.1 pmol of product. In vitras were also carried out as described above except without NADPH, and the ß-keto products were characterized by LC/MS and compared with authentic reference sample. In vitro reactions were also carried out at 37 °C, 250 rpm, and matched with authentic samples from previous studies (17, 26, 27).

**In Vivo Production of Polyketides—**E. coli BAP1 (25) was used as host for polyketide production. [1-14C]Propionic acid feeding was performed as follows. Individual transformants were inoculated into 10 ml of Luria-Bertani (LB) media in the presence of carbencillin (50 μg/ml) and kanamycin (25 μg/ml) at 37 °C and 250 rpm. Cultures were grown to mid-log phase (A660 = 0.6–0.8), cooled to ice for 10 min, and then centrifuged. The cell pellets were resuspended in 1 ml of the remaining supernatant and induced with 100 μM isopropyl-β-D-thiogalactopyranoside and [1-14C]propionic acid was added to a final concentration of 0.625 μM. The culture was stirred at 37 °C to mid-log phase (A660 = 0.6–0.8), cooled to 4 °C for 10 min, and induced with 100 μM isopropyl-β-D-thiogalactopyranoside. Unlabeled or [1-14C]propionic acid was added to a final concentration of 250 μM/liter, and the cultures were incubated at 22 °C for 0.2 h. The sample was then centrifuged and the supernatant extract twice with 300 μl of ethyl acetate. The sample was dried under vacuum and subjected to TLC analysis.

Unlabeled or [1-14C]propionic acid feeding experiments were performed as follows. A single transformant was used to start a 5-ml LB culture with carbencillin (100 μg/ml), and kanamycin (50 μg/ml) at 37 °C and 250 rpm. The starter culture was used to inoculate 200 ml of LB media at the same antibiotic concentrations as above. These cultures were grown at 250 rpm and 37 °C to mid-log phase (A660 = 0.6–0.8), cooled to 4 °C for 10 min, and induced with 100 μM isopropyl-β-D-thiogalactopyranoside. Unlabeled or [1-14C]propionic acid was added to a final concentration of 250 μM/liter, and the cultures were incubated at 22 °C for 20 h. The sample was then centrifuged and the supernatant extract twice with 300 μl of ethyl acetate. The sample was dried under vacuum, resuspended in C18, analyzed using high pressure liquid chromatography (Beckman System Gold, C18 reverse-phase column, 4.6 mm × 25 cm; Beckman Coulter, CH3CN/H2O gradient), and analyzed by atmospheric pressure chemical ionization--or electrospray ionization--mass spectrometry (Thermo-Finnigan LCQ).

**In Vitro Production of Individual Modules—**Individual modules fused with a TE domain were expressed and purified as described previously (15). In vitro analysis was performed in a solution of 400 mM NaH2PO4, 1 mM EDTA, 2.5 mM DTT, 5 mM NaCl, 20% glycerol, and 1% v/v MOPS, pH 7.2, in the presence of 0.1 mM of diketide (product 4), 800 μM [2-14C]methylmalonyl-CoA (or [2-14C]malonyl-CoA), and 4 mM NADPH. After 1 h, the reactions were quenched by the addition of ethyl acetate and vortexing. Extraction twice with ethyl acetate removed the triketide lactone product from the aqueous layer. Triketide product was detected using radio-TLC and compared with authentic reference sample. In vitro reactions were also carried out at described above except without NADPH, and the ß-keto products were compared with authentic ß-keto triketide reference samples.

**Protein Labeling Experiments—**Protein labeling experiments were performed by preincubating individual proteins (20 μg) for 5 min at room temperature with or without 5 mM cerulenin. Thereafter [14C]-labeled product 4 was added, and the sample was incubated at room temperature for an additional 15 min. The reactions were terminated.
FIG. 2. Native polyketide synthase pathways used to generate hybrid systems in this study. In this study selected modules (*) were examined from three different pathways, erythromycin (A), picromycin (B), and rifamycin (C). Polyketide synthase proteins (protein names in boldface type) are made up of modules (module names in boxes), composed of the catalytic domains described in Fig. 1. The postulated biosynthetic product of each module is shown. The substrate for each module is the intermediate attached to the immediate upstream module. LDD, loading didomain; AS, amide synthase.
RESULTS

In Vitro Production of Triketide Lactone—We tested the ability of various KS domains to accept and process a simple diketide substrate in vitro (4, Fig. 3A). Modules were fused to a thioesterase domain and a hexa-His tag and expressed in E. coli under the control of the T7 promoter. Purification by affinity chromatography afforded the individual proteins high purity. The individual proteins were then incubated in vitro with the N-acetylcysteamine thioester of the diketide (product 4), 14C-labeled methylmalonyl-CoA (or malonyl-CoA), and NADPH and assayed for the production of radiolabeled chain elongation products. Because of the availability of authentic standards of the expected triketide products, we were able to determine with certainty in a radio-TLC based assay whether the expected compounds were produced. The high sensitivity of the radio-TLC assay also allowed us to probe for very low levels of product formation (0.1 pmol, which corresponds to a product concentration of 5 nM under typical assay conditions). We found that four modules from DEBS (eryM2, eryM3, eryM5, and eryM6), one module from the rifamycin synthetase (rifM5), and one module from the picromycin synthase (picM6) could elongate 4 as evidenced by a detectable spot at the appropriate retention time on the radio-TLC image. Our assays also showed that eryM4, rifM2, rifM7, rifM8, and picM3 could not elongate 4 since there was no detectable spot on the radio-TLC image at the appropriate retention time for the β-keto or β-hydroxy triketide lactone products.

Dissecting Barriers to Chain Elongation by Individual Modules—We investigated the ability of selected individual modules from the above experiment to accept substrate (product 4) and a methylmalonyl extender unit. To determine whether the substrate was accepted by the protein, 14C-labeled product 4 was incubated in separate experiments with eryM5 and eryM6 (which catalyze elongation of 4), and rifM7 and picM3 (which do not catalyze elongation of 4). To verify that the substrate was acylating the correct active site of these multifunctional proteins, the KS domain, incubations were performed in the presence and absence of cerulenin, a selective KS inhibitor (28). SDS-PAGE autoradiography (Fig. 4, A and B) revealed that the purified modules were acylated and that cerulenin inhibited acylation. Similar observations were also made with other modules that do not catalyze elongation of 4 (data not shown). Separate incubation of the above purified proteins with either 14C-methylmalonyl-CoA or 14C-malonyl-CoA (29) showed that the proteins were selectively acylated by methylmalonyl-CoA, the predicted extender unit (data not shown).

In Vivo Tolerance of Modules for Triketide Substrates—We also tested the ability of various KS domains to accept and
process a triketide substrate (7, Fig. 3B) by developing a two-plasmid in vivo expression system. Because the synthetic triketide N-acetylcycteamine thioester spontaneously cyclizes, we constructed a system that generated the triketide intermediate in situ. By using the expression construct for the protein DEBS1, we were able to generate the desired triketide as an acyl-enzyme intermediate (product 7). We then created a set of plasmids with a complementary resistance marker to the above construct. This set of nine plasmids contained individual modules fused to a TE domain (Fig. 3B). To facilitate the transfer of the acyl intermediate between the two proteins, the native C terminus of DEBS1 and the N terminus of DEBS2 were incorporated into our two-plasmid system. By co-expressing the DEBS1 expression plasmid with plasmid containing either eryM3 or picM6, we discovered radio-TLC and LC/MS the expected triketide product 8 which matched authentic sample from previous studies (26). For the reaction of DEBS1 with eryM6 and rifM5, the expected triketide product 9 was isolated and characterized by radio-TLC and LC/MS. When [1-\textsuperscript{14}C]propionate feeding, the expected products rifM7, rifM8, and picM3 were assayed, by co-expression with tetraketide character of the compound. When eryM5, rifM2, and rifM7, rifM8, and picM3 were assayed, by co-expression with DEBS1 and [14C]propionate feeding, the expected products could not be detected by radio-TLC analysis (<1% as active as ery3).

In Vivo Tolerance of Modules for Pentaketide Substrates—We further extended our in vivo system by testing individual modules toward pentaketide substrates (10, Fig. 3C). Since pentaketide substrates present a significant challenge to synthesize chemically, we developed an in situ method for their production. We used a construct that expresses the first two proteins from erythromycin biosynthesis, DEBS1 and DEBS2. This system produced the pentaketide as acyl-enzyme intermediate (product 10). We then constructed a small library of plasmids containing our modules of interest fused to a TE domain. To facilitate the transfer of the pentaketide intermediate between proteins, we added the N-terminal linker sequence from DEBS3 to the N terminus of our plasmid library (Fig. 3). We selected the modules eryM5, eryM6, and rifM5 to study with this system because we had authentic references of the product. Moreover, the above studies showed that eryM6 and rifM5 were relatively tolerant toward unnatural substrates, whereas eryM5 was the natural recipient of the pentaketide product of DEBS1 and DEBS2. When each individual member of our library was co-expressed with DEBS1 and DEBS2, we were able to detect the formation of compound 11 by comparison to an authentic reference on radio-TLC. We were also able to confirm product formation by mass spectrometry.

In Vivo Tolerance of Modules eryM3 and picM3 for Alternative Triketides—We tested the ability of two different KS domains from eryM3 and picM3 to accept and process two different triketide substrates 7 or 12 (Fig. 3B and Fig. 5). This experiment was based on the two-plasmid system described under "In Vivo Tolerance of Modules for Triketide Substrates." From the above work we had three of the four needed expression vectors. The remaining plasmid was generated from DEBS1 by replacing eryM2 with picM2. Expression of the vector containing eryM2 produced compound 7 as an acyl-enzyme intermediate, and expression of the vector containing picM2 generated 12 as an acyl enzyme intermediate. When eryM3 was co-expressed with the acyl-enzyme intermediate 7 we were able to detect the formation of the expected product by both radio-TLC and LC/MS analysis (Table I, entry 2B). However, when eryM3 was co-expressed with intermediate 12, none of the expected product could be detected (Fig. 5). We were also able to detect the expected product when picM3 was co-expressed with intermediate 12 (Fig. 5) but not with intermediate 7 (Table I, entry 10B). Thus eryM3 and picM3 appeared to have orthogonal recognition for intermediates 7 and 12, respectively.

Role of Linkers in Engineered Multimodular PKSs—We evaluated the importance of having appropriately matching C- and N-terminal linker sequences for the generation of polyketide products. These linker sequences are important for selective intermolecular transfer of the growing polyketide chain to the next protein (13, 14). By using selected constructs we evaluated the relative contributions of matched and mismatched linker sequences. When DEBS1 was co-expressed with eryM6+eryTE fused to the M3N linker (Fig. 3B), the titer of product 5 was substantially (~10 times) reduced relative to the analogous construct in which eryM6+eryTE was fused to the M3N linker. In contrast, when co-expressed with DEBS1 and DEBS2, substantially greater quantities of hexaketide product 11 were obtained from the former eryM6+eryTE construct relative to the latter construct.

**DISCUSSION**

In this study we have investigated the tolerance of individual modules from erythromycin, picromycin, and rifamycin PKSs for substrates of increasing structural complexity. Each substrate examined was a biosynthetic intermediate in the 6-deoxyerythronolide B pathway (Fig. 3, 4, 7, and 10). We identified trends in the substrate specificity of individual modules based on the substitution and stereochemistry at the α and β positions. These results provide the first comparative glimpse into relative substrate tolerance of diverse modules, setting the stage for the rational design of hybrid multimodular PKSs.
Our initial experiments assayed the ability of various purified modules to accept and elongate a diketide substrate (4) \textit{in vitro}. Whereas some modules successfully generated the desired triketide products, others did not. The recombinant proteins were not misfolded or inactivated during purification since all of them were able to discriminate between malonyl- and methylmalonyl-CoA in labeling studies. We therefore interpret the absence of product to indicate intolerance of these modules to either accept or elongate substrate 4.

Substrate selectivity can be achieved through three different mechanisms: (i) a barrier against chain transfer to the KS domain, (ii) inability of the KS to catalyze decarboxylative chain elongation, or (iii) inability of the TE domain to release the product of chain elongation. Since the TE domain has been shown to release equivalent reduced or unreduced products in control experiments with other modules, we can rule out blockage at this step of the catalytic cycle. Unexpectedly, our labeling studies have shown that modules incapable of producing triketide products can be acylated by diketide (4) at the active site cysteine of their KS domains. Thus, their failure to process the substrate lies not in the chain transfer step but in the condensation reaction (29, 30). We speculate that either the electrophilic acyl chain binds in a different binding pocket during the acylation step as compared with the condensation step, or more likely that the requirements for accurate positioning of the electrophile are more stringent during catalysis of the decarboxylative condensation reaction as opposed to the inter-module acyl transfer reaction.

Comparison of the ability of differing modules to accept and process the three substrates studied (Table I) provides us with some insight into the substrate selectivity of these different modules. Based on these results, we can propose a tentative relationship between the native substrate a module processes and the range of non-native substrates that it will accept and elongate. We have studied previously the substrate tolerance of individual modules from erythromycin biosynthesis for different diketide substrates (15, 17–19). These studies found that the relative stereochemistry of the native substrate at the $\alpha$ and $\beta$ positions needs to be conserved for the substrate to be processed. We identified a similar trend. Most of our substrates contained syn $\alpha$-methyl-$\beta$-hydroxy substituents. These substrates were uniformly accepted by modules whose native substrates contain syn $\alpha$-methyl-$\beta$-hydroxy substituents, regardless of their absolute stereochemical configuration (Table I, entries 1A, 2A and B, 5A–C, and 11A and B). Also, modules whose native substrate possessed an anti stereochemical arrangement did not accept the syn substrates used in our study (Table I, entries 9A and B). There was, however, an exception to this trend. rifM7, whose native substrate contains syn $\alpha$-methyl-$\beta$-hydroxy substituents would not process any of the substrates we tested (Table I, entries 5A and B). We also observed that modules whose native substrates contain no $\beta$-substituent and an $\alpha$-methyl group were highly likely to accept and process our substrates (Table I, entries 4A, 4C, and 7A–C).

In addition to stereochemical preferences, our results suggest that maintaining the same level of hybridization as the native substrate is important for the KS domain to accept and process non-native substrates. Modules whose native substrates contain $sp^2$-hybridized carbons ($\beta$-keto or $\alpha$-$\beta$-unsaturated) would not process our $sp^1$-hybridized substrates (entries 3A and 10A and B). To test this hypothesis further, we examined two tetraketide-forming systems. In these systems triketide substrates with $sp^2$ ($\alpha$-$\beta$-unsaturated) and $sp^1$ (syn $\alpha$-methyl-$\beta$-hydroxy)-hybridized centers were assayed to determine whether they could be accepted and elongated by eryM3 or picM3. As we anticipated, eryM3, whose native substrate is $sp^1$-hybridized, could not accept and process the $sp^2$-hybridized substrate but could accept and process the $sp^3$-hybridized substrate. Similarly, picM3, whose native substrate is $sp^2$-hybridized, could not accept and process the $sp^3$-hybridized substrate; however, it could accept and process the $sp^3$-hybridized substrate. Thus the inability of eryM3 to process the $sp^3$-hybridized substrate as well as its inability to process the $sp^1$-hybridized substrate could be the result of substrate selectivity exerted by the KS domain. These results are also consistent with the work of Reynolds and co-workers (31), where a triketide, rather than the expected 12- and 14-membered macrolides, was produced when PikA1 was replaced by DEBS1.

An important aspect of designing hybrid modular polyketide systems is ensuring the correct protein-protein interactions such that the growing acyl chain is transferred and processed to provide the correct final product. Acyl chain transfer has been shown to be facilitated by the C- and N-terminal regions of these multifunctional proteins (13–16). The C-terminal peptide sequence of one PKS subunit binds to the N-terminal region of the next subunit in the multi-enzyme complex (32). In the experiments presented here, we use C and N termini that are known to interact and successfully create functional hybrid multi-enzyme PKS systems. When C- and N-terminal regions that do not interact are used, a substantial decrease in the efficiency of the hybrid PKS systems is observed. These results reinforce the importance of maintaining protein-protein interactions for optimal performance of hybrid PKS systems. However, the growing acyl chain can still be transferred to the next PKS protein, even in systems where the C- and N-terminal regions do not interact. This observation further supports our hypothesis that the substrate specificity of the KS domains is the critical determinant for generating functional hybrid PKS systems.

In conclusion, combinatorial shuffling of PKS modules to generate diverse polyketide products requires not only an understanding of the inter-module acyl chain transfer requirements but also an understanding of the intrinsic substrate specificity of the individual modules themselves. Understanding the mechanistic and structural basis of substrate specificity will allow us to engineer broad substrate tolerance and simplify module shuffling based on biosynthetic engineering.

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Understanding Substrate Specificity of Polyketide Synthase Modules by Generating Hybrid Multimodular Synthases
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