

CREATING POLYKETIDE DIVERSITY THROUGH GENETIC ENGINEERING

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1. ABSTRACT

Modular polyketide synthases (PKS) are large multifunctional enzymes that synthesize complex polyketides, a therapeutically important class of natural products. The linear order and composition of catalytic sites that comprise the PKS represent a “code” that determines the identity of the polyketide product. By re-programming the PKS through genetic engineering, it is possible to alter the code in a predictable manner to create specific structural modifications of polyketides and to produce new libraries of these natural products.

2. INTRODUCTION

Polyketides are natural products produced by bacteria, fungi, and plants. They are synthesized from simple carboxylic acids in biosynthetic reactions mechanistically related to those of fatty acid biosynthesis. As a class, the polyketides exhibit remarkable structural diversity and have been a rich source of human pharmaceuticals targeting diverse therapeutic areas, including infectious disease (erythromycin, **1**, and derivatives), oncology (adriamycin), transplantation /immunosuppression (rapamycin, **3**), and cardiovascular disease (lovastatin/mevacor) (1) (Figure1). It is estimated

that there are about 7000 known natural polyketides, but this number represents only a fraction of what nature is capable of producing. Recently, there has been much interest in expanding nature’s repertoire of natural polyketides by “re-programming” polyketide synthases through genetic engineering. In this paper, I will review recent advances in the field to illustrate the utility and potential of this technology. Several excellent and more comprehensive reviews have recently appeared in the literature ((2-7) (8-10)).

3. POLYKETIDE SYNTHASES

Polyketides are synthesized by large multifunctional enzymes (or assemblies thereof) called polyketide synthases (PKSs). PKSs can be classified into two general types (I and II), using nomenclature borrowed from the fatty acid synthase field. The type I PKSs, which include the modular and fungal PKSs, contain catalytic sites arranged on multifunctional proteins. For modular or “complex” PKSs, the active sites are arranged in modules and the number of modules is equal to the number of condensation reactions necessary to generate the full length polyketide. In some systems, module “skipping” (11) or

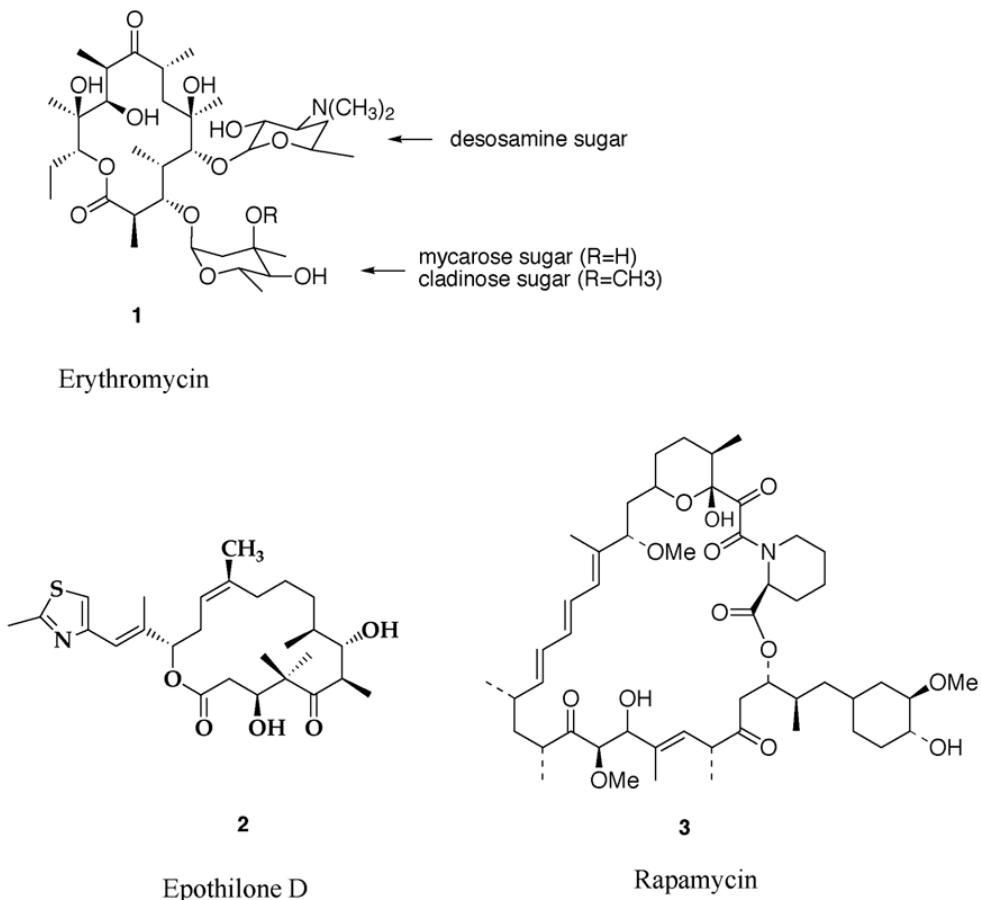


Figure 1. Complex polyketides of clinical or potentially clinical importance. Epothilone D is a mixed polyketide/non-ribosomal peptide with microtubule stabilizing properties (42), (43).

“stuttering” (12, 13) can lead to shorter or longer polyketide products. Polyketides produced by modular PKSs are sometimes referred to as “complex” polyketides. Work from the laboratories of Leadlay and Katz in the early 1990s on the cloning and sequencing of the genes for the *Saccharopolyspora erythraea* (*Sac. erythraea*) PKS that produces the erythromycin precursor, 6-deoxyerythronolide B (6dEB) (4, Figure 2), demonstrated that the primary structure and enzymatic functions of the protein in this modular PKS are co-linear (14), (15). That is, the PKS can be thought of as a molecular assembly line with each individual domain playing a unique role in the build-up of the polyketide. The sequence and composition of the individual modules represent a “code” which specifies the polyketide product, and there is a one-to-one correspondence between the sequence of enzymatic functions and the structure of the polyketide. This type of organization implied the possibility of rationally re-programming the PKS by domain manipulation to generate novel polyketide products (15); hence, a new and exciting area of research emerged.

The catalytic sites in the multifunctional fungal PKSs are used more than once, or iteratively, during

polyketide biosynthesis, a property that distinguishes these synthases from the modular enzymes, where the active sites are used only once during polyketide assembly. The type II bacterial aromatic PKSs have few active sites and these are carried on separate proteins. Like the fungal PKSs, the active sites of the type II PKSs are used iteratively during the course of polyketide synthesis. The fact that the catalytic sites in the fungal and bacterial aromatic PKSs are used iteratively reduces the combinatorial potential of these PKS systems for generation of targeted analogs by genetic manipulation, since a change in an individual domain is manifested at each corresponding step in the iterative cycle. For this reason, the iterative PKSs have been less attractive candidates for genetic engineering. Accordingly, the following discussion will focus on modular systems; specifically, the generation of polyketide diversity through genetic manipulation of the 6-deoxyerythronolide B synthase (DEBS), the prototypical modular PKS.

4. THE DEBS POLYKETIDE SYNTHASE

DEBS is composed of three ~ 350 kDa polypeptides designated DEBS1, 2 and 3, encoded by the genes *eryA1*, *eryAII* and *eryAIII*, respectively (Figure 2). An

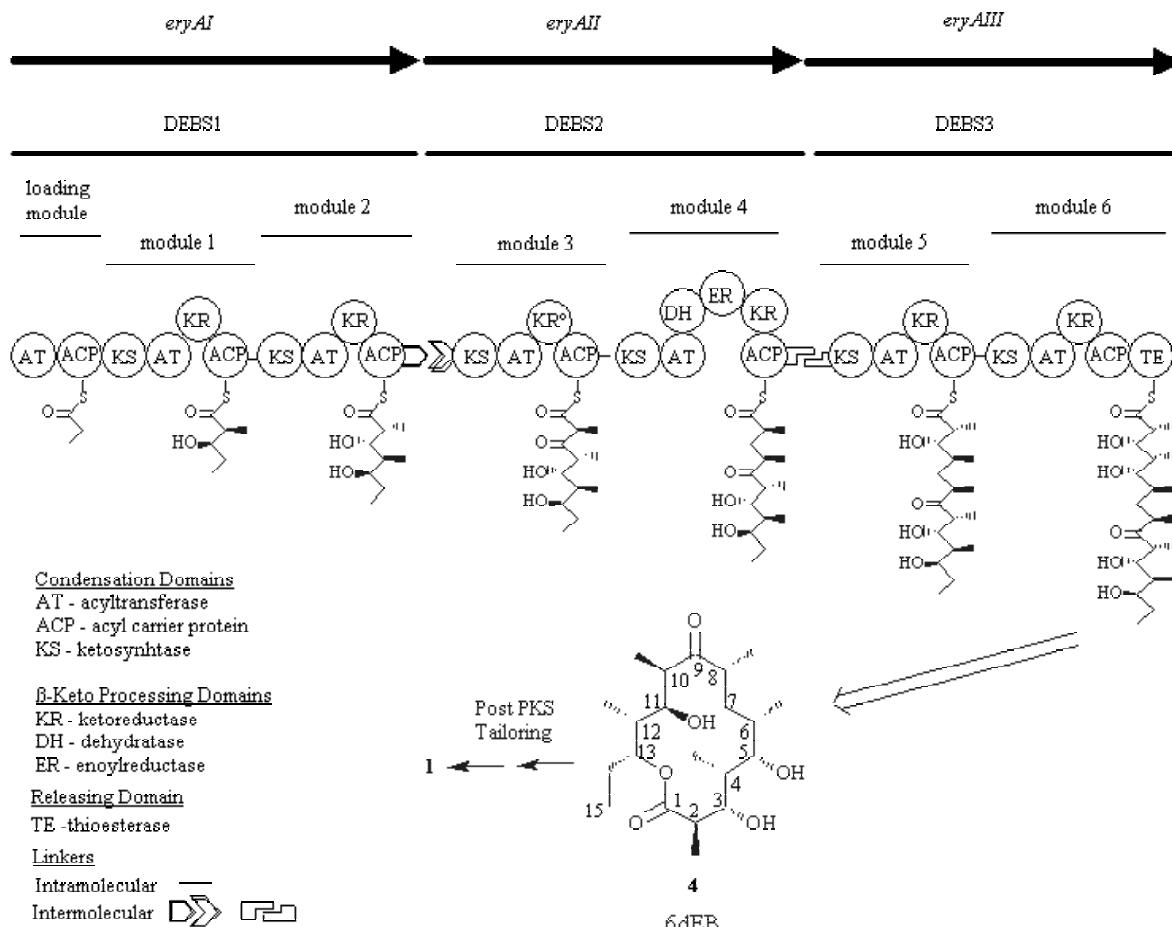


Figure 2. The DEBS PKS. The three *ery* genes (~11 kbp in length each) are represented by arrows, the DEBS proteins by lines, modules by staggered lines, and individual domains by circles. Polyketide intermediates are shown tethered to ACP domains, following condensation reactions in modules 1-6.

individual DEBS protein is made up of 2 modules, each of which catalyzes a condensation reaction followed by up to three cycles of beta-keto reduction. The acyl carrier protein (ACP) domains are post-translationally modified with 4'-phosphopantetheine, catalyzed by a specific phosphopantetheinyl transferase (16) (p-pant transferase, Figure 3). DEBS1 contains an additional N-terminal loading domain consisting of a loading acyltransferase (AT_L) and an ACP (ACP_L). The loading AT selects the acyl-CoA starter unit, propionyl-CoA, and transfers it onto the thiol of the phosphopantetheine moiety of the ACP_L domain. The propionate unit is then transferred to an active site cysteine on the first keto synthase (KS1) domain. The AT of the first module (AT1) binds the (2S)-methylmalonyl-CoA extender unit and transfers it to ACP1. The flexible phosphopantetheine arm “delivers” the methylmalonyl-CoA extender to KS1, which catalyzes the decarboxylative condensation (see Figure 3). The newly formed diketide is then swung into the ketoreductase (KR1) active site, where the beta-keto group is reduced to an alcohol, in an NADPH-dependent reaction. The polyketide chain is then transferred to KS2, and a second round of condensation/reduction occurs. Following transfer of the

triketide to KS3, a fourth condensation reaction takes place, but in this case, the beta-keto group is left intact, due to an inactive KR3 domain (designated KR[°] in Figure 2). In module 4, a full complement of accessory activities is present, and the beta-keto group is fully reduced to the methylene. Following condensation and reduction in module 6, the completed polyketide is transferred to the thioester or releasing domain, which catalyzes lactonization and release of the polyketide. Post PKS “tailoring” reactions, composed of two cytochrome P-450 hydroxylations, two glycosylations and an AdoMet-dependent sugar O-methylation, convert the 6dEB into the antibiotic erythromycin (1).

At all stages of polyketide biosynthesis, the polyketide remains bound to the PKS. Following condensation reactions, the growing chain is tethered to the ACP of the condensing module via the phosphopantetheine arm. There is good evidence that the DEBS proteins function as homo dimers, and it has been proposed that each giant protein pair is organized “head to head” and twisted into a helix (17). The KS, AT, and ACP domains form the core of the helix and the beta-keto modifying

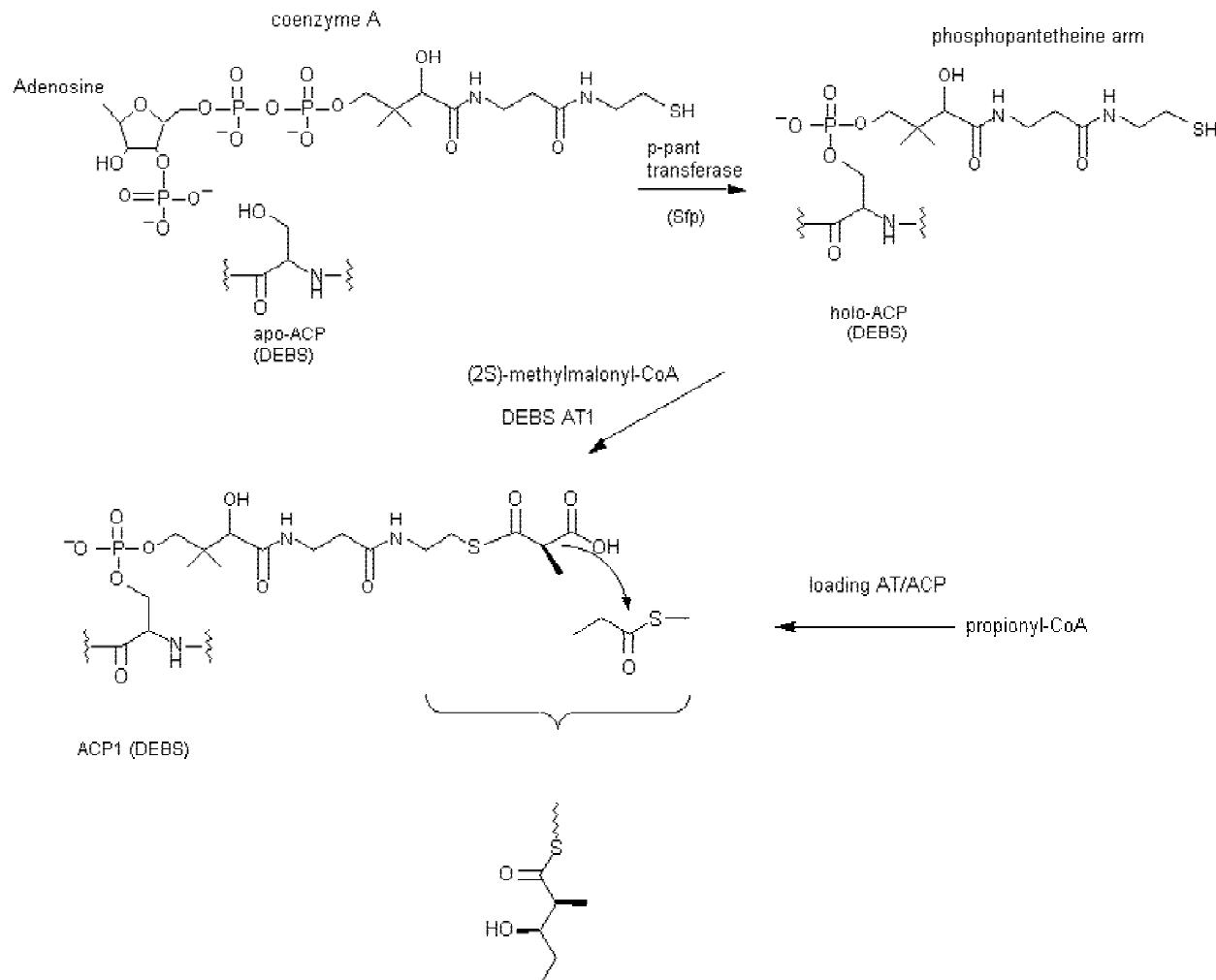


Figure 3. Post-translational phosphopantetheinylation of DEBS ACP domains catalyzed by Sfp and decarboxylative condensation catalyzed by DEBS.

activities (if present) are looped out. The ACP domain carrying the extender methylmalonyl-CoA delivers it to the corresponding KS domain on the opposite protein (i.e. across the dimer). Following condensation, the chain is directed to reductive domains on the same protein, and then passed to the KS of the next module. The linker regions (Figure 2) facilitate communication between covalently (intramolecular) and non-covalently (intermolecular) attached modules to ensure proper transfer of the polyketide chain from the “upstream” ACP to the “downstream” KS (18). The AT domains bind only the (2S)- isomer of methylmalonyl-CoA (19), yet in the 6dEB product, the stereochemistries of the corresponding methyl substituents are mixed (positions 2,4,6,8,10,12 in 4). Assuming inversion of configuration of the methyl upon condensation (20), additional post-condensation epimerization reactions must be invoked to account for the configuration of the methyl substituents at positions 6 (module 4), 8 (module 3) and 12 (module 1) of 6dEB. The KR domains catalyze stereospecific reductions, thereby controlling the stereochemistry of the hydroxyl

substituents. They also indirectly dictate the stereochemistry of the methyl substituent by processing only one of the epimers, in those modules that catalyze epimerization (21).

5. GENETIC MANIPULATION OF DEBS

As discussed above, the modular organization of DEBS lends itself to the rational design of novel polyketide analogs. Genetic manipulation at the domain level leads to precise and predictable (in most cases) changes in the polyketide structure, as illustrated in Figure 4. Note that many of the substitutions enabled by the genetic approach would be extremely difficult or intractable by conventional chemical methods. The host /vector systems developed by Khosla and colleagues ((22), (23)) simplified the construction of mutant PKSs by enabling mutagenesis and vector construction to be carried out in *E. coli*, followed by transfer of the shuttle vector(s) containing the mutant PKS gene(s) to a heterologous “clean” production host. A clean host, such as *Streptomyces coelicolor* CH999, does not

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| Domain | Genetic Manipulation | PK Product | Example |
|---------|--|----------------------------|----------------------------------|
| AT | Domain Swap mm-CoA \leftrightarrow m-CoA | | 5 |
| KR | Domain inactivation/addition Loss of Function/ change in stereochemistry | | 6 7 loss Δ stereo |
| DH | Domain addition | | 8 gain |
| ER | Domain inactivation/addition Loss/Gain of Function | | 9 10 loss gain |
| Loading | Domain inactivation diketide feeding | | 11 |
| TE | Domain relocation | truncated lactone products | 12 |

Figure 4. Combinatorial Elements of the DEBS PKS.

produce erythromycin, but has all other genes/pathways for the production of complex polyketides.

To change the methyl-substitution pattern in 6dEB, the AT domains of DEBS can be substituted with the rapamycin PKS (RAPS) AT2, which is specific for malonyl-CoA, thereby eliminating a methyl branch at the position in 6dEB corresponding to the domain swap. Liu *et al* (24) replaced DEBS AT6 with rapAT2, producing 2-desmethyl-6dEB (**5**) (Figure 5). The 6dEB analog was purified from the heterologous *Streptomyces* expression host CH999 and “fed” to a “bioconversion” strain of *S. erythraea*, which converted the analog to 2-desmethylerythromycin A. The bioconversion strain of the natural host contains a mutation in DEBS that prevents 6dEB production, but harbors all other necessary tailoring activities.

The KR domains can be inactivated to change a hydroxyl to a keto functionality. The experiment that indicated the co-linearity of domain organization and polyketide structure involved inactivation of the DEBS KR5 (15). Since this work was carried out in the natural

host, *Sac. erythraea*, the 5-keto-6dEB analog produced (**6**) was further elaborated by addition of the 6-hydroxyl and mycarose sugar (an intermediate with the mycarose but without the 6-hydroxyl was also observed). The change from hydroxyl to keto at C3 prevented addition of the desosamine sugar at that position. Substitution of KR6 with KR2 from the RAPS gene cluster (rapKR2), which catalyzes ketoreduction with the opposite stereochemistry, produced an analog with altered hydroxyl stereochemistry, 3-(epi)-6dEB (**7**).

The addition of a DH domain, so called “gain of function” mutagenesis, changes a hydroxyl moiety to a vinyl group. To produce 2,3-anhydro-6dEB (**8**), DEBS KR6 was swapped with DH4/KR4 from the rapamycin gene cluster (rapDH4/KR4)(25). The elimination or inactivation of an ER domain also generates a vinyl group. In early experiments (26), Donadio *et al* inactivated the DEBS ER4 by disrupting the NADPH binding site, and succeeded in producing 6,7-anhydro-6dEB (**9**), which was further processed in *Sac. erythraea* to 6,7-anhydroerythromycin C. The latter compound differs from the fully elaborated erythromycin A by the lack of the 6-hydroxyl and

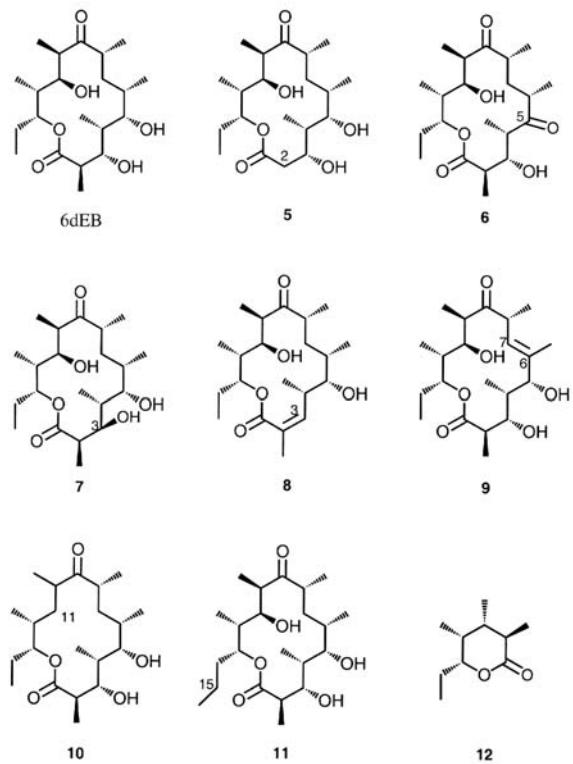


Figure 5. 6dEB analogs produced from genetically modified DEBS.

mycarose sugar O-methyl. The addition of an ER domain converts an enoyl to a methylene. DEBS KR2 was replaced with rapDH1/ER1/KR1 to convert the 11-hydroxyl group first to the enoyl, then to the methylene, producing 11-deoxy-6dEB (**10**) (25).

Production of novel 15-R substituted 6dEB molecules can be achieved by loading domain substitution (27) or by “chemobiosynthesis” (28). The latter method involves supplying an exogenous starter unit in the form of a chemically synthesized diketide thiol ester to a polyketide biosynthetic host in which the DEBS KS1 is inactivated via an active site C129A mutation, generating KS1°. The synthetic diketide effectively mimics the ACP1 bound diketide and is incorporated at KS2, where it is subsequently elongated to the macrolactone. Using the chemobiosynthetic method, 15-methyl-6dEB (**11**) was produced by “feeding” propyl-diketide N-acetylcysteine thioester (propyl-SNac) to a strain of *Streptomyces* sp. containing DEBS KS1° (28).

The chain length of the polyketide can be modified by genetic repositioning of the thioesterase domain (29), (30). Moving the TE domain from the 3'-end of module 6 to the 3'-end of module 2 by constructing an in-frame protein fusion (DEBS1 +TE) resulted in the production of the truncated triketide lactone product, **12**. Likewise, tetraketide lactones can be produced by co-expressing DEBS1 and DEBS modular 3 +TE (31). In addition to its use for producing triketide analogs *in vivo*,

DEBS 1 +TE has also functioned as a valuable model system for the study of PKS enzymology *in vitro* (32, 33).

A limitation of employing a genetic approach to generate “unnatural” polyketide diversity is that not all genetic changes are tolerated, and thus not all envisioned or desired chemical modifications are achievable. Moreover, the titers of polyketide analogs are often lower than those of the parent compound. Part of the problem is surely due to constraints imposed by the catalytic mechanisms of these massive multi-enzyme systems, about which we currently know little. For example, the tolerance of downstream activities toward unfamiliar intermediates with differences in chain length, methyl branching, substituent stereochemistry, beta-keto oxidation level and stereochemistry is a crucial issue that we are only in the early stages of understanding. How do we pick the boundaries of a domain or module substitution, and how will the substitution affect protein structure and stability? Clearly, as our knowledge of PKS enzymology increases so will our ability to more rationally design polyketide analogs and more comprehensive polyketide libraries. With these limitations and caveats in mind, I will now describe the application of genetic engineering of the DEBS PKS for the production of combinatorial polyketide libraries.

6. LIBRARIES OF POLYKETIDES

McDaniel *et al* (25) described the construction of a combinatorial library of 6dEB molecules, prepared by introducing targeted changes in DEBS modules 2, 5, and 6. Productive changes in individual modules were combined on a single vector to generate novel analogs with substitutions in up to three different positions in 6dEB. DEBS AT2 was replaced with rapAT2; DEBS KR modules 2, 5, 6 were replaced with (a) rapDH4/KR4 or (b) rapDH1/ER1/KR1; DEBS KR domains were deleted and inactivated by substitution of an 18-amino acid linker in place of the KR domain. Figure 6 shows examples of 6dEB analogs produced through various combinations of mutations in DEBS. Note that the polyketide titers for the multiple mutants were quite low (0.1- 0.2 mg/L), which the authors attributed to inefficient processing of the analog intermediates by downstream DEBS modules.

The combinatorial potential of the DEBS system was further explored by Xue *et al* (34), who generated a library of 6dEB analogs by expressing mutant DEBS genes from three compatible *Streptomyces* spp. vectors carrying independently selectable antibiotic resistance markers. The power of this approach stems from the ability to combine single mutations by simple transformation, obviating the need to perform a separate genetic construction for each multiple mutant prepared. For example, the combinatorial potential of the DEBS system arising from the permutation of the major combinatorial elements is 262,144 polyketides (2 different ATs x 4 different beta-keto modifying activities per module = 8 combinations per module; 8 x 8 per protein; 64 x 64 x 64 = 262,144 per DEBS). To generate such a library by the multiple plasmid approach requires the construction of only 64 x 3 = 192 different plasmids carrying single mutations (i.e. the number of theoretical

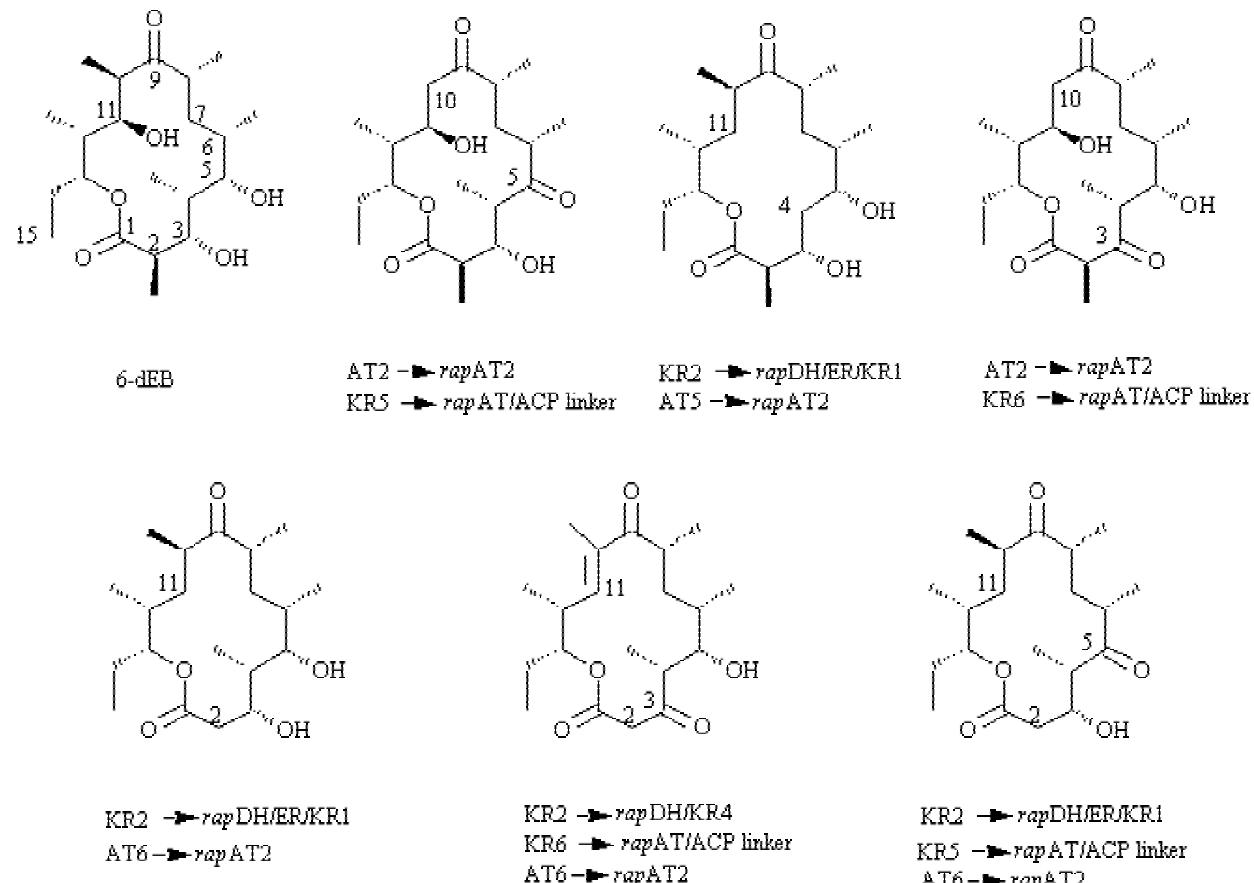


Figure 6. Some representatives in a combinatorial library of 6dEB analogs generated from the combination of single DEBS mutants.

polyketides produced is the product of the number of individual constructions for each DEBS gene). In contrast, the same number of genetic constructions using the single vector combinatorial approach described by McDaniel *et al.* (25) would yield only 192 different polyketides (i.e. the sum of the individual constructions).

Xue *et al* demonstrated the utility of the multiplasmid approach by transforming a *Streptomyces* sp. host (23) with eight variants of DEBS3 (*eryAIII*) on an integrating vector. The resulting strains were co-transformed with four DEBS1 variants (*eryAII*) and 2 DEBS2 variants (*eryAII*) on replicating plasmids. From 14 vectors constructed, 64 transformants were generated. Of the 64 strains tested, 46 produced detectable polyketides, 28 of which had been previously prepared using the single plasmid approach(25), and 15 represented novel structures. The versatility of the system was further demonstrated by combining vectors carrying DEBS2,3 (*eryAII,III*) mutations with the DEBS1 KS^1 system (28) illustrated in figure 4. The vector containing DEBS 1 (KS^1) was co-transformed with one DEBS 2 (*eryAII*) mutant and seven DEBS 3 (*eryAIII*) mutants to generate 16 transformants. These strains were grown in media supplemented with the diketide analog, propyl-SNAC, and 13 novel 15-methyl-6dEB analogs were produced (permutations of structure

11). The approach outlined by Xue *et al* should be applicable to other modular PKSs systems and could possibly be extended to create diverse polyketide libraries from the combinatorial combination of heterologous and hybrid PKSs.

7. PRODUCTION OF POLYKETIDES IN NON-POLYKETIDE PRODUCING HOSTS (*E. COLI* AND *S. CEREVIAE*)

The polyketide production hosts discussed thus far have been either native producers (e.g. the *Sac. erythraea* erythromycin producer) or related heterologous hosts of the genus *Streptomyces* (eg *S. coelicolor* CH999 or *S. lividans* K4-114). Although these hosts have served adequately, they embody several characteristics that could ultimately limit their utility as hosts for PKS engineering and polyketide library construction: they grow slowly, are poorly transformed by DNA, and are often intractable toward genetic manipulation. Recently, two groups have explored the use of *E. coli* and *S. cerevisiae* as polyketide production hosts (35, 36). Due to their well-established genetic systems and fermentation properties, *E. coli* and yeast may offer significant advantages over the typical native polyketide producers. A major drawback, however, is the fact that *E. coli* and *S. cerevisiae* did not evolve to

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produce polyketides and therefore do not possess many of the biosynthetic pathways and ancillary activities necessary for polyketide biosynthesis. Hence, in order to convert these hosts into polyketide producers, requisite pathways and activities must be introduced.

Kealey *et al* (35) first demonstrated that *E. coli* and *S. cerevisiae* could be engineered to produce polyketides. The gene for the fungal PKS 6-MSAS (37, 38) was cloned onto *E. coli* and *S. cerevisiae* expression vectors and transformed into *E. coli* and yeast. Expression of full length 6-MSAS (~ 190 kDa) was confirmed by SDS-PAGE, but the polyketide, 6-MSA, was not produced. When the 6-MSAS was co-expressed with Sfp, a p-pant transferase (Fig 3) of broad specificity from *Bacillus subtilis* (16, 39), yeast produced and excreted copious amounts of the polyketide product (up to 2 g 6-MSA per L of culture in unoptimized shake flask fermentations). Production of 6-MSA in *E. coli* (75 mg/L in shake flasks) required co-expression of Sfp, inclusion of glycerol in the growth medium to boost the malonyl-CoA pool, and low temperature (30°C) expression. The acetyl-CoA and malonyl-CoA substrates used for 6-MSA biosynthesis are also used for fatty acid biosynthesis and therefore are present in *E. coli* and yeast. Evidently, the available malonyl-CoA pool is sufficiently high in yeast to support high level production of 6-MSA, whereas in *E. coli* the malonyl-CoA levels are not sufficient unless the media is altered to increase the malonyl-CoA pool size. This work defined the minimal requirements for polyketide production in heterologous non-polyketide producing hosts: expression of an active, phosphopantetheinylated PKS, an adequate supply of appropriate acyl-CoA precursors, and production of a polyketide that is not toxic to its host.

Pfeifer *et al* have recently succeeded in producing the complex polyketide, 6dEB (4), in *E. coli* (36). The acyl-CoA substrates used for 6dEB synthesis are propionyl-CoA and (2S)-methylmalonyl-CoA (Figure 3), and these substrates are not present in *E. coli* (40). To construct the *E. coli* production system, Pfeifer *et al* integrated the *sfp* gene into the *E. coli* chromosome at the propionate utilizing loci, the *prp* operon, and placed a T7 promoter upstream of *prpE*, the gene for propionyl-CoA ligase, which converts propionate to propionyl-CoA. The DEBS1 gene (*eryA1*) and the genes for *S. coelicolor* propionyl-CoA carboxylase (PCC) (41) were introduced on a pET vector with a kanamycin selectable marker. The PCC enzyme catalyzes the biotin-dependent carboxylation of propionyl-CoA to (2S)-methylmalonyl-CoA. The DEBS 2,3 genes (*eryAII*, *eryAIII*) were introduced on a second pET vector with ampicillin resistance. All introduced genes were under the control of T7 promoters. Following transformation, the T7 promoters were induced and the cultures were grown at 22°C to promote expression and proper folding of the DEBS proteins. After 75 hours of growth, the cell free media was sampled and shown to contain ~20 mg of 6dEB per L of media. This demonstration of complex polyketide production in *E. coli* is a crucial first step towards the potential future development of *E. coli* as a general host for polyketide production and library construction.

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