A functional chimeric modular polyketide synthase generated via domain replacement

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Background: Modular polyketide synthases (PKSs), such as 6-deoxyerythronolide B synthase (DEBS), are large multifunctional enzymes that catalyze the biosynthesis of structurally complex and medically important natural products. Active sites within these assemblies are organized into 'modules', such that each module catalyzes the stereospecific addition of a new monomer onto a growing polyketide chain and also sets the reduction level of the β-carbon atom of the resulting intermediate. The core of each module is made up of a 'reductive segment', which includes all, some, or none of a set of ketoreductase (KR), dehydratase, and enoylreductase domains, in addition to a large interdomain region which lacks overt function but may contribute to structural stability and inter-domain dynamics within modules. The highly conserved organization of reductive segments within modules suggests that they might be able to function in unnatural contexts to generate novel organic molecules.

Results: To investigate domain substitution as a method for altering PKS function, a chimeric enzyme was engineered. Using a bimodular derivative of DEBS (DEBS1+TE), the reductive segment of module 2, which includes a functional KR, was replaced with its homolog from module 3 of DEBS, which contains a (naturally occurring) nonfunctional KR. A recombinant strain expressing the chimeric gene produced the predicted ketolactone with a yield (35 %) comparable to that of a control strain in which the KR2 domain was retained but mutationally inactivated.

Conclusions: These results demonstrate considerable structural tolerance within an important segment found in virtually every PKS module. The domain boundaries defined here could be exploited for the construction of numerous loss-of-function and possibly even gain-of-function mutants within this remarkable family of multifunctional enzymes.

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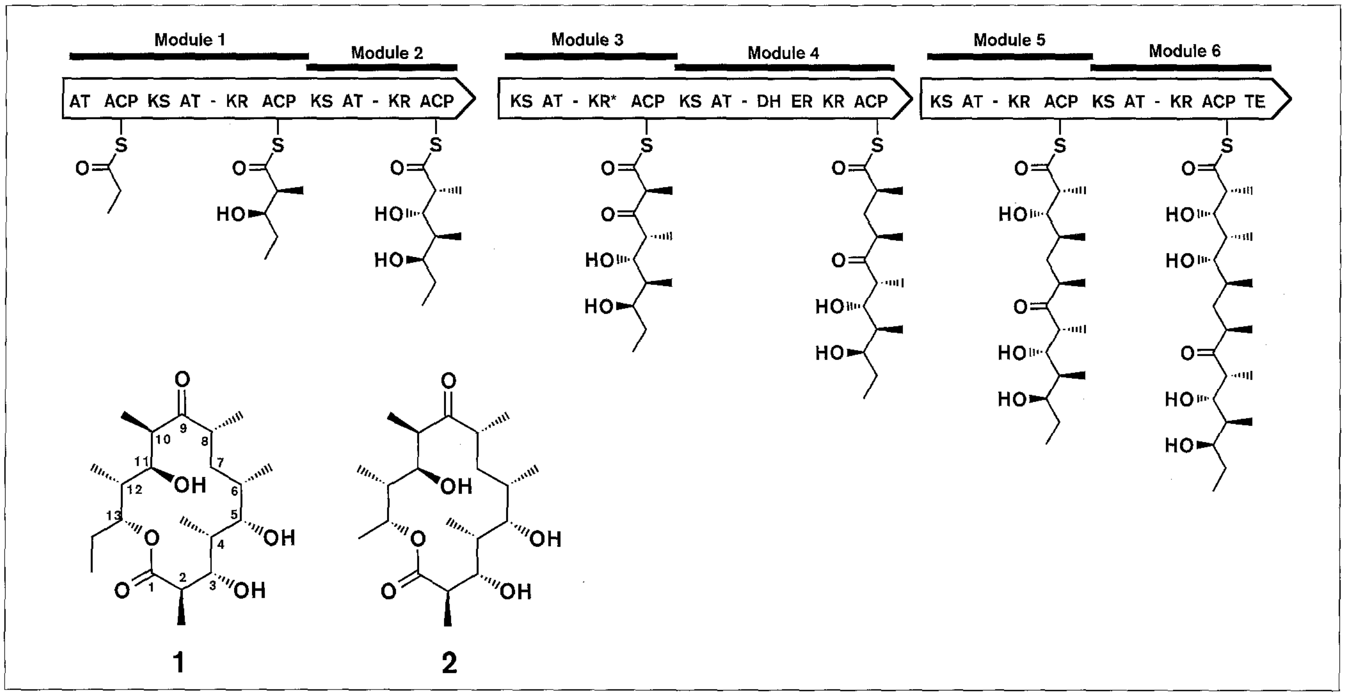
Introduction

Polyketides are a diverse class of natural products which are important as antibiotics, anti-cancer drugs, and other pharmacologically active agents. Biosynthesis of these products is achieved by the repetitive condensation of simple monomers in a process which closely parallels fatty acid biosynthesis [1,2]. In contrast to the fatty acid synthases (FASs), polyketide synthases (PKSs) generate complex products by using different carboxylic acid monomers and varying the degree of β-carbonyl reduction after each condensation. PKSs also control the stereochemistry of chiral carbon centers and the regiochemistry of cyclization(s) after chain synthesis [3,4]. Although the possible combinations of these functions could result in the formation of a vast and diverse collection of compounds, naturally occurring PKSs typically produce a single product, or small group of related products. The possibility of altering PKS function to obtain large numbers of novel compounds has generated considerable interest in the manipulation of PKSs [5,6].

Although this stepwise condensation of building blocks to form oligomers of defined 'sequence' is conceptually similar to the well-studied biosynthesis of nucleic acids and proteins, the synthesis of polyketides is not directed by a modular template. Instead, in at least some cases, the active sites within multifunctional PKSs themselves appear to be modularized. The 6-deoxyerythronolide B synthase (DEBS) from *Saccharopolyspora erythraea* catalyzes the biosynthesis of 6-deoxyerythronolide B (6dEB) (1), the polyketide aglycone of the antibiotic erythromycin [7,8] (Fig. 1). Genetic analysis of this modular PKS revealed three large proteins (each with a molecular weight of >300 kDa) that contain a repetitive series of catalytic centers homologous to FAS active sites: β-ketoacyl-acyl carrier protein synthase (KS), acyltransferase (AT), dehydratase (DH), enoyl reductase

Figure 1



Genetic model for the 6-deoxyerythronolide B synthase (DEBS). Each module includes an acyltransferase (AT), a ketosynthase (KS) and an acyl carrier protein (ACP) domain as well as a reductive segment. The reductive segment minimally consists of an interdomain region (–) and a ketoreductase (KR) domain. Dehydratase (DH) and enoyl reductase (ER) domains are present in the reductive segment of module 4. The KR domain from module 3 (KR*) lacks reductase activity. The thioesterase (TE) domain, present at the carboxy-terminus of module 6, catalyzes lactone formation. In *S. coelicolor*, DEBS produces 6-deoxyerythronolide B (**1**) as well as 8,8a-deoxyoleandolide (**2**) [10].

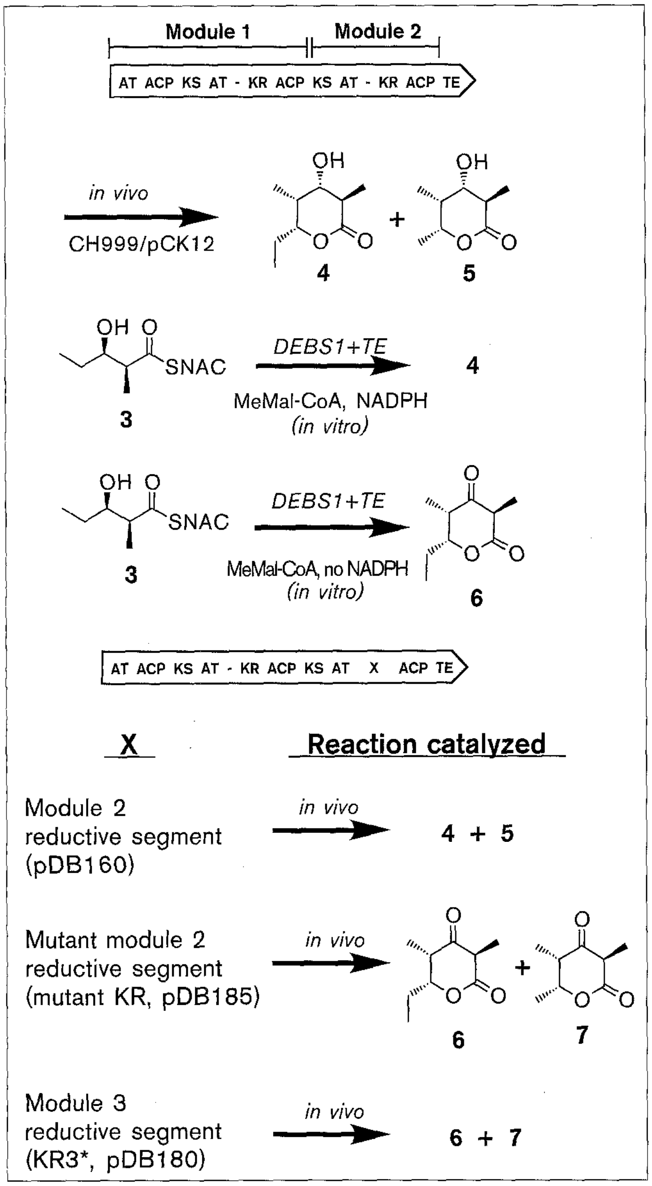
(ER), β-ketoreductase (KR), acyl carrier domain (ACP), and thioesterase (TE). Active sites are organized into groups called 'modules', such that each module catalyzes one cycle of condensation and β-ketoreduction in 6dEB biosynthesis. Thus, modular PKSs are strikingly similar to avian and mammalian FASs with respect to both the identity of active sites as well as the linear arrangement of these domains within each module. However, two key differences exist between modular PKSs and higher eukaryotic FASs. First, whereas a FAS contains a full set of reductive domains (KR, DH, ER), each PKS module contains only the subset of reductive domains required for a particular condensation cycle. Second, whereas a single set of active sites is used iteratively in FASs, the active sites in a PKS module participate only once during the synthesis of a polyketide molecule. This remarkable organization of DEBS and other modular PKSs suggests that it should be feasible to engineer these multienzyme systems to produce novel products. While this notion has been validated to some extent by deletion and site-directed mutagenesis [8–14], its ultimate potential depends upon the feasibility of constructing functional PKSs by domain (or module) replacement. Here we report a first step in this direction.

In previous studies we have shown that a bimodular derivative of DEBS (designated DEBS1+TE [12,14], Fig. 2) can accept an *N*-acetylcysteamine thioester of the diketide intermediate (compound 3, Fig. 2) and convert it into the expected triketide lactone 4 in the presence of NADPH [15]. Furthermore, in the absence of NADPH in the reaction mixture, the same substrate can also be turned over by DEBS1+TE into the expected ketolactone 6 [16]. These *in vitro* results laid the groundwork for the experiments described here, which are aimed at establishing the feasibility of constructing hybrid modules. Specifically, we show here that it is possible to synthesize compound 6 *in vivo* through domain replacement within DEBS1+TE.

Results

DEBS1+TE is an engineered derivative of DEBS, generated by fusion of the DEBS1 protein, which contains the first two modules of DEBS, to the thioesterase domain from the DEBS3 protein. The resulting protein catalyzes the condensation of one equivalent of propionyl-CoA with two equivalents of methylmalonyl-CoA to form the triketide lactone 4. Since loss-of-function mutants within reductase domains have been engineered previously via directed mutagenesis

Figure 2



DEBS1+TE, an engineered bimodular PKS, produces the triketide lactones **4** and **5** *in vivo*. The *N*-acetylcysteamine thioester of the diketide intermediate (**3**) is also a substrate for DEBS1+TE and is incorporated into **4**. Incubation of DEBS1+TE with **3** and methylmalonyl-CoA in the absence of NADPH affords ketolactone **6**. Substitutions to the reductive segment from module 2 were engineered and the compounds produced *in vivo* were isolated. Introduction of the wild-type (module 2) segment resulted in the production of lactones **3** and **4**. Ketolactones **6** and **7** were produced by introduction of a module 2 reductive segment bearing a nonfunctional KR domain. Substitution of the reductive segment from DEBS module 3 (KR3*) also afforded ketolactones **6** and **7**.

[8,9], a similar approach was first taken to test the feasibility of synthesizing 6 *in vivo*. A recombinant strain of *S. coelicolor*, CH999/pDB185, was constructed that expresses a mutant DEBS1+TE gene in which the second ketoreductase domain (KR2) was inactivated by random PCR mutagenesis (Fig. 3). CH999/pDB185 produces the predicted triketide ketolactone 6, which differs from 3 in the degree of reduction at C-3 (Fig. 2). In addition to the propionate primer-derived ketolactone 6, the related acetate primer-derived compound 7 was also obtained. 6 and 7 were produced in yields of 20 mg l^{-1} and 8 mg l^{-1}, respectively. These values are comparable to the yields of 4 and 5 from CH999/pDB160.

To test the feasibility of generating functional chimeric modules, the reductive segment of module 2, which includes a large interdomain linker as well as a KR domain, was replaced with the corresponding region from module 3 (Fig. 3). In its natural context, KR3 leaves the ketone at C-9 of 6-dEB unreduced. Therefore the chimeric PKS thus generated was expected to produce ketolactone 6, in which C-3 remains unreduced. Indeed, *S. coelicolor* CH999/pDB180 was found to produce ~7 mg l^{-1} ketolactone 6. The related ketolactone 7 was also observed in smaller quantities (<2 mg l^{-1}). No production of the reduced triketide lactone 4 could be detected by thin layer chromatography.

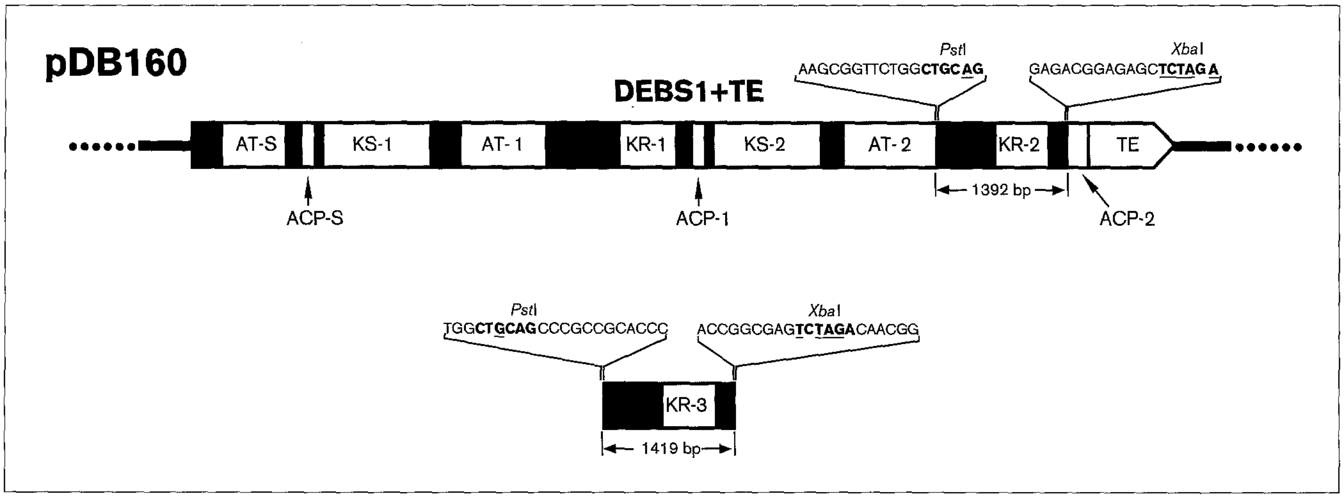
Discussion

The modular structure of DEBS provides a compelling model for the evolution of modular PKSs via domain shuffling, and raises the exciting possibility of generating new products in the laboratory through manipulation of protein domains. Previous experiments had demonstrated that unreduced intermediates generated by DEBS mutants containing inactive reductase domains would be faithfully processed to form the corresponding unreduced analogs of 6-deoxyerythronolide B [8,9]. The substitution of the reductive segment from module 3 for its homolog from module 2 in DEBS1+TE illustrates an alternative strategy for the construction of loss-of-function mutants in modular PKSs. It also demonstrates considerable structural tolerance within an important region found in virtually every PKS module. Based on comparisons with higher eukaryotic FASs, it was earlier speculated that the large interdomain linker between the AT and the KR has a crucial structural role and facilitates the movement of catalytic domains during polyketide synthesis [17]. Our results suggest that either this region is unimportant for PKS assembly and activity, or it lacks specificity and can therefore be substituted with equivalent interdomain linkers from other modules.

Significance

We have constructed a chimeric polyketide synthase by exchange of one protein domain for a homologous domain and demonstrated that this chimera produces the predicted polyketide product. The reductase region from DEBS module 3 was introduced in place of the corresponding region of module 2 in DEBS1+TE, an engineered bimodular PKS. This region consists of the

Figure 3



Construction of module swap mutants. The domains of the DEBS1+TE gene are shown approximately to scale. DNA segments coding for 'linker' segments of unknown function are shaded. PCR mutagenesis was used to generate plasmid pDB160, a derivative of pCK12 [12] which contains restriction sites bordering the reductive segment of module 2. A *Pst*I site was engineered immediately downstream of the AT-2 domain, and an *Xba*I site was engineered immediately upstream of the ACP-2 domain. PCR primers are indicated with the restriction sites in bold face and mutations underlined. pDB185 was generated by amplifying the *Pst*I/*Xba*I fragment by random PCR mutagenesis and recloning the fragment into pDB160. The reductive segment from module 3 was obtained by PCR amplification using primers which introduced *Pst*I and *Xba*I sites at homologous positions. This segment was then cloned into pDB160 to produce pDB180.

ketoreductase domain in addition to a linker region of unknown function. The replacement of this linker region is a crucial prerequisite for gain-of-function mutagenesis, since in modules containing a full complement of reductive domains (e.g. module 4 of DEBS, Fig. 1) this interdomain linker lies nested within catalytic domains. Likewise, the presence of KR3 in DEBS module 3, despite its lack of function, suggests it may be important in the structural arrangement of enzyme domains. Our results suggest that KR3 is also able to fulfill this role within module 2. In conclusion, the domain boundaries defined here could be exploited for the construction of not just other loss-of-function mutants, but possibly even gain-of-function mutants. This bodes well for the future of combinatorial biosynthesis using this remarkable family of multifunctional enzymes.

Materials and methods

Bacterial strains and plasmids

Streptomyces coelicolor CH999 was used as a host for the expression of all recombinant PKS genes. All DNA manipulations were performed in *Escherichia coli* MC1061. Plasmids were passaged through *E. coli* ET12567 (*dam, dcm hsdS* Cm^r) to generate unmethylated DNA prior to transformation into *S. coelicolor*. Plasmid pDB111 is a derivative of pCK12 [12] which carries the DEBS1+TE gene. A Kan^r cassette (Pharmacia GenBlock) was inserted into the unique *Hin*dIII site of pCK12, giving rise to pDB111. Plasmid pDB160 is a derivative of pDB111 in which a *Pst*I site has been engineered immediately downstream of the AT-2 domain (by mutating the sequence AAGCGGTTCTGGCTGCTG into AAGCGGTTCTGGCTGCAG), and an *Xba*I site has been engineered immediately upstream of the ACP-2 domain (by mutating the sequence GAGACGGAGAGCCTGCGC into GAGACGGAGAGCTCTAGA); both restriction sites result in the alteration of a single amino acid residue. Mutations were engineered by standard PCR mutagenesis procedures. The lack of any phenotypic alterations in the mutant DEBS1+TE as a result of these engineered sites was confirmed by *in vivo* and *in vitro* analysis of CH999/pDB160. Both product and protein levels in this strain were indistinguishable from those of CH999/pDB111 or CH999/pCK12. pDB180 was generated by replacing the *Pst*I/*Xba*I fragment of pDB160, which encodes the reductive segment of module 2, with its homolog from module 3. The DNA encoding the reductive segment of module 3 was engineered as a *Pst*I/*Xba*I cassette based on a PILEUP analysis of the amino acid sequences of the DEBS modules (performed using University of Wisconsin, GCG software). To generate pDB185, the *Pst*I/*Xba*I fragment of pDB160 was amplified by random PCR mutagenesis [18] using Taq polymerase for 25 cycles. Two independently generated PCR cassettes were tested by recloning into pDB160 and transforming the resulting plasmids into CH999. One of the clones was found to produce compounds **6** and **7**; this plasmid was designated pDB185. The mutation(s) in the mutant KR2 domain of pDB185 were not characterized via DNA sequencing; however, since a full-length protein has been identified in extracts from this strain, a frameshift or nonsense mutation can be ruled out.

Production and purification of polyketides

Lawns of *S. coelicolor* strains CH999/pDB111, CH999/pDB160, CH999/pDB180, and CH999/pDB185 were grown on R2YE agar plates containing 0.3 mg ml^{-1} sodium propionate. After seven days of incubation at 30 °C, the agar media (300 ml) were homogenized and extracted three times with 500 ml ethyl acetate. The solvent was dried over magnesium sulfate and concentrated. Silica gel chromatography (gradient of 15 to 25 % ethyl acetate in hexane) of the extracts from CH999/pDB180 and CH999/pDB185 afforded two products, **6** and **7**, not found in extracts of the control strains CH999/pDB111 or CH999/pDB160 (both of which produced triketide lactone **4**).

NMR spectroscopy and structural assigments

NMR spectra were recorded on a Varian XL-400. Products **6** and **7** were spectroscopically identical to their synthetic counterparts. The synthesis of the C_9-ketolactone **6** from **4** has been reported previously [16]. To prepare authentic **7**, the C_8 triketide lactone **5**, prepared as previously described [19], was oxidized with methyl(trifluoromethyl)dioxirane [20] as described for the analogous preparation of **6**. [16]. As with **6**, **7** is readily interconverted between the ketolactone and the corresponding enol-lactone tautomer. **7**: $R_f = 0.51$ (enol-lactone, non-uv active, stained yellow by vanillin) and 0.26 (uv active) (1:1 EtOAc/hexanes); ^{1}H NMR ($CDCl_3$): δ 1.12 (d, $J = 7.4$ Hz, 3H, C(4)-CH_3), 1.35 (d, $J = 6.7$ Hz, 3H, C(2)-CH_3), 1.41 (d, $J = 6.5$ Hz, 3H, H-6), 2.59 (dq, $J = 3.1$, 7.6 Hz, 1H, H-4), 3.59 (q, $J = 6.7$ Hz, 1H, H-2), 4.96 (dq, $J = 3.1$, 6.5 Hz, 1H, H-5). ^{13}C NMR: δ 8.4 (C-2 CH_3), 9.5 (C-4-CH_3), 16.7 (C-6), 45.6 (C-4), 50.2 (C-2), 73.1 (C-5), 170.1 (C-1), 205.2 (C-3). HRMS (EI) M^+ calc'd for $C_8H_{12}O_3$: 156.0786, found: 156.0790.

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