and BL can be calculated to any pixel size, we can apply our subpixel algorithm to the bead image using for a PSF, BEAD*BL. The result is then PSF*PIXEL calculated with finer sampling than the original pixel size and with the elimination of error because of the use of a large bead instead of a point source. α = 0.005 and 15 iterations were used. The beads used are manufactured (Molecular Probes) with the dye distributed uniformly throughout their volume.

27. NRK1 cells were fixed and then prepared for immunocytochemistry; microtubules were stained with mouse monoclonal antibodies to β-tubulin (Amersham) and rhodamine-labeled goat antibodies to mouse immunoglobulin (TAGO).
28. The cell was injected with rhodamine-X–labeled hexokinase (dye:protein, ~0.8), which exhibited normal activity, to a final concentration of 0.09 mg/ml at 0 min.
29. We thank D. Fishkein for NRK-1 cells labeled with antibodies to tubulin, D. Bowman and R. Tuft for hardware development, and J. Carmichael for photographic assistance. Supported by grants from NSF (W.A.C., F.S.F.); NIH (W.A.C., F.S.F., R.M.L.); American Heart Association, Massachusetts affiliate (E.D.W.M.); Medical Foundation of Boston (R.M.L.); North Atlantic Treaty Organization (G.I., F.S.F.); and American Diabetes Association (R.M.L.).

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Repositioning of a Domain in a Modular Polyketide Synthase to Promote Specific Chain Cleavage

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Macrocyclic polyketides exhibit an impressive range of medically useful activities, and there is great interest in manipulating the genes that govern their synthesis. The 6-deoxyerythronolide B synthase (DEBS) of *Saccharopolyspora erythraea*, which synthesizes the aglycone core of the antibiotic erythromycin A, has been modified by repositioning of a chain-terminating cyclase domain to the carboxyl-terminus of DEBS1, the multienzyme that catalyzes the first two rounds of polyketide chain extension. The resulting mutant markedly accelerates formation of the predicted triketide lactone, compared to a control in which the repositioned domain is inactive. Repositioning of the cyclase should be generally useful for redirecting polyketide synthesis to obtain polyketides of specified chain lengths.

Complex polyketides are natural products, found predominantly in *Streptomyces* and related filamentous bacteria, that exhibit an impressive range of antibiotic, anticancer, antiparasite, and immunosuppressant activities. Despite their apparent structural diversity, they are synthesized by a common pathway in which units derived from acetate or propionate are condensed onto the growing chain, in a process resembling fatty acid biosynthesis (*1*). The intermediates remain bound to the polyketide synthase (PKS) during multiple cycles of chain extension and (to variable extent) reduction of the β-keto group formed in each condensation (Fig. 1). The structural variation between naturally occurring polyketides arises largely from the way each PKS controls the number and type of units added, and the extent and stereochemistry of reduction at each cycle. In addition, the product of the PKS is frequently acted upon by regiospecific glycosylases, methyltransferases, and oxidative enzymes, to produce still greater diversity.

Sequencing of the structural genes for PKSs has revealed fundamental differences in the organization of the synthases producing aromatic polyketides and complex, reduced polyketides. For aromatic polyketides, the PKS consists of a single set of enzyme activities (*2*), housed either in a single polypeptide chain (type I) or on separate polypeptides (type II), that act in every cycle. In contrast, complex polyketides are synthesized on multifunctional PKSs that contain a distinct active site for every catalyzed step in chain synthesis (*3*–*5*). For example, the clinically important antibiotic erythromycin A is derived from propionyl–coenzyme A (propionyl-CoA) and six molecules of (2*S*)-methylmalonyl-CoA (*6*) through the sequential action of six such sets of enzymes housed in the three multienzyme polypeptides (*7*) of 6-deoxyerythronolide B synthase (DEBS) (Fig. 1).

The discovery of the organization of such "modular" PKSs has prompted attempts, with mixed success, to delete the active sites of individual domains in order to produce an altered product of predicted structure. Deletion of the entire ketoreductase (KR) domain from cycle 5 of DEBS (*4*) and mutation of active site residues in the enoylreductase (ER) active site from cycle 4

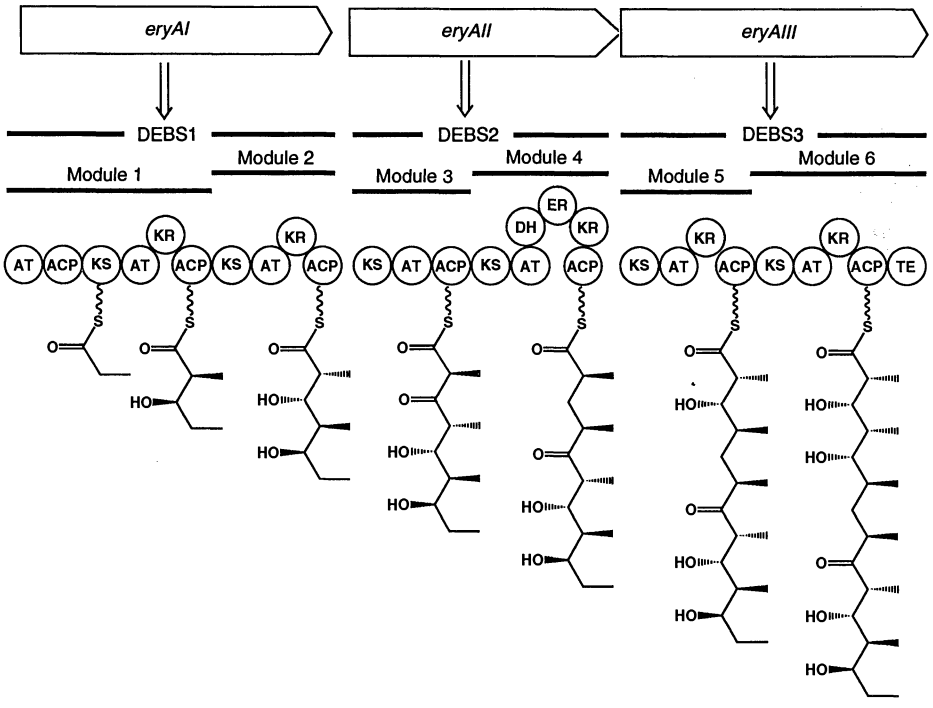

Fig. 1. The domain organization of 6-deoxyerythronolide B synthase (DEBS) [adapted from (*9*) with permission]. Each protein module contains an acyl carrier protein (ACP), an acyltransferase (AT), and a β-ketoacyl-ACP synthase (KS), and some modules also contain activities for reduction [β-ketoacyl-ACP reductase (KR), dehydratase (DH), and enoyl reductase (ER)]. In the mutants described in this report, the thioesterase-cyclase (TE) at the end of module 6, which normally cyclizes the full-length chain to 6-deoxyerythronolide B, is fused to the COOH-terminus of DEBS1.

of DEBS (*8*) in each case gave rise to the expected altered product. In contrast, mutation of a single active site histidine residue in the dehydratase domain from cycle 4 of DEBS abolished all macrolide synthesis, without detectable accumulation of any new product (*9*). Mutation of the equivalent histidine in fatty acid synthase also leads to loss of activity (*10*).

We now report the successful repositioning of a catalytically active domain into a new context within a modular PKS multienzyme. Our strategy should accelerate both detailed enzymological studies and also the rational engineering of PKS multienzymes for synthesis of artificial polyketides. We have exemplified our approach using the DEBS of *S. erythraea* (Fig. 1). The COOH-terminal domain of the multienzyme component DEBS3 appears to act as a chain-terminating cyclase (*3, 11*). The cyclase is not absolutely required for release of the triketide chain from DEBS1, when the multienzyme is heterologously expressed at high level in *Streptomyces coelicolor* (*12*). However, it would be preferable to have an assured, specific method of directing efficient release of a polyketide chain from the synthase in vivo, rather than relying on the intrinsic chemical reactivity of the bound polyketide intermediate or on adventitious hydrolysis by cellular hydrolases. The DEBS3 cyclase, together with the adjacent acyl carrier protein (ACP) domain, has been expressed in *Escherichia coli* as a stable di-domain that reacts appropriately with an active site–directed inhibitor or acyltransferases (*13*), indicating that its structural integrity is not dependent on interactions with the rest of the DEBS. This prompted us to attempt to alter the chain length of the polyketide product of DEBS by repositioning this domain.

We chose to fuse the cyclase domain to the COOH-terminus of DEBS1, where it would be correctly positioned to intercept the growing polyketide chain after only two cycles of chain extension. The genetic strategy (Fig. 2) used standard protocols to permit in vivo recombination between a mutagenic plasmid and the chromosomal copy of the *eryA* genes in *S. erythraea*. A single recombination event gave the disrupted strain TED8, whereas subsequent selection for a second recombination event produced strain TER43 (Fig. 2), in which the structural genes for DEBS2 and (most of) DEBS3 are deleted (*14*).

When grown on a defined sucrose-succinate medium (*7*), neither *S. erythraea* TED8 nor TER43 produced detectable amounts of macrolide (Fig. 3). Analysis of ethyl acetate extracts of the crude culture broth by ^{1}H nuclear magnetic resonance (NMR) showed the presence of essentially a single compound whose structure was confirmed, after rigorous purification, as the expected triketide lactone **1** (Fig. 3). No trace was found of erythromycins or of any biosynthetic intermediate more advanced than the triketide (*15*). The two ACP domains that contribute to the hybrid ACP domain normally bind residues with opposite configurations at the two centers adjacent to the carbonyl group of their normal substrates. The correct functioning of the hybrid domain, which contains the NH_2-terminal half of ACP2 fused to the COOH-terminal half of ACP6, shows that ACP domains are unlikely to exert decisive control of the configuration at newly formed chiral centers in the growing polyketide chain. Since the sequence similarity between the ACPs of DEBS1 and DEBS3 is no greater than between the COOH-terminal ACP of DEBS3 and the ACPs of other modular PKSs, the relocation of a chain-terminating activity is likely to be a general way of truncating polyketide chain synthesis at a chosen point on these multienzymes.

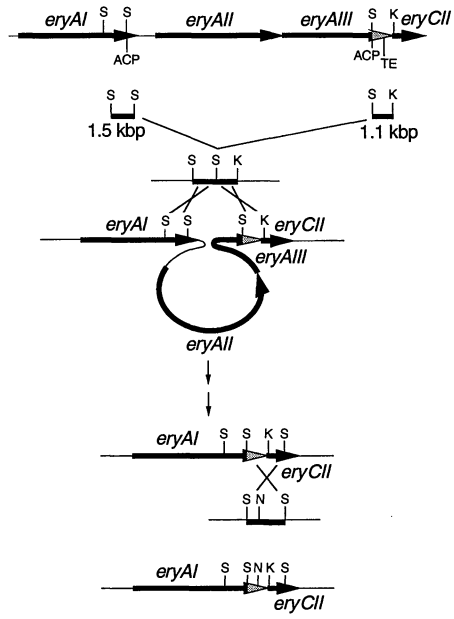

Fig. 2. Genetic strategy for the construction of triketide lactone synthase in *S. erythraea*. The 3′ end of the *eryAIII* gene, containing part of the coding sequences for the ACP from module 6 (ACP6) and the thioesterase-cyclase, was introduced into the 3′ end of the *eryAI* gene. A 1.5-kbp Sac I fragment containing domain KR2 and the ACP3 domain (Fig. 1) (up to the proposed attachment site for 4′-phosphopantetheine) was ligated to a 1.1-kbp Sac I–Kpn I fragment containing the region from the 4′-phosphopantetheine attachment site of ACP6 to the stop codon of *eryAIII*. The resulting construct was subcloned into a bifunctional vector made by ligating together pIJ702 and pUC18 (*19*), and this plasmid (pTED101) was used to transform *S. erythraea* protoplasts (*19*). Recombinants were selected by plating on thiostrepton, and restriction mapping was used to identify those in which the plasmid had undergone recombination with the chromosomal *eryAI* gene. One such transformant, TED8, was used in further experiments. After subculturing four times in M1-102 liquid medium (*20*) in the absence of thiostrepton, protoplasts were made from TED8 and after regeneration were checked for loss of thiostrepton resistance. Restriction mapping was used to identify recombinants such as TER43, which had undergone loss of the *eryAII* and *eryAIII* genes by a further recombination event. To obtain a triketide lactone synthase containing an inactive thioesterase-cyclase domain, a plasmid containing a specifically mutagenized (*21*) 2.2-kbp Sac I fragment from the 3′-end of *eryAIII* was linearized with Bam HI and cloned into the Bgl II site of pIJ702. The resulting construct was used to transform *S. erythraea* TER43 to thiostrepton resistance. Restriction mapping was used to identify recombinants such as TED3029A, in which the inactive cyclase had replaced the wild-type domain in the triketide lactone synthase. S, Sac I; K, Kpn I; N, Nae I.

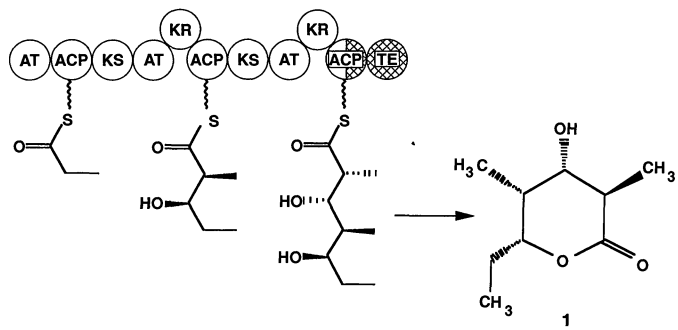

Fig. 3. Domain organization of the triketide lactone synthase. The hatched areas denotes the region derived from DEBS3. *S. erythraea* mutant TED8 was grown in sucrose-succinate medium (*7*) at 30°C. After 5 days, the broth was extracted with ethyl acetate. The extract was chromatographed twice on a silica gel column, eluted the first time with diethyl ether and the second time isocratically with 7:3 ether:hexane, to yield 89 mg of pure triketide lactone **1**, from a 10-liter fermentation. The product was characterized by infrared, high-resolution electrospray– and electron impact–mass spectrometry, and ^{1}H and ^{13}C NMR. Physical data were identical to those obtained for synthetic (3*S*,5*R*)-dihydroxy-(2*R*,4*R*)-dimethyl-*n*-heptanoic acid-δ-lactone (*22*).

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The efficient production of **1** by *S. erythraea* mutants TED8 and TER43 proves that the multienzyme components of modular PKSs are capable of functioning independently (*14*). In addition, production of **1** by these strains demonstrates that the attachment of an additional COOH-terminal domain has not perturbed the other enzyme activities in the DEBS1 multienzyme. We also wished to determine whether an active thioesterase domain was required for efficient chain termination and cyclization or whether the mere presence of an additional COOH-terminal domain would suffice to disrupt the normal chain transfer between DEBS1 and DEBS2 and hence promote adventitious cyclization. This was done by mutagenesis of the DNA from *eryAIII* encoding the cyclase domain, so that the active site serine residue, Ser3029 (DEBS3 numbering) (*3*, *12*), was altered to an alanine (Fig. 2) (*16*). The mutant domain was used to replace the wild-type cyclase domain in *S. erythraea* strain TER43, by recombination (Fig. 2), to create strain TEDS3029A. This strain produced, under comparable conditions of fermentation, less than 1% of the amount of triketide lactone accumulated by strain TED8, as judged by mass spectrometry (Fig. 3), providing evidence that the cyclase directly accelerates chain termination. On a molar basis, the amount of lactone (10 to 15 mg/liter) produced by the DEBS1 fused to active cyclase was only slightly less than the amount of erythromycin produced by the wild-type strain. In contrast, the unmodified DEBS1, even when very highly expressed in *S. coelicolor*, has been reported to produce only 1 to 3 mg/liter of the lactone, by unknown mechanisms (*12*). Cyclase-promoted cleavage is therefore the method of choice to redirect polyketide synthesis to the formation of chains of specified length.

We have previously shown that the NH_2-terminal acyltransferase-acyl carrier protein di-domain of DEBS1 also behaves as an autonomous functional unit after limited proteolysis under native conditions (*17*), but it remains to be determined whether this domain may also be relocated so as to initiate polyketide chain synthesis at a desired point within a modular PKS. Meanwhile, the recombinant DEBS1-cyclase functional unit is much smaller and more amenable to structure-function analysis than the entire PKS, and its study, either in *S. erythraea* or in heterologous systems (*11*, *18*), should provide further detailed insights into the chemistry and stereochemistry of polyketide chain growth.

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14. DEBS2 and DEBS3 were absent from strains TED8 and TER43, as judged by immunoblots of proteins in cell-free extracts.
15. Trace amounts of **1** have been previously isolated from the mother liquor of an *S. erythraea* mutant specifically deleted in DEBS3 (*4*).
16. A strain of *S. erythraea* bearing this single mutation in *eryAIII* is unable to synthesize any macrolides (G. A. Roberts, unpublished data).
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21. The TCC codon for serine-3029 in the active site of the cyclase was substituted by GCC (alanine). A second mutation was also introduced to convert the GGT codon for glycine at position 3027 to a GCC codon, a silent mutation that introduces an Nae I site as a marker for mutant identification. Two partly complementary oligonucleotides 5′-GGAGTGGCCGGCCACCACGAACGG-3′ and 5′-TGGTGGCCGGCCACGCCGCGGGGGCACT-3′ were used to create the mutations. Each was used in a separate polymerase chain reaction (PCR) with, as template, a 627–base pair Sma I fragment of *eryAIII* in pUC18 that contains codon 3029, and with the appropriate pUC sequencing primer as the second primer. The two PCR products were each cut with Sma I, cloned into pUC18, cut with Nae I, and ligated together. The Sma I insert from a 2.2-kbp Sac I DNA fragment containing the 3′ end of *eryAIII* and cloned into pUC18 was then exchanged with the mutant Sma I fragment, to provide a larger target for recombination (Fig. 2).
22. A. Cutter and J. Staunton, unpublished data.

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Crystal Structure of a Purple Acid Phosphatase Containing a Dinuclear Fe(III)-Zn(II) Active Site

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Kidney bean purple acid phosphatase (KBPAP) is an Fe(III)-Zn(II) metalloenzyme resembling the mammalian Fe(III)-Fe(II) purple acid phosphatases. The structure of the homodimeric 111-kilodalton KBPAP was determined at a resolution of 2.9 angstroms. The enzyme contains two domains in each subunit. The active site is located in the carboxyl-terminal domain at the carboxy end of two sandwiched βαβαβ motifs. The two metal ions are 3.1 angstroms apart and bridged monodentately by Asp^{164}. The iron is further coordinated by Tyr^{167}, His^{325}, and Asp^{135}, and the zinc by His^{286}, His^{323}, and Asn^{201}. The active-site structure is consistent with previous proposals regarding the mechanism of phosphate ester hydrolysis involving nucleophilic attack on the phosphate group by an Fe(III)-coordinated hydroxide ion.

Purple acid phosphatases (PAPs) catalyze the hydrolysis of activated phosphoric acid esters and anhydrides like adenosine triphosphate at a pH range from 4 to 7 (*1*). The characteristic purple color results from a tyrosine→Fe(III) charge transfer transition at ~560 nm. The intensively studied monomeric mammalian enzymes contain an antiferromagnetically coupled Fe(III)-Fe(II) center, whereas KBPAP is dimeric with an Fe(III)-Zn(II) center in both subunits. The physiological function of these enzymes has yet to be established. In addition to a hydrolytic function, a role in the activation of dioxygen by the two-metal center has been discussed at least for the mammalian PAPs (*2*, *3*). PAPs have also attracted considerable interest with respect to their active site structure in comparison to other diiron proteins that use a two-metal-ion mechanism in the activation and transport of dioxygen.

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