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Skipping in a Hybrid Polyketide Synthase: Evidence for ACP-to-ACP Chain Transfer

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Summary

A tetraketide synthase containing a loading module (LM), the extension modules erythromycin module 1, rapamycin module 2, and erythromycin module 2 (LM-Ery1-Rap2-Ery2-TE), when expressed in *Saccharopolyspora erythraea* strain JC2, produced as previously reported a mixture of tetraketide lactones (minor products) and triketide lactones (major products). Several alternative plausible mechanisms by which this "skipping" phenomenon might occur may be proposed. Site-directed mutagenesis of the ketosynthase (KS) and acylcarrier protein (ACP) domains in the interpolated module has shown that skipping within the hybrid PKS involves passage of the growing polyketide through the interpolated module, by direct ACP-to-ACP transfer of the polyketide chain.

Introduction

Complex polyketides are a large and structurally diverse class of natural products that include many compounds possessing antibiotic or other clinically useful properties. They are produced by stepwise chain assembly on a modular polyketide synthase (PKS). Such PKSs are giant multienzymes containing a different set or "module" of enzyme domains to accomplish each successive cycle of polyketide chain extension [1, 2]. The evident modularity of these systems, along with numerous reports that productive hybrid polyketide synthases may be engineered by swapping either one [3–6] or more [7–13] domains or modules between different natural PKSs, has generated increasing interest in the degree to which this modularity might be exploited to make large numbers of new, potentially valuable natural products. Another recent approach, which greatly extends the scope and potential for combinatorial engineering of polyketide synthases, is the insertion of heterologous modules into a preexisting PKS multienzyme assembly between two other extension modules [14]. However, the hybrid PKSs so far produced by this approach have been poor yielding compared to their parent synthases, largely because they continue to produce polyketides that are the result of chain extension and processing by the native PKS modules, the interpolated module of enzyme activities apparently being ignored. This phenomenon has been termed "skipping" [14].

Here, we report the results of experiments designed to investigate the molecular basis of skipping. In particular, we wished to determine whether the domains of the interpolated module play an active role in the process that gives rise to the skipped product and whether it is required to aid the channeling of enzyme-bound polyketide chains between the native modules that flank it in the hybrid PKS. The model system we chose was the tetraketide synthase Ery1-Rap2-Ery2-TE, whose construction has been previously described [14] and which contains rapamycin PKS module 2 [15, 16] inserted between erythromycin modules 1 and 2 in the truncated PKS DEBS1-TE ([17]; Figure 1). The major products of this synthase are the triketide lactones **1** and **2** (Figure 1) with tetraketide **3** as a minor product. The tetraketide synthase and mutant versions of it are conveniently expressed in *S. erythraea* JC2 (a strain from which the native erythromycin PKS [DEBS] genes have been almost wholly deleted) [17].

We initially considered four alternative mechanisms (Figure 2) by which skipping might occur within Ery1-Rap2-Ery2-TE. These mechanisms can be distinguished through the differing predictions that they make about the involvement in the process of skipping of the ketosynthase (KS) and acylcarrier protein (ACP) active sites within Rap2.

Mechanism 1: KS(Rap2) to ACP(Rap2)

In this mechanism, the diketide chain is passed as expected from ACP(Ery1) to KS(Rap2) and then transferred without chain extension to ACP(Rap2) in a *trans*-thioesterification that requires that ACP(Rap2) be unprimed with a chain extension malonyl group. Once the polyketide chain is transferred to the 4′-phosphopantetheinyl prosthetic group of ACP(Rap2), extension is no longer possible within Rap2, and the chain can be passed directly to KS(Ery2). Consequently, KS(Ery2) is loaded with the diketide with which it is normally presented in its native environment. The active sites of KS(Rap2) and ACP(Rap2) are both required for skipping to occur.

Mechanism 2: ACP(Ery1) to ACP(Rap2)

Here, the diketide chain at ACP(Ery1) is passed directly to ACP(Rap2) by *trans*-thioesterification. The phosphopantetheinyl arms of ACP domains in adjacent modules both interact with the same intervening KS active site to effect normal chain extensions. It is therefore possible that these same groups are able to become close enough for the growing chain to be passed directly from

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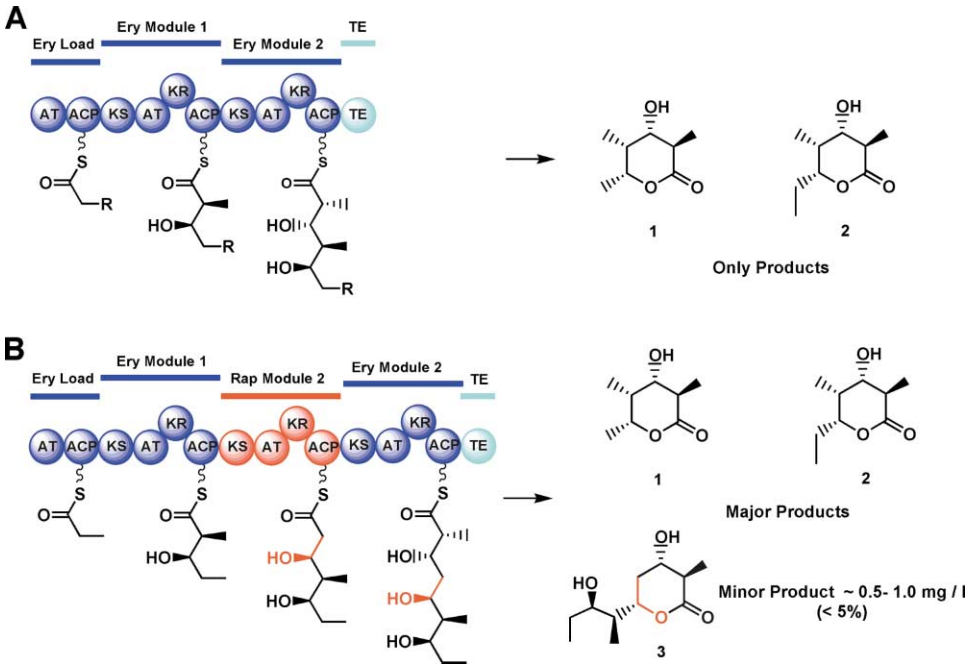

Figure 1. The Lactone Products Produced by the Truncated Model PKS DEBS1-TE and by the Tetraketide Synthase LM-Ery1-Rap2-Ery2-TE

(A) The lactone products produced by the truncated model PKS DEBS1-TE.

(B) Those products produced by the tetraketide synthase LM-Ery1-Rap2-Ery2-TE.

Abbreviations are as follows: LM, loading module; KS, ketosynthase; AT, acyltransferase; KR, ketoreductase; ACP, acylcarrier protein; and TE, thioesterase/cyclase.

ACP(Ery1) to ACP(Rap2). Only the ACP(Rap2) active site is utilized and not the KS(Rap2) active site.

Mechanism 3: KS(Rap2) to KS(Ery2)

Here, the diketide chain at KS(Rap2) is passed directly to KS(Ery2) by *trans*-thioesterification. Although this direct transfer is a formal possibility, current models of modular PKS structure [18, 19] suggest that it is unlikely. Only the KS(Rap2) is utilized and not the ACP(Rap2).

Mechanism 4: ACP(Ery1) to KS(Ery2)

The polyketide chain might also be transferred directly (or via a carrier molecule such as coenzyme A) from ACP(Ery1) to KS(Ery2), and the activities of the Rap2 module are not required at all. Again, from current models [18, 19] it seems likely that for the prosthetic group of ACP(Ery1) to transfer a polyketide moiety directly to KS(Ery2), (hitherto unsuspected) structural flexibility would be required within these modular PKSs.

Site-directed mutagenesis was used to knock out essential residues in either one or both of the KS(Rap2) and ACP(Rap2) active sites in LM-Ery1-Rap2-Ery2-TE, and the effect of these mutations on triketide and tetraketide production in recombinant *S. erythraea* was then monitored.

Results and Discussion

Construction of the Tetraketide Synthase LM-Ery1-Rap2(KS°)-Ery2-TE, in which the Active Site Cysteine Residue of Rapamycin KS2 Is Mutated to Alanine

Standard cloning protocols were used to make a version of rapamycin module 2 in which the active site cysteine is mutated to alanine in the ketosynthase domain. This module was introduced into the interdomain region N-terminal to the KS2 in DEBS1TE to yield the tetraketide synthase LM-Ery1-Rap2(KS°)-Ery2-TE. The resulting plasmid, pIPT62, contains the tetraketide synthase expressed from the *actI* promoter, under the control of *actII*-ORF4 in pCJR24 [14].

Construction of the Tetraketide Synthase LM-Ery1-Rap2(ACP°)-Ery2-TE, in which the Active Site Serine Residue of the Rapamycin ACP2 Is Mutated to Alanine

A similar cloning procedure was used to introduce a mutation at the rapamycin module 2 ACP. The mutation encoded for a change from serine to alanine. The mutant rapamycin module 2 ACP° was cloned into DEBS1TE, N-terminal to KS2. pIPT63 contains the tetraketide synthase LM-Ery1-Rap2(ACP°)-Ery2-TE under the *actI* promoter.

Construction of the Tetraketide Synthase LM-Ery1-Rap2(KS°/ACP°)-Ery2-TE, in which the Active Site residues of Rapamycin KS2 and ACP2 Are Both Mutated to Alanine

The two mutations described above were combined to make a doubly mutated rapamycin module 2 (KS°/ACP°). This module was cloned into DEBS1TE, N-terminal to KS2. pIPT64 contains the tetraketide synthase LM-Ery1-Rap2(KS°/ACP°)-Ery2-TE, expressed under the *actI* promoter.

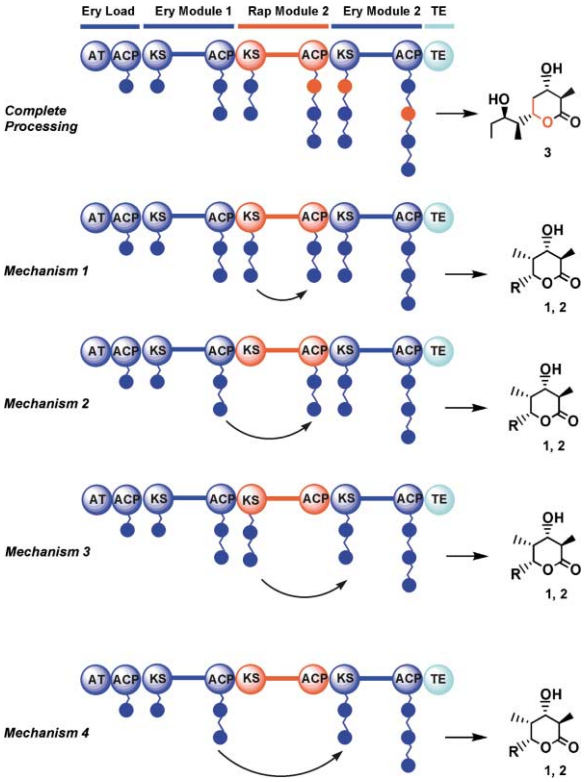

Figure 2. Alternative Proposed Mechanisms for Skipping of an Interpolated Module in a Hybrid Polyketide Synthase

In mechanism 1, the polyketide chain is passed between modules Ery1 and Ery2 via the KS and ACP active sites of the inserted Rap2 module; in mechanism 2, the chain is passed via the Rap2 ACP active site only; in mechanism 3, the chain is passed via the Rap2 KS active site only; and in mechanism 4, the chain is passed between modules Ery1 and Ery2 without any involvement of the Rap2 KS and ACP active sites (perhaps via a carrier molecule). The inserted Rap2 module is shown in red.

Analysis of the Tri- and Tetraketide Products Produced by Expression of pCJR54, pIPT62, pIPT63, and pIPT64 in *S. erythraea* JC2

GC-MS analysis of extracts of fermentation broths revealed that the control strain *S. erythraea* JC2/pCJR54 (LM-Ery1-Rap2-Ery2-TE) produced about 10 mg/liter of triketide lactones **1** and **2**, with the molar ratio of triketide **2**:triketide **1** (as judged by GC analysis) being approximately 10:1, together with about 0.5–1.0 mg/liter of tetraketide lactone **3** (Figure 3). This is in agreement with previously published findings [14]. SDS-polyacrylamide gel electrophoresis of extracts of this strain revealed the presence of polypeptides of a subunit molecular mass consistent with the proposed tetraketide synthase (C.J.M., unpublished data). The strain containing pIPT63 [LM-Ery1-Rap2(KS°)-Ery2-TE] produced triketide lactones **1** and **2** in quantities similar to those produced by the control strain (total 10 mg/liter, molar ratio **2**:**1** of 10:1), but no tetraketide. The strain that contained pIPT62 [LM-Ery1-Rap2(ACP°)-Ery2-TE] and the strain that contained pIPT64 [LM-Ery1-Rap2(KS°/ACP°)-Ery2-TE] yielded neither the tetraketide lactone nor the triketide lactones **1** and **2** (Figure 3).

Module Insertion Yields Full-Length Polyketide Products of the Predicted Structure and a Triketide Lactone by a Pathway in which the Inserted Rap2 Module Is Skipped

The tetraketide synthase LM-Ery1-Rap2-Ery2-TE synthesizes both the tetraketide lactone **3** and the triketide lactones **1** and **2**, and the triketide:tetraketide ratio is approximately 20:1, confirming previous observations [14]. Rapamycin module 2 selects an acetate extension unit and gives rise to a stereochemistry at the hydroxyl functionality that is opposite to that produced by *ery* module 2 [15, 16]. The structure of **3** is that predicted by the sequential action of erythromycin module 1, rapamycin module 2, and erythromycin module 2. As previously noted [14], the exclusive incorporation of propionate chain extension units in the triketide lactones **1** and **2**, and the stereochemistry at C4 in these products, demonstrates conclusively that formation of **1** and **2** did not involve the AT or KR enzymatic activities of the interpolated rapamycin module 2.

Skipping Mechanism in LM-Ery1-Rap2-Ery2-TE Involves Active Sites within Rap2

The lack of tri- and tetraketide lactone production by LM-Ery1-Rap2(ACP°)-Ery2-TE or LM-Ery1-Rap2(KS°/ACP°)-Ery2-TE and the contrasting production of triketide lactones by LM-Ery1-Rap2-Ery2-TE (control) and LM-Ery1-Rap2(KS°)-Ery2-TE demonstrate clearly that the interpolated Rap2 module actively participates in the skipping of the polyketide chain from Ery1 to Ery2. Mechanism 4 (Figure 2) can therefore be safely discarded. It might have been argued that alternative factors, such as an effect of the point mutation(s) on expression levels or the proper folding to a functional dimer, underlie the inactivity of LM-Ery1-Rap2(ACP°)-Ery2-TE and LM-Ery1-Rap2(KS°/ACP°)-Ery2-TE. However, the results from recent work on DEBS1-TE [20] strongly imply that these particular point mutations do not affect expression or folding. Mechanism 3 (Figure 2) can also be discarded because it predicts that LM-Ery1-Rap2(KS°)-Ery2-TE would be inactive in triketide production. We are left with mechanisms 1 and 2 (Figure 2), in each of which the interpolated module must possess an active ACP. Because the levels of triketide production from LM-Ery1-Rap2(KS°)-Ery2-TE were the same as those from the control strain LM-Ery1-Rap2-Ery2-TE, within experimental error, it appears that if mechanism 1 does operate, its contribution is rather small, and the major or sole pathway for skipping is revealed to be an ACP(Ery1)-to-ACP(Rap2) chain transfer process (mechanism 2).

Skipping Requires that the ACP of the Interpolated Module Not Be Primed with an Extender Unit

A skipping process that involves ACP-to-ACP chain transfer (mechanism 2) requires that the ACP of the skipped module must be free and not primed with an extender malonyl group. The priming of an ACP domain is normally accomplished by the acyltransferase (AT) domain within the module [21]. Three alternative plausible mechanisms by which an ACP domain might become unprimed are (1) the priming of an ACP domain by the

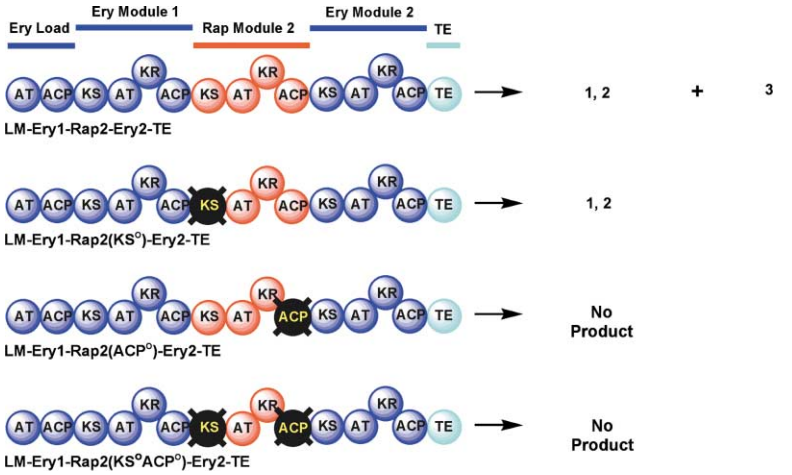

Figure 3. The Lactone Products Produced by Mutagenized Versions of the Tetraketide Synthase LM-Ery1-Rap2-Ery2-TE

A cross indicates the replacement of a single essential active site residue within a Rap2 domain.

action of the AT domain may itself be reversed; (2) the hydrolysis of the malonyl extender unit [22, 23]; and (3) adventitious decarboxylation of an ACP bound malonyl group [24] to give an ACP bound acetyl group may be followed by hydrolysis of this group by the discrete thioesterase TEII [25]. These effects will certainly be reinforced if the AT domain of the interpolated module is not functioning optimally, perhaps because of unfavorable protein-protein contacts within the hybrid PKS.

The Role of KS-Mediated Chain Transfers

Although not directly implicated in skipping, KS(Rap2) could play an important role in facilitating ACP-to-ACP polyketide chain transfer. For effective chain transfer to occur, the thiols of the two 4′-phosphopantetheinyl arms of ACP(Rap2) and ACP(Ery1) must come together to allow *trans*-thioesterification. A KS active site recognizes the prosthetic groups of both the preceding and following ACPs, and it might be able to dock both these units simultaneously. If so, the thioester group of the polyketide-bearing prosthetic group and the thiol terminus of the following 4′-phosphopantetheinyl unit will naturally be brought into proximity. It is plausible that an intervening inactive or retarded KS(Rap2) will also be able to mediate such transfers (Figure 4).

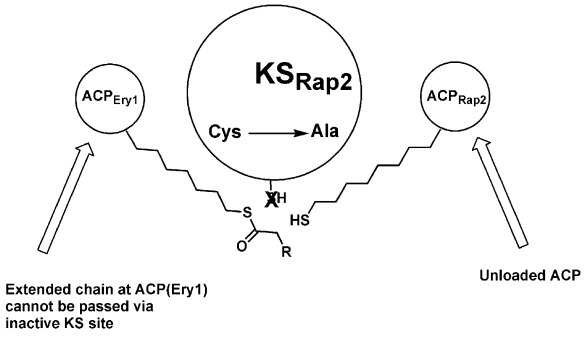

Figure 4. A Model for ACP(Ery1)-to-ACP(Rap2) Transfer Mediated by the Intervening KS(Rap2) Domain

The polyketide acyl chain (represented by R-CH_2-CO-) can be transferred to the 4′-phosphopantetheine "swinging arm" ACP(Rap2) within the KS(Rap2) site, even when the active site cysteine residue is replaced by alanine.

Skipping by mechanism 2 might also be enhanced if the rate of polyketide chain transfer from ACP(Ery1) to KS(Rap2) of the interpolated module is slowed significantly with respect to the rate of formation of unprimed ACP and the transfer to it of unextended chain. A slower rate of ACP(Ery1)-to-KS(Rap2) transfer compared with ACP-to-KS transfer between normally adjacent modules would be consistent with poorer substrate recognition by a heterologous KS domain and would be in accord with recently published results [12] that suggest that maintaining native ACP-KS interactions is important in the design of functional hybrid PKSs.

Evolution of Modular PKS Function

The evolution of modular polyketide synthases has presumably come about by gene and hence module duplication. However, extensive work on hybrid polyketide synthases, reviewed for example by Staunton and Weissman [21], clearly shows that mixing, matching, and inactivation of domains and modules frequently yields inefficient synthases and may result in the abolition of polyketide production. Such obstacles to effective polyketide synthesis are likely to arise within a polyketide synthase after most evolutionary changes. This would act as a barrier to further evolution. Skipping would allow any new duplicated modules to be bypassed on most occasions and would ensure production of the original polyketide metabolite for which there is existing selective pressure. However, it would also allow the intermittent production of alternative novel product(s). If such products confer an evolutionary advantage, then further mutation will select for the relative levels of production. On this model, the skipping process therefore makes viable the required intermediates in the evolutionary process. There has been no deliberate search for such truncated products but, in addition to the well-known example of the *Streptomyces venezuelae* pikromycin PKS, which synthesizes both a pentaketide and a hexaketide (see below) [26], other reports have described the presence of polyketide metabolites whose structures show that they may have arisen by skipping on a natural modular polyketide synthase [27]. The converse situation, aberrant repeated use of an extension module ("stuttering") within a modular PKS, has also been seen at low level on a natural modular PKS [28, 29].

Pikromycin

The pikromycin polyketide synthase from *Streptomyces venezuelae* contains six extension modules distributed between four multienzyme polypeptides PikAI–PikAIV and is responsible for the production of both the 14-membered macrocycle narbonolide and the 12-membered macrocycle 10-deoxymethynolide [26]. The latter compound is the result of chain extension and processing by the first five modules of the pikromycin polyketide synthase and represents in formal terms a natural example of a skipping process. It has been shown that the premature termination of the polyketide synthase is achieved via a wholly novel mechanism, the production of an N-terminally truncated form of PikAIV (housing module 6), and it has been proposed that this allows the pikromycin chain-terminating thioesterase (TE) to dock directly onto the ACP of module 5 rather than after module 6 [30, 31]. However, skipping mechanisms analogous to those described here, through which the pentaketide chain traverses the module 6 within the truncated PikAIV to reach the C-terminal thioesterase domain, are also possible. Indeed, such mechanisms are under active investigation (D.H. Sherman, personal communication).

Significance

Modular polyketide synthases are responsible for the biosynthesis of a wide range of clinically important natural products. The modular nature of the polyketide synthases has made them attractive targets for use in the synthesis of new related bioactive compounds and for eventual use in combinatorial biosynthesis. Polyketide synthases have been the recipients of many modifications, including even the interpolation of an entire heterologous PKS module into the erythromycin PKS and the model DEBS1-TE system. This system yields the predicted extended polyketide chains but is limited by the propensity of the hybrid PKS to ignore the presence of the inserted module, i.e., the module is skipped. We have shown here that the interpolated module, and specifically the active site of the ACP domain, is required for skipping to occur. We propose that a major (if not the sole) pathway for skipping involves direct ACP-to-ACP chain transfer and note that the phenomenon of skipping may have relevance for natural modular polyketide synthases and for their evolution.

Experimental Procedures

Chemical Analysis

Gas chromatography mass spectrometry (GC-MS) was performed on a Finnigan MAT GCQ instrument. Analytical and preparative reverse-phase high-performance liquid chromatography mass spectrometry (HPLC-MS) analyses were carried out on a Finnigan MAT LCQ instrument, with either an ES or API-MS interface and switching between positive and negative ion modes as required.

Bacterial Strains and Culture Conditions

Escherichia coli DH10B (GibcoBRL) was used in all standard cloning procedures and was grown in 2× TY medium [32]. Electrocompetent cells of DH10B were made as described previously [33]. *Saccharopolyspora erythraea* NRRL2338 (red variant) [34] was the kind gift of Dr J.M. Weber. *S. erythraea* mutant strain JC2 [35] has been described. *S. erythraea* strains were routinely maintained on R2T20 agar plates [36] and TSB medium (Difco) for liquid cultures at 30°C. After approximately 2 weeks on R2T20 agar at 30°C, spores were harvested and stored in 10% glycerol at −80°C. TSB and SM3 expression media have been described elsewhere [37].

Plasmids and DNA Manipulation Procedures

Construction of Plasmid pIPT62 Containing the Hybrid Tetraketide Synthase LM-Ery1-Rap2(KS°)-Ery2-TE

DNA encoding parts of the rapamycin module 2 KS domain were cloned between each of the EcoRI and BlpI sites that flank the KS active site cysteine residue and an AflIII site introduced over the active site with concomitant mutation of the cysteine codon to an alanine codon as follows. A 245 bp fragment encoding the N-terminal region up to the mutated active site was amplified by PCR with the oligonucleotides 5′-ATGATTAC*GAATT*CGAGCTCGGTA-3′ (reverse) and 5′-TGG*ACGCGT*TGACTGCGGTGGAACTACGGAA-3′ (forward). A 459 bp fragment encoding the C-terminal region up to the mutated active site was amplified by PCR with the oligonucleotides 5′-AGTCA*ACGCGT*CCACACCCAGATCCCTGAA-3′ (reverse) and 5′-GGACGCGCTG*GCTGAG*CAGCGTCACGCTGA-3′ (forward) (restriction sites shown in italics); each of these fragments was cloned into dephosphorylated SmaI-cut pUC18 (where the DNA sequences were confirmed by sequencing). The EcoRI-AflIII and AflIII-BlpI fragments and EcoRI-BlpI-cut pCJR53 were assembled by ligation to give the plasmid pIPT59, which contained the DNA encoding the rapamycin module 2 with a mutation at the KS active site. A pIPT59 Sse8387I fragment housing the mutated rapamycin module 2 was cloned into pIB103 that had previously been linearized with the same enzyme and dephosphorylated. The plasmid pIPT62 that contained the correctly oriented Sse8387I fragment was identified by restriction analysis.

Construction of Plasmid pIPT63 Containing the Hybrid LM-Ery1-Rap2(ACP°)-Ery2-TE

DNA encoding parts of the rapamycin module 2 ACP domain were cloned between the MluI and XbaI sites that flank the ACP active site serine residue and a PvuII site introduced over the active site with concomitant mutation of the serine codon to an alanine codon as follows. A 1567 bp fragment encoding the N-terminal region up to the mutated active site was amplified by PCR with the oligonucleotides 5′-ATACGGGCGGAATGGA*ACGCGT*GACT-3′ (reverse) and 5′-TTCACGGTCGACA*CAGCTG*CTTCGTCGTCGTT-3′ (forward). A 504 bp fragment encoding the C-terminal region up to the mutated active site was amplified by PCR with the oligonucleotides 5′-ACGAC GAAG*CAGCTG*TGTCGACCGTGAA-3′ (reverse) and 5′-TCGAC*TC TAGA*GGATCCCCGTATGGCCT-3′ (forward) (restriction sites shown in italics); each of these fragments was cloned into dephosphorylated SmaI-cut pUC18 (where the DNA sequences were confirmed by sequencing). The MluI-PvuII and PvuII-XbaI fragments and MluI-XbaI-cut pCJR53 were assembled by ligation to give the plasmid pIPT60, which contained the DNA encoding the rapamycin module 2 with the ACP active site mutated from serine to alanine. A pIPT60 Sse8387I fragment housing the mutated rapamycin module 2 was cloned into pIB103 that had previously been linearized with the same enzyme and dephosphorylated. The plasmid pIPT63 that contained the correctly oriented Sse8387I fragment was identified by restriction analysis.

Construction of Plasmid pIPT64 Containing the Hybrid Tetraketide Synthase LM-Ery1-Rap2(KS°/ACP°)-Ery2-TE

DNA encoding part of rapamycin module 2 KS including the mutated sequence at the active site was excised as an EcoRI-BlpI fragment from pIPT59 and cloned into pIPT60 that had been cut with these enzymes to give plasmid pIPT61. A pIPT61 Sse8387I fragment housing the mutated rapamycin module 2 in which both KS and ACP active site sequences had been mutated was cloned into pIB103 that had been linearized with the same enzyme and dephosphorylated. The plasmid pIPT64 that contained the correctly oriented Sse8387I fragment was identified.

Construction of S. erythraea *Strains Housing pCJR54, pIPT62, pIPT63, or pIPT64*

Protoplasts of *S. erythraea* NRRL2338 JC2 were transformed with pCJR54, pIPT62, pIPT63, or pIPT64 by adaptation [37] of a published procedure [36]. Transformants were selected on solid medium by overlaying with thiostrepton (25 mg/l) after 24 hr.

Growth and Extraction

Aliquots (10 ml) of TSB media containing thiostrepton (5 μg/ml) were inoculated with mycelia from a single transformant colony of *S. erythraea* NRRL2338 JC2:pCJR54, pIPT62, pIPT63, or pIPT64, and the culture was incubated for 2–4 days. Plates of SM3 agar containing thiostrepton (50 μg/ml) were individually inoculated with 500 μl from one of the liquid cultures and incubated for 10–14 days.

Regions of approximately 1 cm^2 were excised from each plate, extracted with 1.2 ml ethyl acetate containing 2% formic acid at 50°C for 15 min, and vortexed at room temperature for 30 min, and the supernatant was removed. The solvent was removed in vacuo, and the sample was dissolved in 50 μl ethyl acetate.

Analysis of Culture Extracts

The ethyl acetate extracts were analyzed for tri- and tetraketide lactones by GC-MS as previously described [12, 14]. Triketide lactones **1** and **2** and tetraketide lactone **3** were identified by comparison with traces for authentic samples of these compounds. Extracts from nine different transformants of each strain were analyzed and found to give comparable results.

Acknowledgments

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