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Context-Dependent Behavior of the Enterocin Iterative Polyketide Synthase: A New Model for Ketoreduction

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Summary

Heterologous expression and mutagenesis of the enterocin type II polyketide synthase (PKS) system suggest for the first time that the association of an extended set of proteins and substrates is needed for the effective production of the enterocin-wailupemycin polyketides. In the absence of its endogenous ketoreductase (KR) EncD in either the enterocin producer "*Streptomyces maritimus*" or the engineered host *S. lividans* K4-114, the enterocin minimal PKS is unable to produce benzoate-primed polyketides, even when complemented with the homologous actinorhodin KR ActIII or with EncD active site mutants. These data suggest that the enterocin PKS requires EncD to serve a catalytic and not just a structural role in the functional PKS enzyme complex. This strongly implies that EncD reduces the polyketide chain during elongation rather than after its complete assembly, as suggested for most type II PKSs.

Introduction

Aromatic (type II) polyketide synthases (PKSs) are comprised of several mostly monofunctional proteins and are responsible for the biosynthesis of bacterial aromatic natural products such as actinorhodin, tetracenomycin, doxorubicin, and oxytetracycline [1–3]. These enzymes are related to type II fatty acid synthases and form complexes containing a "minimal" set of four proteins [two β-ketosynthase subunits KS_α and KS_β (alternatively referred to as the chain length factor [4]), acyl carrier protein (ACP), and malonyl-CoA:ACP acyltransferase (MAT)] that are required for polyketide chain assembly. Additional PKS subunits, including ketoreductases and cyclases, have been proposed to convert the fully elongated, linear poly-β-ketoacyl thioester intermediate to the cyclized polyketide molecule. A large series of novel polyketides has been generated by manipulating type II PKS systems that express a variety of combinations of a minimal PKS with associated proteins [2].

Most type II PKSs utilize malonyl-CoA as both priming and elongating molecules in the synthesis of polyaromatic compounds that typically range in size from octaketides to dodecaketides. The KS_β subunit, in coordination with the KS_α subunit [5], has been shown to assist in the decarboxylative priming of the malonate starter unit [6] as well as in controlling polyketide chain length [7]. Despite the widespread occurrence of acetate-primed polyketides, several aromatic PKSs deviate from the decarboxylative-priming mechanism and utilize different starter molecules [8]. In R1128 [9–11], daunorubicin [12–14], and frenolicin [15] biosynthesis, where the respective PKS is primed with short-chain carboxylates, KSIII and in some cases an additional ACP are involved, leading to a functional crosstalk between fatty acid and polyketide metabolism. A different priming mechanism is involved in oxytetracycline [16] and enterocin [17] biosynthesis, as their associated biosynthetic gene clusters contain just one ACP and no KSIII homolog. Rather, a monofunctional CoA ligase and a dedicated AT may function to activate and transfer the starter units malonamate and benzoate, respectively, onto the PKS.

The bacteriostatic agent enterocin from "*Streptomyces maritimus*" is a novel type II PKS-derived octaketide product [18] which originates from an intermediate assembled from an uncommon benzoyl-CoA starter unit [17, 19–21] and seven malonate molecules that has undergone a rare Favorskii-like oxidative rearrangement [22] (Figure 1). Sequence analysis of the 20 open reading frame *enc* biosynthetic gene cluster revealed that the centrally located minimal PKS genes *encABC* are flanked by a number of genes encoding polyketide tailoring enzymes as well as enzymes involved in starter unit biosynthesis [23]. Here, we describe the construction and expression of a series of expression plasmids carrying different arrangements of *enc* genes and show that the *enc* minimal PKS requires an additional protein component, namely the ketoreductase (KR) EncD, as well as the substrate benzoyl-CoA in order to constitute a fully functional PKS. Gene knockout and complementation experiments corroborate the heterologous biosynthesis results and lend support to the notion that the timing of ketoreduction in the *enc* type II PKS may take place on the growing polyketide chain rather than on a fully extended octaketide linear intermediate. This proposed pathway represents a new mechanism for type II PKS assembly that may be operative in other systems as well.

Results

Characterization of the *enc* Minimal PKS by Heterologous Biosynthesis

Despite its homology to the acetate-primed *act* PKS, the *enc* PKS must suppress the decarboxylative chain initiation by malonyl-S-ACP in favor of the benzoyl-CoA starter unit, as all natural *enc*-derived polyketides described to date are benzoate primed [24]. To test whether the *enc* minimal PKS functions in the absence

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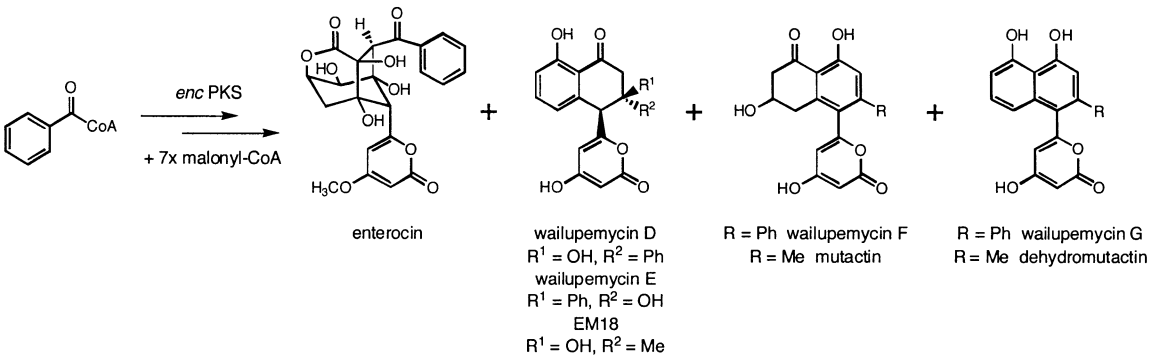

Figure 1. Structure and Biogenesis of Enterocin and Wailupemycins D–G

The structures of the *act* PKS-derived products EM18, mutactin, and dehydromutactin are provided for comparison with wailupemycins D–G. The stereochemistry of wailupemycin D and E is relative and is unknown in EM18.

of the natural starter unit and unnaturally accepts an acetyl primer unit in its place, we constructed the pSEK4-derived plasmid pCH8 by introducing the *enc* minimal PKS genes *encABC* downstream of the *act*I promoter (Table 1). This expression plasmid was introduced via transformation into the engineered host strain *Streptomyces lividans* K4-114 [25]. Although we anticipated acetate-primed polyketides such as the octaketides SEK4 [4] and SEK4b [26], the nonaketide PK8 [27], or the decaketide SEK15 [28], HPLC-MS analysis of the resulting organic extract did not reveal any polyketide metabolites.

To evaluate whether the ActIII homologous enterocin KR EncD is required by the *enc* minimal PKS for activity, we next coexpressed the genes *encABC* with *encD*. Two related expression systems were constructed and evaluated. First, pCH8 was coexpressed with pCH20 [23], a previously constructed *encD*-containing derivative of the integrating *E. coli-Streptomyces* shuttle plasmid pSET152 [29]. Second, pCH8 was reengineered to give pMP5, in which the *encD* gene was introduced on the same plasmid downstream of the divergent *act*III promoter. In both cases, inclusion of *encD* with the *encABC* minimal PKS gene cassette did not facilitate *enc*-based polyketide production. Supplementation with sodium benzoate did not facilitate polyketide production either, presumably because of the host's inability to activate benzoate as its CoA thioester in the absence of the benzoate:CoA ligase EncN.

The failure of the *enc* minimal PKS to function in vivo is in stark contrast to other type II PKS systems examined to date [2]. Even in the case of the malonamide-primed aromatic polyketide antibiotic oxytetracycline (*otc*), expression of the *otc* minimal PKS genes with *actIII* gave a reduced decaketide derived from acetate only [30]. To test the effect of the natural *enc* starter unit on polyketide assembly, we constructed another pSEK4-derived plasmid carrying the *encABCLMN* gene cassette (pCH16). This construct contains the machinery for the activation of benzoic acid to benzoyl-CoA [24] and its anticipated loading onto the enterocin ACP EncC by the products of the *encN* and *encL* genes, respectively. In addition, the oxygenase *encM*, previously shown to be involved in the Favorskii-like oxidative rearrangement reaction [22], is present in pCH16. HPLC-MS analysis of *S. lividans* K4-114 (pCH16) grown in the presence of benzoic acid, however, did not reveal any benzoate-primed molecules.

To our surprise, the expression of the full complement, including both the benzoate-priming machinery (*encL* and *encN*) and the KR *encD* together with the minimal PKS genes *encABC*, provided benzoate-primed polyketides when *S. lividans* K4-114 (pCH16 + pCH20 or pMP6) was supplied with sodium benzoate. The *enc* PKS prod-

Table 1. Plasmid Constructions and Resulting Polyketide Products in the Host Strain *S. lividans* K4-114

Plasmid(s)[a]	Genes	Products
pCH8	*encABC*	none
pCH8 + pCH20	*encABC* + *encD*	none
pMP5	*encABC/encD*	none
pCH16	*encABCLMN*	none
pCH16 + pCH20	*encABCLMN* + *encD*	wailupemycins D–G[b]
pMP6	*encABCLMN/encD*	wailupemycins D–G[b]
pBM19	*encABCLMN/actIII*	none

Transformants were grown in duplicate in the presence and absence of sodium benzoate.

[a] Plasmids pCH8 and pCH16 are derivatives of pSEK4 [4], pBM19 is a derivative of pRM5 [4] that contains the *actIII* KR gene downstream of the divergent *actIII* promoter, and pMP5 and pMP6 are pRM5 derivatives that rather contain the *encD* KR. The integrative plasmid pCH20 for multiplasmid expression was described previously [23].

[b] Only in the presence of supplied benzoic acid. Wailupemycins D and E were each produced at ~5 mg/l, whereas wailupemycins F and G were produced at ~10 mg/l.

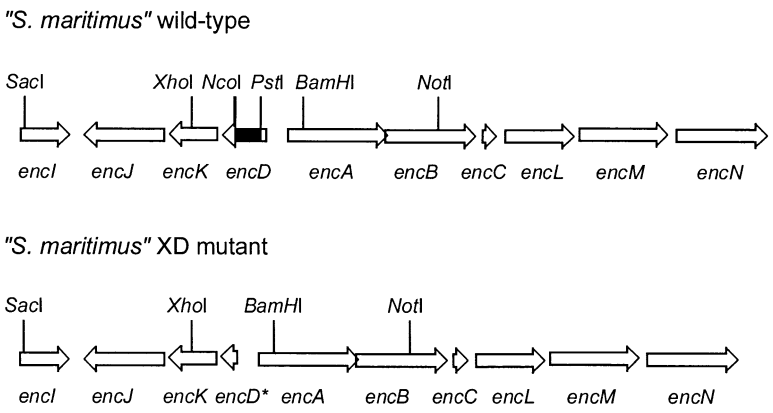

Figure 2. Partial Maps of the Enterocin Biosynthesis Gene Cluster in the Wild-Type "*S. maritimus*" and the Mutant "*S. maritimus*" XD

ucts wailupemycins D–G (Figure 1), which are minor wild-type metabolites that have not undergone the oxidative, Favorskii-like rearrangement reaction [22], were identified through comparison with authentic standards by HPLC-MS as the major polyketides produced by the resulting transformants (Table 1). These benzoate-primed wailupemycins are structurally analogous to the acetate-primed *act* PKS shunt products EM18, mutactin, and dehydromutactin [2] (Figure 1). However, we were not able to detect through HPLC comparison with standard compounds these or any other acetate-primed molecules from an organic extract of *S. lividans* K4-114 (pMP6) when this transformant was grown in the absence of benzoic acid. The requirement of the *encD* gene product was further illuminated when the substitution of *encD* with the actinorhodin homolog *actIII* in *S. lividans* K4-114 (pBM19) resulted in the loss of polyketide production, thereby suggesting a definite association of EncD in the functional enterocin PKS complex.

Construction and Complementation of the *encD* Knockout Mutant "*S. maritimus*" XD

In order to further examine the required role of the KR EncD in the functional *enc* PKS complex, the encoding gene was disrupted in "*S. maritimus*" by double-crossover homologous recombination. Conjugal transfer of the pKC1139-based [31] temperature sensitive plasmid pBM24 from *E. coli* to "*S. maritimus*" and growth of the resulting exconjugants under selective conditions resulted in a 512 bp in-frame deletion in the gene *encD* (Figure 2). PCR amplification of genomic DNA from the wild-type strain and the Δ*encD* mutant "*S. maritimus*" XD confirmed the gene knockout. HPLC-MS analysis of an organic extract from the mutant verified the loss of production of the benzoate-primed polyketides enterocin and the wailupemycins, thereby corroborating the in vivo expression results. Enterocin production could be restored in the mutant upon complementation with the wild-type *encD* gene in the modified pKC1139 vector pBM26 when expressed under the control of the constitutive *ermE** promoter. Complementation of the Δ*encD* mutant with the homologous actinorhodin KR gene *actIII* (pBM27), however, did not restore enterocin production, once again suggesting a specific association of the enterocin minimal PKS with its endogenous KR. To evaluate whether EncD is an essential component of a functional enterocin PKS primarily due to its catalytic or structural properties, the Δ*encD* mutant was next complemented with modified *encD* genes carrying mutations corresponding to the conserved active site residues Ser-148 and Tyr-161 (Figure 3) [32]. Single S148A and double S148A-Y161F EncD mutations were constructed in the vectors pBM28 and pBM29, and production profiles of the complemented Δ*encD* mutants were examined by HPLC-MS analysis. No polyketides, neither reduced nor nonreduced, were detected. Assuming that the mutations in EncD did not perturb protein structure, this experiment established that the *enc* minimal PKS requires the KR to serve a catalytic and not just a structural role as a "linker" in the functional *enc* PKS enzyme complex.

Discussion

In this study, the enterocin PKS was used as a model system to address the interactions among type II PKS components that constitute a functional enzyme complex. The heterologous expression of various combinations of *enc* genes demonstrated that, in addition to the typical elements of an aromatic minimal PKS, including the KS_α-KS_β heterodimer EncA-EncB and the ACP EncC, the functional *enc* PKS complex must be extended to also include the KR EncD. While the heterologous expression of *encABCDLMN* in *S. lividans* K4-114 (pMP6) yielded the metabolic shunt products wailupemycins D–G in the presence of exogenous benzoate, knockout experiments suggest that the AT *encL* (L.X. and B.S.M., unpublished observations), the oxygenase *encM* [22], and the benzoate:CoA ligase *encN* [21] are not required for minimal PKS activity. In each case, the resulting mutants were able to biosynthesize benzoate-primed polyketides, signifying that these enzymes are not required in the minimal PKS complex and thus do not additionally coordinate in a functional cage of linked enzymes. These and other experiments did, however, demonstrate that the starter unit benzoyl-CoA is indeed an essential substrate, as in its absence, either in the Δ*encP* mutants "*S. maritimus*" KP [20] and XP [24] or in *S. lividans* K4-114 (pMP6) without supplemental benzoate (this study), *enc*-based polyketides were not produced.

The functional *enc* minimal PKS most likely consists of EncABCD plus benzoyl-CoA to prime its function.

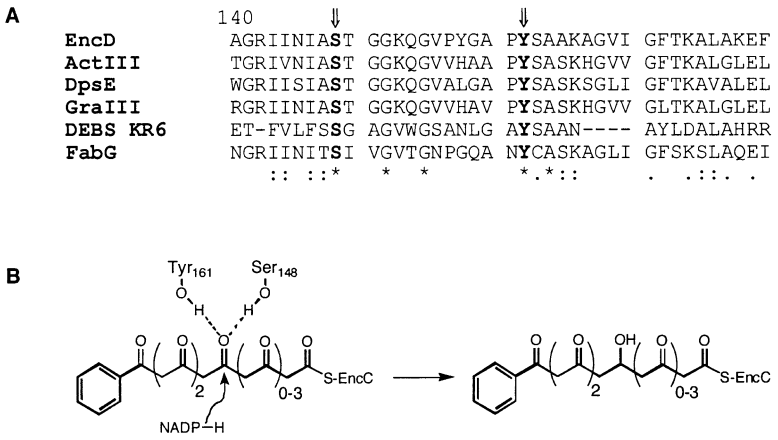

Figure 3. Proposed Mechanism for EncD-Catalyzed Ketoreduction

(A) Partial alignment of ketoreductases showing the predicted EncD active site residues Ser-148 and Tyr-161 that were mutated in this work (labeled with arrow). The amino acid numbering (140–179) relates to that of EncD. KRs from type II PKSs include EncD (enterocin), ActIII (actinorhodin), DpsE (daunorubicin/doxorubicin), and GraIII (granaticin). Others include the KR domain from the type I modular erythromycin PKS (DEBS KR6) and the FAS KR from *Escherichia coli* (FabG).

(B) Based on homology modeling of short chain dehydrogenases, the EncD active site residues Ser-148 and Tyr-161 are predicted to orient targeted carbonyl group in the polyketide substrate for NAD(P)H reduction. The exact chain length (pentaketide→octaketide) of the polyketide substrate is unknown.

However, expression of EncABCD alone in *S. lividans* K4-114 (pMP5) or (pCH8 + pCH20) when grown in the presence of benzoate failed to produce polyketides, presumably because of the host's inability to convert benzoate to its CoA thioester. Thus, at present the smallest set of enzymes that constitutes a functional *enc* PKS heterologously is EncABCDLMN. Further work is ongoing to characterize the *enc* minimal PKS in vivo and in vitro.

The *enc* PKS is the first reported iterative type II PKS that has an absolute requirement for its endogenous KR for PKS activity and hence has an extended minimal PKS that is context dependent. This requirement was initially observed with the heterologous biosynthetic experiments, in which the expression of *encABCLMND* (pMP6) yielded polyketides (wailupemycins D–G) in the presence of exogenous benzoate, whereas the expression of *encABCLMN* (pCH16) did not. This dependency was corroborated in the Δ*encD* mutant "*S. maritimus*" XD. Polyketide productivity could only be restored in this *encD* null mutant by complementation with the wild-type *encD* gene but not with the homologous *actIII* gene nor with a mutated *encD** gene with single (S148A) or double (S148A and Y161F) mutations. Based on homology modeling of short chain dehydrogenases [32], these active site residues orient the targeted carbonyl group in the polyketide substrate for NAD(P)H reduction (Figure 3). Since nonreduced polyketides of any chain length were not generated in "*S. maritimus*" XD (pBM28) or in *S. lividans* K4-114 (pCH16), these observations strongly imply that the *enc* minimal PKS requires EncD to serve a catalytic and not just a structural role in the functional *enc* PKS enzyme complex.

In the current model for aromatic polyketide synthesis, the timing of ketoreduction in type II PKS assembly has been proposed after the complete synthesis of the linear poly-β-ketide (Figure 4, path A) [1–3]. Like enterocin and the wailupemycins, all ketoreduced aromatic PKS products, including actinorhodin, frenolicin, oxytetracycline, and aclacinomycin A, are similarly reduced at the ninth carbon from the carboxyl terminus of the assembled polyketide irrespective of the polyketide chain length (octaketide to decaketide) or the nature of the starter unit (acetate, butyrate, malonamate, benzoate) [2]. Ketoreductases from these different systems have been interchanged and shown to function with hybrid PKSs [23, 28, 33, 34], thereby leading to the hypothesis that "the polyketide chain is completely assembled before reduction of the keto group takes place" [33]. In light of the data presented here, we propose that the enterocin KR acts at an earlier stage in polyketide assembly, such as when the target carbonyl group is in the β position relative to the thioester carbonyl (Figures 3B and 4, path B). In analogy to fatty acid assembly by the dissociated system fatty acid synthase II [35], polyketide biosynthesis may stall to allow for the FabG-related EncD to reduce at the predestined pentaketide stage. Absolutely no aberrant polyketides were observed in the constructs without EncD or with inactive EncD, however, suggesting that if chain growth terminates at this stage, truncated pentaketides are not released from the PKS complex or are simply catabolized. The likely mode of catalysis by EncD involves its association with the KS and reduction of the KS-bound pentaketide intermediate. The alternative, in which the ACP-bound pentaketide dissociates away from the KS heterodimer followed by EncD-catalyzed reduction and reassociation with the KS for further chain extension without significant spontaneous cyclization, appears less likely.

One advantage of this scenario over the current model of type II PKS assembly typified in path A (Figure 4) is that ketoreduction at an early stage (path B) facilitates proper aldol cyclization of the first ring by limiting nonspecific cyclizations due to the deactivation of the reduced carbonyl carbon and the adjacent methylene groups (path B). This proposed model is indirectly supported by the observation that all type II PKS-derived polyketides engineered to date that have undergone ketoreduction share the same regiochemistry about the first cyclized ring, regardless of chain length [2]. In contrast, when KRs are omitted from type II PKSs that normally associate with KRs, the resulting engineered polyketides include appreciable products of mixed first ring cyclization events, as evident in the correctly folded *act* octaketide SEK4 versus the aberrantly folded SEK4b [26].

The *act* minimal PKS, as well as most other type II PKSs, can, however, function in the absence of its associated KR ActIII [28, 36] as well as with KRs from other systems, including EncD [23]. In fact, inactivation of

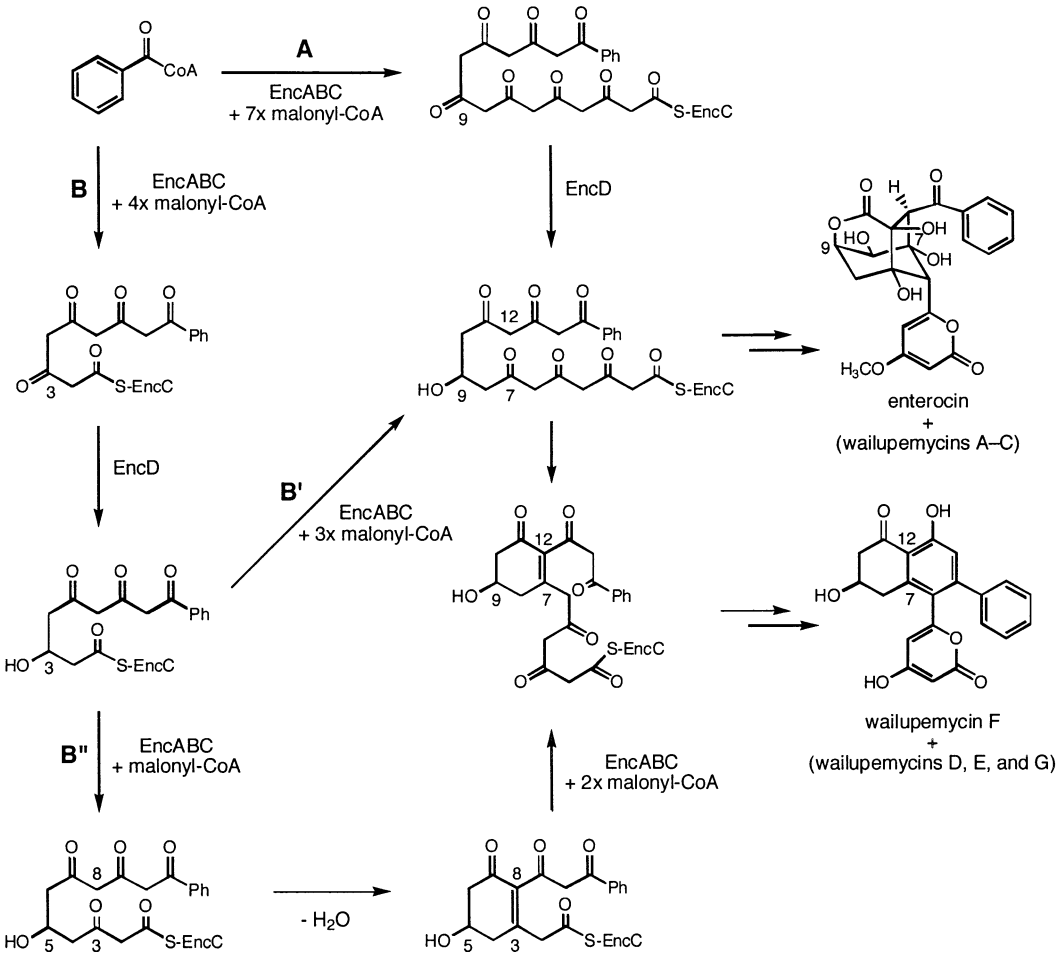

Figure 4. Proposed Biosynthetic Pathways to *enc*-Based Polyketides in "*S. maritimus*" Depicting the Relative Timing of Ketoreduction during the Polyketide Assembly Process

Based on the new evidence (this study) that EncD is essential for turnover of the *enc* PKS, path A can be excluded, while paths B′ and B″ cannot yet be distinguished.

actIII in *S. coelicolor* A3(2) resulted in the loss of actinorhodin biosynthesis with concomitant production of an as yet uncharacterized diffusible pigment [37], thereby indicating that KR-less minimal type II PKSs can still function and assemble the complete polyketide chain. The observed differences between the *enc* and *act* KRs suggest that there may be two pathways for ketoreduction in type II PKS systems, either during polyketide assembly (EncD) or post-polyketide assembly (ActIII). The enterocin system strongly implies that, due to the requirement of only a functional EncD for in vivo activity, its KR may act before the entire octaketide is biosynthesized and thereby direct the cyclization regiochemistry. It remains to be seen whether this new model for ketoreduction is operative in other aromatic PKSs as well. The in vitro reconstitution of the *enc* PKS system is currently being pursued to address this working hypothesis regarding the timing of ketoreduction in type II PKS systems.

Significance

Iterative type II polyketide synthases (PKSs) are responsible for the production of most aromatic polyketides in bacteria, including the antibiotic oxytetracycline and the anticancer agent daunorubicin. The biosynthesis of the octaketide actinorhodin has served as a model system in type II PKS assembly, where it has been shown that the minimal PKS is comprised of a ketosynthase heterodimer, an acyl carrier protein (ACP), and a malonyl-CoA:ACP acyltransferase. The all malonyl-CoA-derived linear octaketide product then undergoes post-PKS modifications, including ketoreduction, cyclization, aromatization, and oxidation reactions, to yield the polyketide product. Several biochemical processes involving the selectivity and attachment of starter units other than acetate and the timing of the ketoreduction and subsequent cyclization reactions during aromatic polyketide assembly remain unsettled and were addressed in this study with the benzoate-primed enterocin PKS. Heterologous expression and mutagenesis of the enterocin type II PKS system suggest for the first time that the association of an extended set of proteins (ketoreductase) and substrates (benzoyl-coenzyme A) is needed for the effective production of polyketides in the enterocin-wailupemycin structural family. The observed dependency of the enterocin PKS on its endogenous

ketoreductase EncD strongly implies that the enterocin minimal PKS requires EncD to serve a catalytic and not just a structural role in the functional PKS enzyme complex. As a consequence, the apparent timing of ketoreduction in the enterocin PKS system occurs during the polyketide chain elongation process rather than post-PKS assembly, as proposed for most other type II PKS systems, suggesting a new mechanism in aromatic PKS assembly.

Experimental Procedures

Bacterial Strains and Culture Conditions

"*S. maritimus*" strain BD26T (GenBank accession number AF233338) was grown as previously described [23]. A1 medium was used for sporulation, and R2YE medium was used for isolation of genomic DNA. *S. lividans* K4-114 was used as a host for transformation with all expression plasmids [25]. *Escherichia coli* XL1-Blue and DH5α was used for subcloning and grown in LB medium supplemented with ampicillin (100 μg/ml) or apramycin (50 μg/ml) for selection of plasmids. *E. coli* S17-1 was used as the host for *E. coli*-"*S. maritimus*" conjugation [38]. For polyketide production, wild-type and mutant strains were cultivated on A1 agar plates for 5 days at 28°C. *S. lividans* K4-114 was cultured on R2YE agar and YEME liquid medium [39] for protoplast transformation and on 2CM for all other experiments. Transformants were selected with thiostrepton and/or apramycin in both solid and liquid medium. Individual plates were enriched with sodium benzoate (1 mM). Metabolite production was monitored by LC-MS analysis of crude plate extracts and compared with known standards [22].

Plasmids and General Techniques for DNA Manipulations

"*S. maritimus*" total genomic DNA was isolated as described [23]. Cosmids pSS9A6 and pJP15F11 [23], which harbors the entire *enc* biosynthesis gene cluster, were used as the source of DNA in the construction of expression plasmids. The *E. coli-Streptomyces* shuttle vectors pSEK4 and pRM5 [4] were used for all expression experiments in *Streptomyces*. Recombinant DNA procedures were performed by standard techniques [39, 40]. Restriction enzyme-digested DNA fragments were recovered from agarose gels with the Qiaquick DNA Purification Kit (Qiagen). Oligonucleotides were obtained from Sigma Genosys. PCR was carried out on a PTC-2000 Peltier thermal cycler (MJ Research) with PfuTurbo (Stratagene) DNA polymerase. DNA sequencing by BigDye terminator cycle sequencing reaction using an ABI 377 sequencer was performed at the Laboratory of Molecular Systematics and Evolution at the University of Arizona.

Construction of *enc* Expression Plasmids

pCH8 is a derivative of pSEK4, in which the *act* genes were replaced by a 3.6 kb PacI-EcoRI *encABC* fragment downstream of the *act*I promotor. The *encABC* cassette was constructed by PCR amplification of an *encA* fragment (forward primer CHPA1, CCCC**TTAAT TAA**GGAGCAGAGGATGAGCGGC; bold, PacI site; reverse primer, T7), with introduction of a PacI site upstream of the ribosomal binding site and ligation of the 0.4 kb PacI-BamHI fragment and a 3.2 kb BamHI-EcoRI genomic fragment into the PacI-EcoRI sites of pNEB193 (New England Biolabs), yielding pCH6.

pCH16 is a derivative of pSEK4 containing a contiguous 7.3 kb PacI-EcoRI *encABCLMN* cassette. In order to introduce an EcoRI site downstream of *encN*, a 5.4 kb NotI-FspI (blunted) *encCLMN* genomic fragment from cosmid pSS9A6 was subcloned into NotI-SmaI of pBluescript, and the resulting NotI-EcoRI fragment was cloned into pCH6. pBM19 is a derivative of pRM5 in which the 7.3 kb PacI-EcoRI *encABCLMN* gene cassette was cloned into the analogous site downstream of the *actI* promoter.

pMP5 and pMP6 are derivatives of pCH8 and pCH16, respectively, in which *encD* was cloned downstream of the divergent *act*III promotor. FseI-NsiI sites of the 0.9 kb *encD* fragment from cosmid pSS9A6 were changed into XbaI-SwaI sites by subcloning into a modified polylinker, then *encD* was cloned into the XbaI-SwaI sites of pHGF7505 [41], and the HindIII-PacI fragment including the activator was exchanged with the HindIII-PacI fragments of pCH8 and pCH16, respectively.

Construction and Characterization of the *encD* Mutant "*S. maritimus*" XD

This mutant was obtained by gene disruption as follows. A 5.538 kb SacI-NotI fragment containing genes *encIJKDAB* in the natural sequence of the *enc* cluster from the cosmid pJP15F11 [23] was subcloned into pGEM.5ZF (Pharmacia) to make pBM20. The 1.493 kb BamHI-XhoI fragment containing *encD* and partial *encAK* in pBM20 was subcloned into XhoI-BamHI-digested pGEM.7ZF to create pBM21. After a 512 bp NcoI-PstI internal fragment from *encD* was deleted in pBM21, the remaining plasmid was blunt ended by mung bean nuclease and then self-ligated to give pBM22. The 916 bp XhoI-BamHI fragment from pBM22 was used to replace the analogous 1.493 kb fragment in pBM20 to create pBM23. HindIII and XbaI restriction sites were introduced on the ends of the 4.92 kb NotI-SacI-containing *encIJKD*AB* fragment in pBM23 for cloning into the *E. coli*-streptomycete conjugal transfer vector pKC1139 [31] digested with HindIII-XbaI to create pBM24.

The plasmid pBM24 was introduced into "*S. maritimus*" by conjugal transfer via *E. coli* S17-1, selected on 50 μg/ml apramycin at 37°C to get the single crossover mutant [22]. The exconjugant clone was inoculated into A1 broth at 37°C with shaking at 250 rpm for 48 hr. The resultant mycelia were used as inoculum for the next round of culturing in the same fashion, and this process was repeated for 3–4 rounds, at which time the cultures were diluted with water and spread on A1 plates at 37°C. The colonies were randomly plated out on A1 agar plates with and without apramycin (100 μg/ml) and incubated at 37°C. Genomic DNAs from apramycin-sensitive colonies were PCR amplified with the primers 5′-GGTTAATTAACC GGCCGCCCCGACGAAGG-3′ (forward) and 5′-GGTGGCCCCGGAC GTCATGC-3′ (reverse). 1.6 kb PCR products were indicative of revertants (wild-type) carrying a complete *encD* gene, whereas 1.0 kb products corresponded to a truncated *encD* gene from the double-crossover mutant, designated "*S. maritimus*" XD (Figure 2).

The plate culture of the Δ*encD* mutant fermented at 30°C for 3 days was exhaustively extracted with 5% MeOH in EtOAc, dried over $MgSO_4$, filtered, and evaporated to dryness. The extract was redissolved in approximately 1 ml of MeOH, and 10 μl was used for analysis by reversed-phase HPLC.

Complementation of "*S. maritimus*" XD

The mutant "*S. maritimus*" XD was complemented with wild-type *encD*, *actIII*, and two mutated *encD* genes in the pBM25-based plasmids pBM26 through pBM29, respectively. pBM25 is a derivative of pKC1139 containing a 0.4 kb EcoR1 fragment from pBM10 [20] containing the promoter *ermE**. For pBM26, *encD* was PCR amplified from the cosmid clone pJP15F11 with the primers 5′-AGTACTAGTCGACGAAGGGAGAAAGTCTCAC-3′ (forward) and primer 5′-GCTTCTAGATGGTCATGCGCGTGTCCC-3′ (reverse), cloned into pCR^R-Blunt, sequence verified, digested with EcoRV and HindIII, and cloned downstream of the promoter *ermE** in pBM25 to create pBM26. For pBM27, *actIII* was PCR amplified from the genomic DNA of *S. coelicolor* A3(2) with the primers 5′-ATGACTAGTAGGGAC CACGAGGCAGGG-3′ (forward) and 5′-ATGTCTAGACCGGCCGGC GTCAGTAGTAG-3′ (reverse), cloned into pCR^R-Blunt, sequence verified, digested with EcoRV and HindIII in the vector, and cloned downstream of the promoter *ermE** in pBM25 to give pBM27. For pBM28 and pBM29, mutations in the gene *encD*, corresponding to the S148A single mutant and the S148A-Y161F double mutant, respectively, were created by PCR using the QuickChange Multi-mutagenesis Kit (Stratagene) with the primers 5′-GATCATCAA CATCGCGGCCACCGGCGGCAAGC-3′ for exchanging S to A and 5′-CGTACGGGGCCCCGT*T*CTCGGCGGCCAAGG-3′ for exchanging Y to F (changed bases are italicized), with pBM26 as the DNA template to give pBM28 and pBM29 after sequence confirmation.

pBM26 through pBM29 were introduced into "*S. maritimus*" XD by plasmid conjugal transfer method via *E. coli* S17-1 as previously described [22] to yield "*S. maritimus*" XD (pBM26), "*S. maritimus*" XD (pBM27), "*S. maritimus*" XD (pBM28), and "*S. maritimus*" XD (pBM29), respectively. The exconjugants were grown on A1 plates

with 100 μg/ml aparamycin at 30°C for 3 days, extracted, and analyzed by HPLC as previously described [22].

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