The Acyltransferase Homologue from the Initiation Module of the R1128 Polyketide Synthase Is an Acyl-ACP Thioesterase That Edits Acetyl Primer Units†

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Received April 26, 2004; Revised Manuscript Received May 28, 2004

ABSTRACT: Type II polyketide synthases (PKSs) synthesize polyfunctional aromatic polyketides through iterative condensations of malonyl extender units. The biosynthesis of most aromatic polyketides is initiated through an acetate unit derived from decarboxylation of malonyl-acyl carrier protein (ACP). Modification of this primer unit represents a powerful method of generating novel polyketides. We have demonstrated that recombination of the initiation module from the R1128 PKS with heterologous elongation modules afforded regioselectively modified polyketides containing alternative primer units. With the exception of the role of the acyltransferase homologue ZhuC, the catalytic cycle of the initiation module has been well explored. ZhuC, along with the ketosynthase III homologue ZhuH and the ACP_p ZhuG, is essential for the in vivo biosynthesis of aromatic polyketides derived from non-acetate primer units. Here we have studied the role of ZhuC using PKS proteins reconstituted in vitro. We show that the tetracenomycin (*tcm*) minimal PKS can be directly primed with non-acetate acyl groups. In the presence of ∼10 μM hexanoyl-ZhuG or ∼100 μM hexanoyl-CoA, the *tcm* minimal PKS synthesized hexanoyl-primed analogues of octaketides SEK4 and SEK4b, as well as acetate-primed decaketides SEK15 and SEK15b at comparable levels. Addition of ZhuC abolished synthesis of the acetate-primed decaketides, resulting in exclusive synthesis of the hexanoyl-primed octaketides. In the absence of alternative acyl donors, ZhuC severely retarded the activity of the *tcm* minimal PKS. The editing capabilities of ZhuC were directly revealed by demonstrating that ZhuC has 100 times greater specificity for acetyl- and propionyl-ACP as compared to hexanoyl- and octanoyl-ACP. Thus, by purging the acetate primer units that otherwise dominate polyketide chain initiation, ZhuC (and presumably its homologues in other PKSs such as the doxorubicin and frenolicin PKSs) allows alternative primer units to be utilized by the elongation module in vivo. The abilities of other alkylacyl primer units to prime the *tcm* minimal PKS were also investigated in this report.

Type II polyketide synthases (PKSs,[1] also known as bacterial aromatic PKSs) synthesize polycyclic aromatic polyketides from primary metabolites such as malonyl-CoA through repeated decarboxylative condensations (*1*). The gene clusters of numerous type II PKSs have been sequenced, revealing a minimal set of enzymes required for the biosynthesis of a complete polyketide chain (*2*). The minimal PKS includes the heterodimeric ketosynthase−chain length factor (KS-CLF) (*3*), an acyl carrier protein (ACP), and a malonyl-CoA:ACP acyltransferase (MAT). The MAT (*4*), which is shared between the PKS and the fatty acid synthase (FAS), transfers malonyl groups from malonyl-CoA to the phosphopantetheinyl arm of an ACP. Malonyl-ACP is then recruited by the KS-CLF for chain elongation. For many aromatic polyketides such as actinorhodin (*act*) (*5*) and tetracenomycin (*tcm*) (*6*), polyketide biosynthesis is initiated by an acetyl-ACP, which can arise through the decarboxylation of a malonyl-ACP (Figure 1, pathway I).

Recently, we have demonstrated that, in addition to priming by an acetyl-ACP, the *act* and *tcm* KS-CLFs can be primed by alkylacyl groups of various sizes when a suitable initiation module is present (*7*). We showed that polyketides primed with pentanoyl, hexanoyl, or isohexanoyl primer units can be synthesized in good yields by *Streptomyces coelicolor* when minimal PKSs (also referred to as elongation modules throughout the text) and the R1128 initiation module are coexpressed. The R1128 initiation module (part of the R1128 PKS cloned from *Streptomyces* sp. R1128) consists of three unique proteins: ZhuG, ZhuH, and ZhuC (*8*). ZhuG is a dedicated ACP (ACP_p) that carries the primer unit through the steps of the initiation module (*9*). ZhuH is a ketosynthase III (KSIII) homologue that condenses short-chain acyl-CoAs, such as propionyl-CoA and isobutyryl-CoA, with malonyl-ZhuG to yield a β-ketoacyl-

† This work was supported by a grant from the NIH (CA 77248 to C.K.). Y.T. and A.T.K. were both supported by postdoctoral fellowships from the NIH.

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[1] Abbreviations: PKS, polyketide synthase; FAS, fatty acid synthase; CoA, coenzyme A; KS, ketosynthase; CLF, chain length factor; ACP, acyl carrier protein; ACP_p, acyl carrier protein found in initiation modules; MAT, malonyl-CoA:ACP transacylase; KSIII, ketoacyl synthase III; KR, ketoreductase; DH, dehydratase; ER, enoylreductase; TCA, trichloroacetic acid; TE, thioesterase; MWCO, molecular weight cutoff; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography.

10.1021/bi049157k CCC: $27.50
Published on Web 07/07/2004

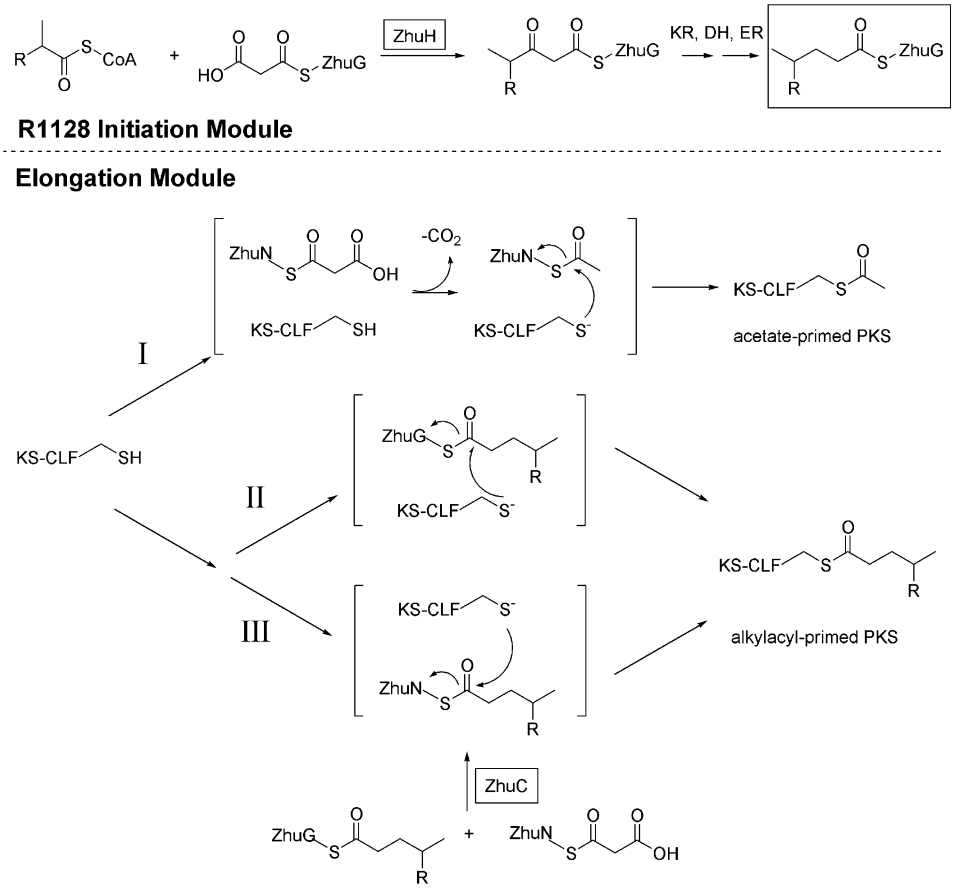


FIGURE 1: Alternative mechanisms for intermodular chain transfer in a bimodular type II polyketide synthase. Shown at the top is the R1128 initiation module, which produces alkylacyl-ZhuG. Shown at the bottom is the elongation module. The KS-CLF can be primed with either alkylacyl-ZhuG (pathways II and III) or acetyl-ZhuN (pathway I), which is derived from decarboxylation of malonyl-ZhuN. It is unknown how the alkylacyl group is transferred from ZhuG to KS-CLF. One possible mode is via a direct thioester exchange between alkylacyl-ZhuG and KS-CLF (pathway II). Alternatively, the acyltransferase homologue ZhuC can transfer the alkylacyl group onto the elongation module ACP (ZhuN), followed by a thioester exchange between alkylacyl-ZhuN and KS-CLF (pathway III).

ZhuG (Figure 1) (*10*). The β-ketoacyl-ZhuG is subsequently converted to an alkylacyl-ZhuG moiety by ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) associated with the FAS. The alkylacyl-ZhuG group is able to prime the KS-CLF of an elongation module through an undetermined mechanism. Transferring the alkylacyl group to the active site cysteine of KS-CLF completes polyketide priming and commits the PKS to the biosynthesis of alkylacyl-primed polyketides.

The function of the third component of the initiation module, ZhuC, has not been resolved. ZhuC displays high sequence similarity (25% identity) to MAT and was initially proposed to be an acyltransferase enzyme (*10*). Homologues of ZhuC are found in other PKSs that utilize non-acetate primer units, including DpsD from the daunorubicin (*dnr*) PKS (which uses a propionyl starter unit) (*11*), FrenK from the frenolicin (*fren*) PKS (which uses a butyryl starter unit) (*12*), and EncL from the enterocin (*enc*) PKS (which uses a benzoyl starter unit) (*13*). The functions of ZhuC homologues in the priming steps of their respective PKSs are not clear. DpsD was shown to be nonessential for propionyl priming during the biosynthesis of daunorubicin in *Streptomyces peucetius* (*14*). We initially assigned ZhuC as a malonyl-CoA:ACP transferase dedicated to the initiation module (i.e., to convert holo-ZhuG into malonyl-ZhuG) (*10*). Subsequently, we observed that holo-ZhuG is an excellent substrate of the FAS MAT in vitro (k_{cat} > 8000 min^{-1}) (*9*), whereas ZhuC is sluggish at malonylating ZhuG (at least 10 times slower than MAT). Therefore, under in vivo conditions, MAT alone should be sufficient for the malonylation of ZhuG. However, we noted that expression of ZhuC is required for the in vivo biosynthesis of alkylacyl-primed polyketides in *S. coelicolor (7)*, which suggested that ZhuC has an essential function in the R1128 initiation module and may be involved in the cross-talk between the initiation and elongation modules.

The KS-CLF may be loaded with the alkylacyl moiety by two distinct mechanisms. In one model, the alkylacyl-ZhuG binds to the KS-CLF and a direct thioester exchange between the two proteins can occur, resulting in a non-acetate-primed KS-CLF (Figure 1, pathway II). This model alone does not explain why ZhuC is essential for non-acetate priming of the KS-CLFs in vivo. Alternatively, the alkylacyl group can be first transacylated onto a minimal PKS ACP (e.g., ZhuN). The KS-CLF can subsequently capture the starter unit from alkylacyl-ZhuN (Figure 1, pathway III) and proceed with chain extension. In the second model, ZhuC assumes the role of a transacylase that transfers the alkylacyl group from an initiation ACP to an elongation ACP.

In this work, we studied the priming properties of the KS-CLF in vitro and the functional role of ZhuC in the initiation process. We observed that the *tcm* KS-CLF can be primed efficiently by medium-chain-length CoAs (hexanoyl and octanoyl) at ~100 μM acyl-CoA concentration and by the

corresponding acyl-ZhuGs at low micromolar concentration in the absence of ZhuC (hence validating pathway II in Figure 1). We also observed that whereas acetyl- and propionyl-ACP are rapidly hydrolyzed by ZhuC, long-chain acyl-ACPs are not. Together, these results suggest that ZhuC serves as an acetyl-ACP thioesterase that attenuates the acetyl priming pathway in favor of non-acetate priming. Finally, we show that although ZhuC does catalyze the transacylation between acyl-ZhuG and holo-ZhuN, the rate of acyl transfer is too slow to be physiologically relevant in vivo.

MATERIALS AND METHODS

Materials. [1-^{14}C]Propionyl-CoA (55 mCi/mmol) and [1-^{14}C]acetyl-CoA (50 mCi/mmol) were purchased from Moravek Biochemicals; [2-^{14}C]malonyl-CoA, [1-^{14}C]butyryl-CoA, [1-^{14}C]hexanoyl-CoA, and [1-^{14}C]octanoyl-CoA (all at 55 mCi/mmol) were from American Radiolabeled Chemicals. Nonradioactive acyl-CoAs were from Sigma Chemical Co. and were stored as 100 μL aliquots (20 mM) in 50 mM NaH_2PO_4 (pH 3.5) at −80 °C. Phenyl-Sepharose resin and HiTrap Q anion-exchange column were purchased from Amersham Biosciences. Precast SDS−PAGE gels were from Bio-Rad.

Protein Expression and Purification. The *tcm* KS-CLF was expressed from *S. coelicolor* strain CH999/pYT127. Purification of *tcm* KS-CLF was performed as described before (*9*). MAT was expressed from *Escherichia coli* strain BL21(DE3)/pGFL16 and was purified as described previously (*15*). His_6-ZhuG was expressed from BL21(DE3)/pEMS10 and was purified as described (*10*). Untagged ZhuN and FrenN (an elongation ACP from the frenolicin PKS) were expressed from BL21(DE3)/pYT221 and BL21(DE3)/pFren, respectively, and were purified in two chromatographic steps as described (*9*, *15*). His_6-ZhuC was expressed from BL21(DE3)/pESM8. ZhuC was first purified via Ni-NTA chromatography under native conditions as recommended by Qiagen. Fractions containing ZhuC were pooled, concentrated, and buffer exchanged into buffer A (100 mM NaH_2PO_4, pH 7.4, 2 mM DTT, 2 mM EDTA) using a 10000 molecular weight cutoff (MWCO) spin column (Amicon Ultra, Millipore). The concentrated ZhuC was loaded onto a HiTrap Q anion- exchange column equilibrated with buffer A. A linear gradient of increasing NaCl concentration was applied to the column at a flow rate of 2 mL/min. ZhuC was eluted at 300 mM NaCl. Pure fractions were combined, concentrated, and buffer exchanged into buffer A. Aliquots were flash frozen with liquid nitrogen and stored at −80 °C.

Modification of Apo-ACP. The conversion of apo-ACP to holo-ACP was carried out in buffer S (75 mM MES−acetate buffer, pH 6.0, 10 mM $MgCl_2$, 5 mM DTT) using the phosphopantetheinyl transferase Sfp (*16*). The molar ratios of CoASH, apo-ACP, and Sfp were maintained at 5:1:0.1. The reaction was incubated at 30 °C overnight and loaded directly onto a HiTrap Q column equilibrated with buffer A. All holo-ACPs eluted at ∼350 mM NaCl. Purified holo-ACP was exchanged into buffer A, concentrated, and stored at −80 °C. For the conversion of apo-ACP into radiolabeled acyl-ACP, buffer S without DTT was used. Apo-ACP and Sfp were added to final concentrations of 100 and 10 μM, respectively, and radiolabeled acyl-CoA (27 mCi/mmol) was added to a final concentration of 300 μM. The final volume of the reaction was 300 μL. After overnight incubation at 30 °C, precipitated protein was removed via centrifugation. The reaction mixture was diluted to 2.5 mL with buffer S (no DTT) and loaded onto a PD-10 desalting column (Amersham). Acyl-ACPs were eluted with 3.5 mL of buffer S (no DTT) and concentrated with a 5000 MWCO spin column (Amicon Ultra, Millipore). The residual solution was diluted with the same buffer and concentrated again to remove as much of the radiolabeled acyl-CoA as possible. One microliter of the final concentrate was applied to a SDS−PAGE gel, and the amount of radioactivity was quantified by autoradiography. The effective concentration of acyl-ACP was calculated from the specific activities of the acyl-CoA used. Labeled ACP proteins were stored at −20 °C.

PKS Product Assay with Radio-TLC. Assays are usually performed at 30 °C in a final volume of 10 μL. The assay buffer consists of 100 mM NaH_2PO_4, 2 mM DTT, and 10% glycerol. [^{14}C]Malonyl-CoA (4.5 mCi/mmol) was added to the final concentration of 2 mM. All reactions contained 1 μM KS-CLF, 200 nM MAT, and 30 μM holo-ZhuN. ZhuC, acyl-CoAs, or acyl-ACPs were added where necessary. After 15 or 30 min of incubation, the reaction mixture was extracted with 200 μL of ethyl acetate containing 1% acetic acid. The organic phase was evaporated and the residual dissolved in 20 μL of ethyl acetate. The reaction products were separated by thin-layer chromatography (TLC) (ethyl acetate/methanol/acetic acid, 89:10:1) and quantified with a phosphoimager as described before (*17*).

PKS Product Assay with HPLC. The reaction conditions were essentially the same as above, except nonradioactive malonyl-CoA was used. The reaction volume was increased to 100 μL to facilitate product detection. After 90 min of incubation, the polyketides were extracted with 600 μL of ethyl acetate containing 1% acetic acid. The organic phase was evaporated to dryness, and the residual was dissolved in 30 μL of dimethyl sulfoxide (DMSO). The polyketide products were separated and detected by analytical reverse-phase HPLC using a diode array detector at 280 and 410 nm [Alltech Econosphere C18 column (50 mm × 4.6 mm); linear gradient, 20% acetonitrile in water (0.1% TFA) to 60% acetonitrile in water (0.1% TFA) over 30 min; 1 mL/min]. Alternatively, the DMSO solution was directly analyzed via LC/MS, performed at the Vincent Coates Foundation Mass Spectrometry Laboratory at Stanford University using a ThermoFinnigan quadrupole ion trap LC-MS system and electrospray ionization (both positive and negative ionization).

ZhuC Hydrolysis Assay. The hydrolysis assay was performed using either TCA precipitation or gel autoradiography. The reaction buffer consisted of 100 mM NaH_2PO_4, 2 mM DTT, and 10% glycerol. Five ACP concentrations (between 1 and 20 μM) were used in the assays. The concentrations of ZhuC used in the assays varied with the substrate. For acetyl- and propionyl-ACPs, 10 nM ZhuC was used to observe a time-dependent decrease of ACP radiolabel. For sluggish substrates such as hexanoyl- and octanoyl-ACP, 1 μM ZhuC was used. Addition of ZhuC to the mixture containing acyl-ACP initiated the reaction. In the TCA assay, aliquots (7 μL) were removed at four time points and spotted on filter disks (3MM chromatography paper, Whatman)

presoaked with 10% TCA. At the end of the assay, the filter disks were rinsed with ice-cold 10% TCA twice, followed by 95% ethanol twice. Dried disks were counted using a scintillation counter.

In the gel autoradiography assay, aliquots (10 μL) were removed at three time points and quenched with 5 μL of the 3× SDS−PAGE loading dye without any reducing agents. The quenched samples were heated briefly (30 s) at 70 °C and loaded onto a 4−20% gradient SDS−PAGE gel (Ready Gel, Bio-Rad). After electrophoresis, the gel was stained, destained, and dried. The radioactivities of ACPs were counted using autoradiography.

For either assay, the radioactivities measured at the time points were converted to micromolar acyl-ACPs using internal calibration standards. The data for each substrate were fitted to Michaelis−Menten kinetics to yield the reported k_{cat} and K_m values shown in Table 1. We were not able to reach saturating concentrations for some of the acyl-ACP substrates, thus precluding the measurement of k_{cat} and K_m values separately. For these substrates, we fitted the linear regions of the V vs [S] plot and determined the ratios of k_{cat}/K_m.

Transacylation Assay. The transacylation assay was performed similarly to the hydrolysis assay, except two ACPs were added to the reaction mixture. Hexanoyl- or octanoyl-ZhuG was added to a final concentration of 3 μM, while holo-ZhuN was added to a final concentration of 30 μM. At specific time points (Figure 8), aliquots (10 μL) were removed from the reaction mixture and quenched with 5 μL of the 3× SDS−PAGE loading dye, heated briefly, and loaded onto a 16.5% Tris−tricine gel (Ready Gel, Bio-Rad). The transfer of the radiolabel was visualized by autoradiography.

RESULTS

Protein Expression, Purification, and Modification. The expression and purification of *tcm* KS-CLF from *S. coelicolor* has been previously described in detail (*9*). ZhuC was expressed with an N-terminal His_6 tag from *E. coli* strain BL21(DE3)/pESM8. Overexpressed ZhuC was purified by a nickel affinity chromatography step followed by an anion-exchange chromatography step. ZhuC without the His_6 affinity tags was not prepared in this work. All ACPs were expressed as apo proteins from *E. coli* and were purified as described previously (*15*). His-tagged ZhuG was purified with a Ni-NTA metal affinity chromatography step to homogeneity. Elongation ACPs without His tags (ZhuN and FrenN) were first purified with phenyl-Sepharose chromatography, followed by anion-exchange chromatography. Apo-ACPs were converted to the holo forms by using the phosphopantetheinyl transferase Sfp, as previously described (*16*).

Labeling of apo-ZhuG with various acyl-CoAs was achieved with Sfp. The efficiency of the modifications varied significantly with the acyl substrate. Apo-ZhuG could be quantitatively labeled with acetyl-, propionyl-, or malonyl-CoA, while only 10−20% of ZhuG could be labeled with butyryl-, hexanoyl-, or octanoyl-CoA after overnight incubation at 30 °C. Excess acyl-CoAs were removed with a PD-10 size-exclusion column, followed by repeated washing and concentration in a 5000 MWCO spin column. Unlabeled apo-ZhuG and Sfp were not removed from the labeled ZhuG for subsequent assays.

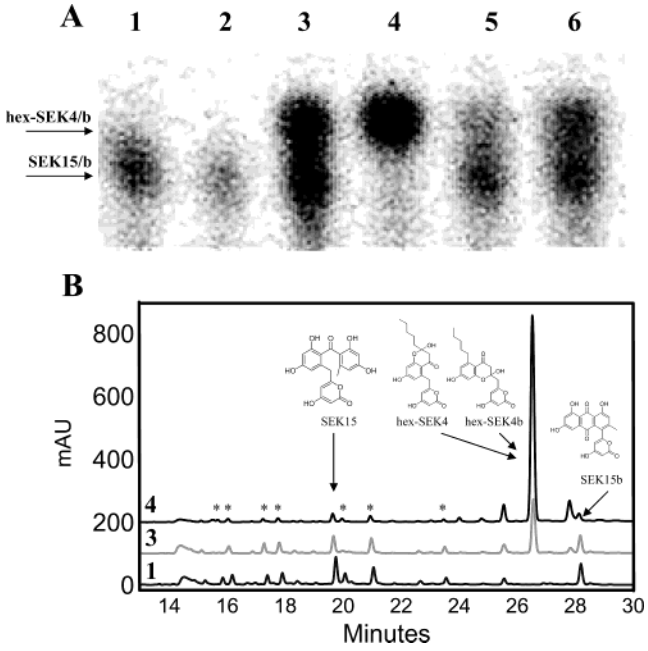


FIGURE 2: Hexanoyl-ZhuG/hexanoyl-CoA can prime *tcm* KS-CLF in the absence of ZhuC. (A) Radio-TLC analysis of polyketides produced by minimal *tcm* PKS [1 μM KS-CLF, 200 nM MAT, 2 mM [^{14}C]malonyl-CoA (4.5 mCi/mmol), and 30 μM holo-ZhuN]. Lane 1: Minimal PKS alone produced SEK15 and SEK15b, as expected. Lane 2: Addition of 2 μM ZhuC resulted in 3-fold reduction in polyketide yield. Lane 3: Minimal PKS + 500 μM hexanoyl-CoA. The new spot corresponds to a mixture of hexanoyl-primed SEK4 and SEK4b (see panel B). Lane 4: Minimal PKS + 500 μM hexanoyl-CoA + 2 μM ZhuC. Hex-SEK4/SEK4b are the dominant products. Lane 5: Minimal PKS + 1 μM hexanoyl-ZhuG. Lane 6: Minimal PKS + 4 μM hexanoyl-ZhuG. Reaction conditions: 10 μL reaction volume; 30 °C incubation for 30 min. Under these radio-TLC conditions, both acetate-primed polyketides as well as both hexanoyl-primed polyketides comigrated. (B) HPLC analysis of polyketide products. The reactions are the same as in (A) (nonradiolabeled malonyl-CoA only) in 100 μL reaction volume and were incubated at 30 °C for 90 min before analysis. The numbers on the left side of the traces correspond to the lane numbers in (A). Asterisks indicate unidentified decaketides (m/z = 384). Hex-SEK4 and hex-SEK4b coelute under the HPLC conditions (see Materials and Methods) but could be resolved as two separate peaks with the same mass (m/z = 374) using LC-MS.

Hexanoyl-CoA and Hexanoyl-ZhuG Can Prime the tcm KS-CLF Directly. To investigate whether KS-CLF could be directly primed by alkylacyl-ZhuG-derived primer units (Figure 1, pathway II), an in vitro assay was employed in which polyketides produced by the *tcm* KS-CLF were quantified by either radio-TLC (using [^{14}C]malonyl-CoA) (*17*) or HPLC. Alkylacyl-primed polyketides could readily be distinguished from the acetate-primed products. Both hexanoyl-CoA and hexanoyl-ZhuG were used as acyl donors in these assays. Hexanoyl-ZhuG represents the most similar substrate to pentanoyl-ZhuG and isohexanoyl-ZhuG, the main products of the R1128 initiation module (neither pentanoyl-CoA nor isohexanoyl-CoA were commercially available) (*10*). The results of these assays are shown in Figure 2.

In the presence of malonyl-CoA, the minimal *tcm* PKS (consisting of 1 μM KS-CLF, 200 nM MAT, and 30 μM holo-ZhuN) produced SEK15 and SEK15b as the main products (*18*), along with several unidentified decaketides (m/z = 384) (Figure 2A, lane 1, and Figure 2B, trace 1).

When either hexanoyl-CoA (500 μM, lane 3, and Figure 2B, trace 3) or hexanoyl-ZhuG (1 and 4 μM, lanes 5 and 6) was added to the minimal PKS, a new product was observed. The product was labeled with either [^{14}C]hexanoyl-CoA or [^{14}C]malonyl-CoA, confirming its identity as a hexanoyl-primed polyketide. Analysis of this reaction mixture by LC-MS facilitated resolution of the new peak into two peaks with identical masses (m/z = 374). The identity of these compounds as hexanoyl-primed analogues of SEK4 (hex-SEK4) and SEK4B (hex-SEK4b) was established in comparison with authentic samples obtained biosynthetically (*19*). The effect of ZhuC on the activity of the minimal PKS was also investigated (lanes 2 and 4). Addition of 2 μM ZhuC resulted in a 3-fold reduction in the amount of polyketide produced by the minimal *tcm* PKS (Figure 2A, lane 2). In contrast, when 2 μM ZhuC was added to the minimal PKS along with 500 μM hexanoyl-CoA (Figure 2A, lane 4), the yield of hex-SEK4 and hex-SEK4b increased substantially, thereby becoming the dominant polyketide products (>90%).

To further analyze the mechanism by which KS-CLF is primed by either hexanoyl-ZhuG or hexanoyl-CoA, we performed titration experiments by varying the concentrations of the acyl donors and ZhuC in the presence of the minimal *tcm* PKS. The relative amounts of SEK15/SEK15b (minimal PKS products) and hex-SEK4/SEK4b (bimodular PKS products) were quantified by radio-TLC (Figure 3). In the absence of ZhuC, hexanoyl-CoA was able to prime the KS-CLF at a concentration as low as 50 μM; equal amounts of both sets of polyketides were synthesized when 500 μM hexanoyl-CoA was added (Figure 3A, upper). The amounts of SEK15/SEK15b stayed relatively the same with increasing hexanoyl-CoA concentration. Addition of ZhuC led to increased hex-SEK4/SEK4b at lower hexanoyl-CoA concentrations. More importantly, the amount of SEK15/SEK15b was significantly decreased when ZhuC was introduced. The relative amounts of each set of polyketides at different hexanoyl-CoA concentrations are plotted in Figure 3B. At 100 μM hexanoyl-CoA concentration, addition of 500 nM ZhuC doubled the yield of hex-SEK4/SEK4b from 35% of total products to nearly 70%. At higher hexanoyl-CoA concentrations, addition of 500 nM ZhuC in the reaction mix led to nearly exclusive synthesis of the hexanoyl-primed octaketides.

A similar trend of product distribution using hexanoyl-ZhuG as the acyl donor is observed in Figure 3A. In the presence of excess holo-ZhuN and malonyl-CoA, approximately half of the polyketides produced by the *tcm* minimal PKS containing 1.3 μM hexanoyl-ZhuG was primed with the hexanoyl starter unit. Higher concentrations of hexanoyl-ZhuG did not lead to an increase in the relative amounts of hex-SEK4/SEK4b in the absence of ZhuC. Identical to that observed in the presence of hexanoyl-CoA, addition of 500 nM ZhuC led to the exclusive synthesis of hex-SEK4/SEK4b at concentrations of hexanoyl-ZhuG shown in Figure 3A.

Three important conclusions can be drawn from these assays: (1) The *tcm* KS-CLF has remarkable affinity for hexanoyl-ZhuG even in the absence of ZhuC, suggesting that direct thioester exchange occurs between acyl-ZhuG and KS-CLF (Figure 1, pathway II). We have previously shown that malonyl-ZhuG is a poor substrate of KS-CLF (k_{cat}/K_m = 5.2 min^{-1} mM^{-1}) (*9*). The results reported here demonstrate that the acyl substrate is important in the recognition between

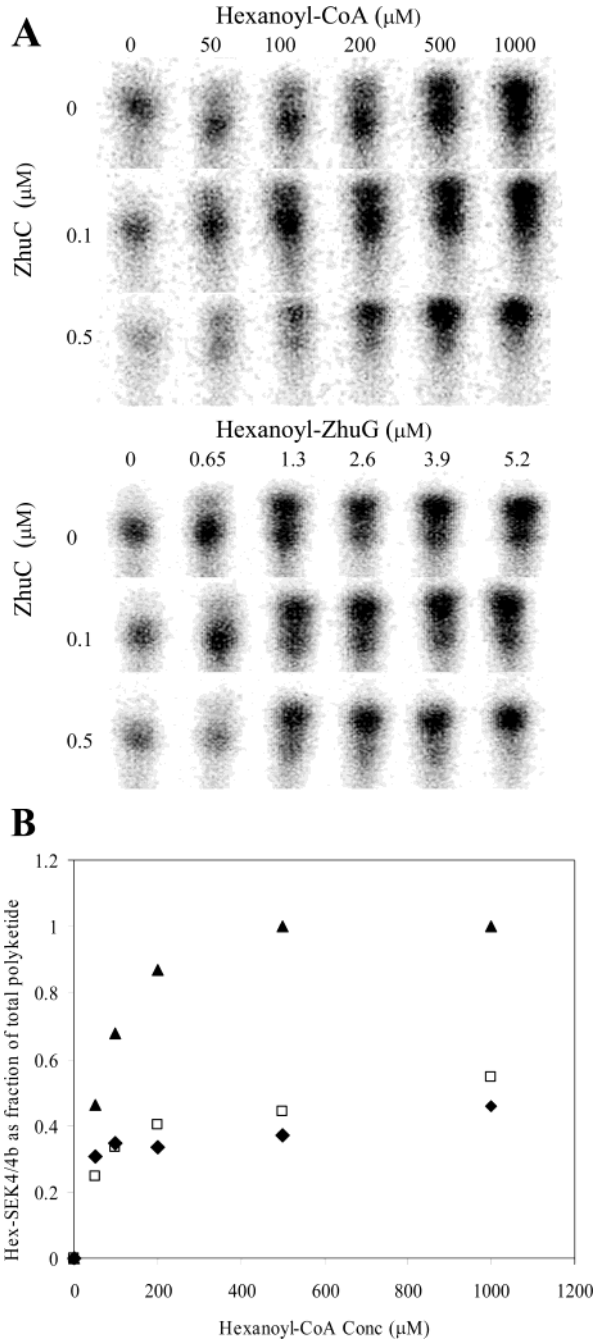


FIGURE 3: Titration of ZhuC with hexanoyl-CoA and hexanoyl-ZhuG in the presence of *tcm* minimal PKS. (A) Radio-TLC containing SEK15/SEKb and hex-SEK4/SEK4b is shown. Minimal PKS [1 μM KS-CLF, 200 nM MAT, 2 mM [^{14}C]malonyl-CoA (4.5 mCi/mmol), and 30 μM holo-ZhuN] in the presence of different concentrations of hex-CoA/hex-ZhuG and ZhuC produced different ratios of SEK15/SEKb (lower spot) to hex-SEK4/SEK4b (upper spot). ZhuC decreases the amount of acetate primed products and increases the relative amounts of the hexanoyl primed polyketide. Reaction conditions: 10 μL total reaction volume; 30 °C incubation for 30 min. (B) Hex-SEK4/SEK4b as a fraction of total polyketide produced as the concentration of hexanoyl-CoA is varied. Polyketides are quantified by radio-TLC. Each curve represents a different concentration of ZhuC (◆, no ZhuC; □, 100 nM ZhuC; ▲, 500 nM ZhuC).

KS-CLF and ACP. (2) At high (nonphysiological) concentrations (~100 μM), hexanoyl-CoA can prime the *tcm* KS-CLF without the aid of an ACP_p. In contrast, hexanoyl-ZhuG can prime KS-CLF at significantly lower (physiological) concentrations, a feature that is presumably facilitated by

Table 1: Hydrolysis Kinetics of Various Acyl-ACPs by ZhuC[a]

	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})	k_{rel}[b]
acetyl-FrenN[c]	92.5 ± 9.5	6.39 ± 1.65	14.5	1.00
acetyl-ZhuN[c]	—[e]	—[e]	10.0 ± 0.29	0.69
acetyl-ZhuG[d]	212 ± 16.0	7.99 ± 1.32	26.5	1.83
propionyl-ZhuG[d]	171 ± 13.6	6.787 ± 1.08	25.1	1.74
butyryl-ZhuG[d]	—	—	3.17 ± 0.4	0.22
hexaonyl-ZhuG[d]	—	—	0.126 ± 0.007	0.009
octaonyl-ZhuG[d]	—	—	0.200 ± 0.012	0.014
malonyl-ZhuG[d]	—	—	0.065 ± 0.003	0.004

[a] Reaction conditions: 100 mM NaH_2PO_4, pH 7.4, 2 mM DTT, 25 °C. The concentrations of ZhuC ranged between 10 nM and 1 μM, depending on the activities of the substrates. [b] The relative k_{cat}/K_m values normalized against that of acetyl-FrenN. [c] These assays were performed using the TCA precipitation method (see Materials and Methods). [d] These assays were performed with gel autoradiography. [e] k_{cat} and K_m values not separately determined for these substrates because we cannot obtain saturating concentrations of the radioactive ACPs.

protein−protein interactions between the KS-CLF and ACP (*20*). (3) ZhuC decreases the level of acetate-primed polyketides produced by the *tcm* minimal PKS. Interestingly, ZhuC does not appear to affect the activity of the PKS if alkylacyl starter units are present. ZhuC therefore inhibits the acetate-priming pathway (Figure 1, pathway I) in favor of the alkylacyl-priming pathway (Figure 1, pathway II). ZhuC also inhibited the activities of other minimal PKSs, including the *act* PKS (octaketide synthase) and *sch* PKS (*21*) (a dodecaketide synthase from the *Streptomyces halstedii* spore pigment PKS) (data not shown).

ZhuC Is a Short-Chain CoA Thiolase. Because ZhuC selectively suppressed the formation of acetate-primed polyketides, we anticipated that ZhuC is an editing enzyme that deacylates acetyl-ACP prior to its binding to the KS-CLF. ZhuC therefore must hydrolyze the acyl group from acetyl-ACP rapidly, while leaving longer chain acyl-ACPs (e.g., pentanoyl-ZhuG) intact for priming of the KS-CLF. To investigate this possible role of ZhuC, we performed a quantitative study on acyl-ACP hydrolysis using either a TCA precipitation assay or a gel autoradiography assay. The results of the assays are shown in Table 1.

As expected, ZhuC rapidly hydrolyzed all acetyl-ACPs examined, regardless of the ACP identity. ZhuC concentration was lowered to 10 nM for both [^{14}C]acetyl-FrenN (an elongation ACP from the frenolicin PKS) and [^{14}C]acetyl-ZhuN in order to observe a time-dependent decrease in the amounts of radiolabeled ACPs. Both acetyl-ZhuG and propionyl-ZhuG were hydrolyzed slightly more rapidly than acetyl-FrenN. Butyryl-ZhuG was hydrolyzed by ZhuC at an 8-fold slower rate than propionyl-ZhuG. This is expected since *Streptomyces* sp. R1128, the native host of R1128 PKS, produces the butyryl-primed R1128 analogue (R1128A) at appreciable quantities (*22*). Hexanoyl-ZhuG and octanoyl-ZhuG were hydrolyzed approximately 200-fold and 120-fold slower than acetyl-ZhuG, respectively. ZhuC displayed a very slow hydrolysis rate toward malonyl-ACP, consistent with the observation that once the *tcm* PKS is primed with a hexanoyl group, ZhuC does not affect the rates of subsequent chain elongation steps (hydrolyzing malonyl-ACP at higher rates would deplete the polyketides extender units).

The high specificity toward acetyl-ACP and the potent thioesterase activity displayed by ZhuC show that it serves as a scavenger of acetyl-ACPs that can compete with the alkylacyl priming of the KS-CLF. The mechanism of decarboxylative priming is debated in the literature (*23*, *24*). We have proposed that the active site of the KS subunit is the location of decarboxylation (*24*), while others have proposed that decarboxylation takes place in the CLF, facilitated by the conserved glutamine residue (*23*). In our model, the acetyl-ACP generated at the KS subunit can be directly transferred to the KS active site cysteine or the acetyl-ACP can first dissociate from the KS-CLF dimer after decarboxylation, followed by reassociation to the KS active site. In the alternative model (*23*), the acetyl-ACP must completely dissociate away from the CLF "active" site, followed by subsequent binding to the KS subunit. In this model, we would anticipate a complete loss of minimal PKS activity in the presence of high concentrations of ZhuC since all dissociated acetyl-ACPs would be immediately deacylated. We were able to observe ~30% residual activity of the minimal PKS in the presence of 10 μM ZhuC (Figure 4). This is consistent with the partial dissociation of the acetyl-ACP from the KS active site. The residual minimal PKS activity arises from acetyl units directly transferred to the KS active site cysteine, which are inaccessible to the thioesterase activities of ZhuC.

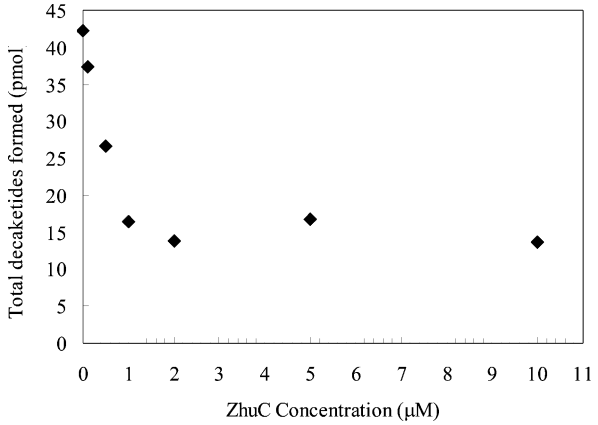


FIGURE 4: Incomplete inhibition of *tcm* minimal PKS by ZhuC. The amount of polyketides produced by the minimal *tcm* PKS (1 μM KS-CLF, 200 nM MAT, 2 mM [^{14}C]malonyl-CoA, and 30 μM holo-ZhuN) is measured in the presence of increasing levels of ZhuC. Reaction conditions: 10 μL total reaction volume; 30 °C incubation for 15 min. The incomplete inhibition of the minimal PKS by ZhuC suggests that only a fraction of the acetyl-ACP formed via decarboxylation dissociates from the KS active site prior to transfer of the acetyl primer to the KS active site.

The Role of ZhuC in Vivo. We demonstrated that, in the absence of ZhuC, hexanoyl-CoA and hexanoyl-ZhuG can prime the *tcm* KS-CLF and initiate synthesis of hex-SEK4 and hex-SEK4b in vitro (Figure 2A). This is in apparent contrast to our in vivo results in which exclusion of *zhuC* from an expression plasmid containing *tcm* KS-CLF, *zhuN*, *zhuH*, and *zhuG* led to the sole production of SEK15/SEK15b in *S. coelicolor* (*7*), whereas coexpression of ZhuC led to biosynthesis of alkyl-SEK4/SEK4b at a combined yield comparable to that of SEK15/SEK15b (*19*). Thus there must be other intracellular conditions that render ZhuC essential for alkylacyl priming in vivo. We hypothesized that since acetyl-CoA is a highly abundant metabolite in vivo (~100 μM) (*25*), the relative concentrations of acetyl-ZhuN must also be appreciable. Acetyl-ZhuN can accumulate through

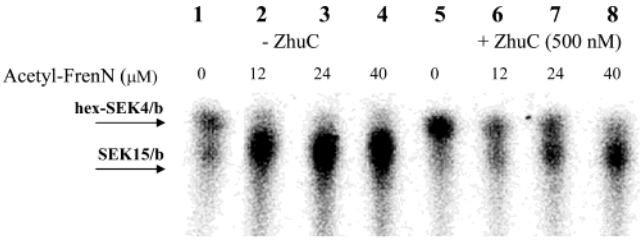


FIGURE 5: Efficiency of hexanoyl priming in the presence of acetyl-FrenN. Hexanoyl-CoA (500 μM) cannot prime the minimal PKS (1 μM KS-CLF, 200 nM MAT, 2 mM [^{14}C]malonyl-CoA, and 30 μM holo-ZhuN) in the presence of acetyl-FrenN (0, 12, 24, 40 μM). Addition of 500 nM ZhuC restores hexanoyl priming. Reaction conditions: 10 μL total reaction volume; 30 °C incubation for 15 min.

the actions of endogenous acetyltransferases, decarboxylation of malonyl-ZhuN, or even noncatalytic inter-thiol exchange in the presence of high concentrations of acetyl-CoA. Elevated concentrations of acetyl-ZhuN may lead to attenuation of alkylacyl priming in the absence of ZhuC. To assess this hypothesis, we performed the polyketide product assay in the presence of acetyl-FrenN (an equivalent substrate as acetyl-ZhuN). The results are shown in Figure 5.

Without acetyl-FrenN, addition of 500 μM hexanoyl-CoA resulted in equal levels of SEK15/SEK15b and hex-SEK4/SEK4b (lane 1), as shown previously. Upon addition of acetyl-FrenN (12, 24, and 40 μM), the synthesis of hex-SEK4/SEK4b was completely abolished (lanes 2−4). This showed that *tcm* KS-CLF has strong affinity for an acetyl-ACP, which is expected since tetracenomycin itself is derived from an acetate unit. Hexanoyl priming can be restored in the presence of acetyl-FrenN by the addition of 500 nM ZhuC. Hex-SEK4/SEK4b is produced as 58%, 48%, and 33% of all polyketides in the presence 12, 24, and 40 μM acetyl-FrenN, respectively, when ZhuC is included. These results demonstrate that ZhuC is indeed required for alkylacyl priming when micromolar concentrations of acetyl-ACP are present along with the KS-CLF (Figure 6).

Priming Properties of KS-CLF. To investigate the intrinsic preference of *tcm* KS-CLF for non-acetate primer units, we presented the *tcm* minimal PKS with 2 mM propionyl-, butyryl-, octanoyl-, decanoyl-, and lauroyl-CoAs. The reaction mixture (100 μL) was incubated at 30 °C for 90 min. Polyketide products were extracted with ethyl acetate, dried, redissolved in 30 μL of DMSO, and injected onto a HPLC column. The effect of ZhuC (2 μM) on KS-CLF priming by each acyl-CoA substrate was also analyzed. The results of the assays are shown in Figure 7. The reaction mixtures were also analyzed by LC-MS, and the masses of new compounds are listed in Table 2.

The *tcm* KS-CLF was primed with octanoyl-CoA readily as evident by the appearance of new polyketides during HPLC analysis (Figure 7A). Three new products (**1**, **2**, and **3**) were synthesized at approximately the same levels as the acetate-primed decaketides. Compound **1** has a mass of 378, corresponding to an octanoyl-primed heptaketide from which one water equivalent has been eliminated (presumably via cyclorelease of the polyketide chain). Compounds **2** and **3** have masses of 360, from which an additional water molecule was presumably eliminated. The structures of these compounds were not elucidated. Analogous to that observed with hexanoyl-CoA (Figure 2B), addition of both octanoyl-CoA

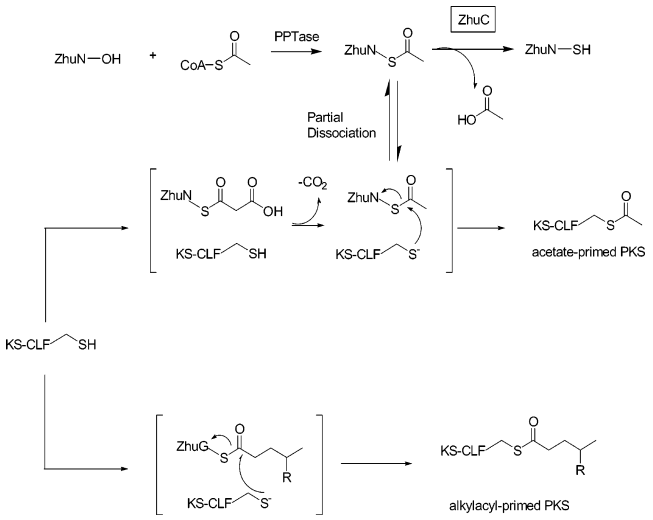


FIGURE 6: Priming mechanism for bimodular type II polyketide synthase in vivo. The KS-CLF can be primed by either acetyl-ACP or alkylacyl-ACP$_p$. Acetyl-ACP can accumulate by either decarboxylative priming of malonyl-ACP or by the action of endogenous PPTases. The presence of high levels of acetyl-ACP can prevent the binding of alkylacyl-ACP$_p$ to KS-CLF. ZhuC serves as an acetylthioesterase that hydrolyzes acetyl-ACP. Decreased levels of acetyl-ACP allow alkylacyl-ACP$_p$ to prime the KS-CLF. Acetate priming is not completely suppressed by ZhuC because acetyl-ACP derived from decarboxylative priming can presumably be transferred directly to the KS-CLF active site cysteine without dissociation.

and ZhuC to the minimal PKS resulted in the synthesis of the three octanoylheptaketides as the predominant products.

When presented with decanoyl-CoA, the *tcm* KS-CLF was unable to synthesize any acetate-primed decaketides. A new compound (**4**) with a mass of 364 was observed. This corresponds to a monodehydrated, decanoyl-primed hexaketide. We have previously determined the structures of pentanoyl- and isohexanoyl-primed hexaketides (*19*). Compound **4** can be expected to be the decanoyl-primed analogue of these polyketides. Since acetate priming is completely suppressed by decanoyl-CoA, addition of ZhuC had no incremental influence on product distribution. It must be noted, however, that the yield of **4** by *tcm* KS-CLF is significantly lower than either hex-SEK4/SEK4b or compounds **1**−**3**. Addition of lauroyl-CoA (C12) to the *tcm* minimal PKS completely abolished all polyketide synthesis (data not shown). These results suggest that the polyketide binding pocket of the KS-CLF is a lipophilic environment in which the presence of a poly-β-carbonyl backbone facilitates chain extrusion. A KS-CLF primed with a decanoyl starter unit turns over at a substantially slower rate, resulting in the observed reduction in polyketide synthesis (Figure 7A). A KS-CLF primed with a lauroyl starter unit is unable to release the polyketide with a long hydrophobic tail, resulting in complete inhibition of the PKS.

Figure 7B shows the products synthesized by the *tcm* minimal PKS in the presence of propionyl-CoA and butyryl-CoA. In the absence of ZhuC, most of the polyketides produced remained acetate-primed decaketides. New compounds **5** and **6** were present at low levels when propionyl-CoA was included without ZhuC, whereas no new compounds were detected when butyryl-CoA was included. Upon addition of 2 μM ZhuC, the levels of acetate-primed

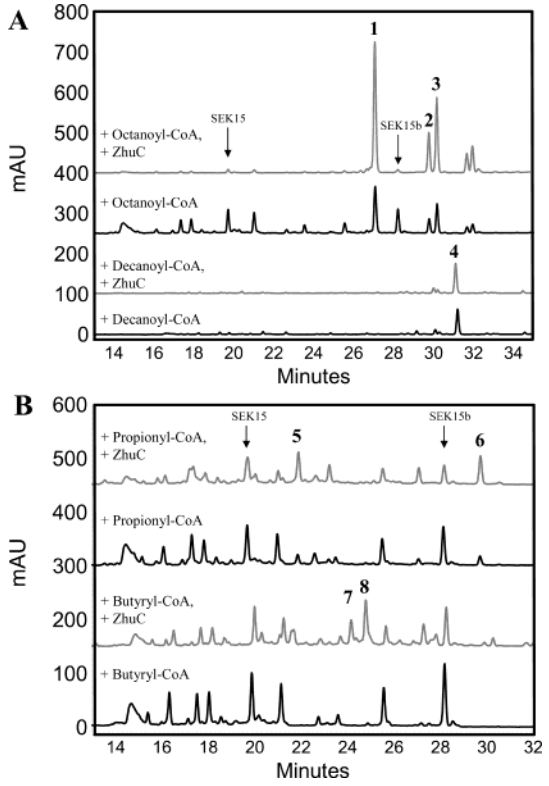


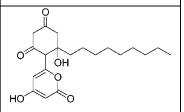
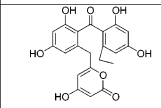
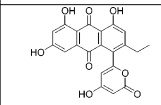
FIGURE 7: KS-CLF can be primed with a variety of acyl-CoAs. HPLC analysis of polyketides produced by the minimal *tcm* PKS (1 μM KS-CLF, 200 nM MAT, 2 mM malonyl-CoA, and 30 μM holo-ZhuN) in the presence of (A) octanoyl-CoA and decanoyl-CoA and (B) propionyl-CoA and butyryl-CoA. Acyl-CoAs are added to a final concentration of 2 mM. The effect of adding ZhuC (2 μM) is also shown for each acyl-CoA addition. Reaction conditions: 100 μL total reaction volume; 30 °C incubation for 90 min. SEK15 and SEK15b are shown with arrows. New compounds are numbered and tabulated in Table 2.

decaketides are lowered, and new polyketides derived from either propionyl- or butyryl-CoA can be detected. LC-MS analysis confirmed that **5** and **6** were indeed propionyl-primed analogues of SEK15 and SEK15b. Thus the *tcm* KS-CLF is able to accommodate a polyketide backbone as long as 21 carbon atoms. No butyryl-primed analogues of SEK15 and SEK15b were observed, suggesting the KS-CLF cannot tolerate a 22-carbon backbone. Instead, two butyryl-primed nonaketides (**7** and **8**) were detected. The mass of both compounds was 370, corresponding to the loss of three water molecules from the nascent polyketides.

ZhuC Catalyzes the Slow Transacylation between Acyl-ZhuG and ZhuN. An alternative role proposed for ZhuC is an ACP_p:ACP transacylase that catalyzes the transacylation between alkylacyl-ZhuG and holo-ZhuN (Figure 1, pathway III). To investigate this possibility, we performed an acyl-ZhuG:ZhuN thiol exchange assay. Radiolabeled His_6-tagged ZhuG (MW 11.5 kDa) was used as the acyl donor, and untagged holo-ZhuN (8.6 kD) was used as the acyl acceptor. The two proteins could be separated on a 16.5% Tris−tricine polyacrylamide gel, and the radiolabel was visualized by autoradiography.

Figure 8 shows the results of the transacylation assay for [^{14}C]hexanoyl-ZhuG (3 μM) and [^{14}C]octanoyl-ZhuG (3 μM). A 10-fold excess of holo-ZhuN (30 μM) was added to shift the transacylation equilibrium toward acyl-ZhuN. Transacylation was indeed observed for both hexanoyl-ZhuG (Figure 8A) and octanoyl-ZhuG (Figure 8B) at ZhuC concentrations of 0.5 and 1 μM. No transfer is observed in the absence of ZhuC or in the presence of MAT. The transacylation rates of both hexanoyl- and octanoyl-ZhuG were, however, very slow (~0.3 min^{-1}), as the majority of

Table 2: New Polyketides Produced by the *tcm* Minimal PKS through Non-Acetate Priming in Vitro[a]

Compound	Mass	Backbone[b]	Dehydration[c]/ Oxidation	Structure[d]
1	378	octanoyl-primed heptaketide (C20)	-1 H_2O	Unknown
2	360		-2 H_2O	Unknown
3	360		-2 H_2O	Unknown
4	364	decanoyl-primed hexaketide (C20)[e]	-1 H_2O	
5	398	propionyl-primed decaketide (C21)	-3 H_2O	
6	394		-4 H_2O, 1 [O]	
7	370	butyryl-primed nonaketide (C20)	-3 H_2O	Unknown
8	370		-3 H_2O	Unknown
Hex-SEK4/SEK4b	374	hexanoyl-primed octaketide (C20)	- 2 H_2O	See Figure 3B

[a] Compounds are numbered as shown in Figure 8. For assay conditions, see Figure 7 legend. [b] The term backbone represents the nascent polyketide before any cyclization or oxidation. The size of the backbone is inferred from the mass of the molecule. [c] In this table, we included hydrolysis of the enzyme-bound polyketide (thioester hydrolysis) as one dehydration event. [d] All structures are tentative since no NMR spectra of compounds have been collected. [e] This compound should rapidly dehydrate as in the case of YT84b (*19*).

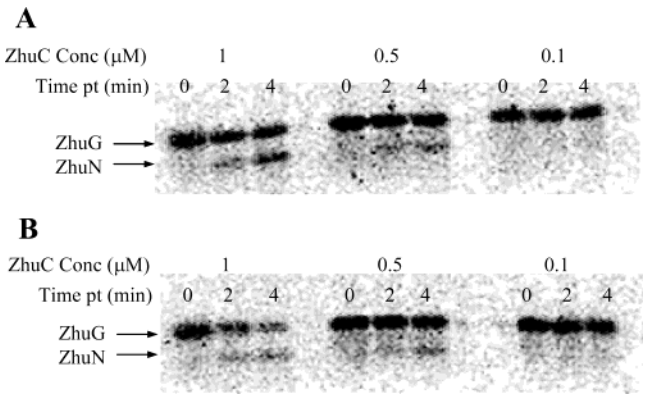


FIGURE 8: ZhuC catalyzes slow transacylation between ZhuN and ZhuG. Acyl-ZhuG (3 μM) and holo-ZhuN (30 μM) are incubated in the presence of ZhuC (1, 0.5, and 0.1 μM). At indicated times, aliquots were quenched and loaded onto 16.5% gel. The transfer of radiolabel from ZhuG to ZhuN is visualized by autoradiography of the SDS−PAGE gel. (A) ZhuC catalyzes transfer of the [^{14}C]-hexanoyl unit from ZhuG to ZhuN. (B) ZhuC catalyzes transfer of the [^{14}C]octanoyl unit from ZhuG to ZhuN. The rates of acyl transfer for both acyl-ZhuG are estimated to be less than 0.3 min^{-1}. No transfer is observed at 0.1 μM ZhuC concentration.

acyl groups remained on ZhuG. Under in vivo conditions, the slow rate of transacylation is unlikely to generate sufficient amounts of acyl-ZhuN for priming the KS-CLF. Most holo-ZhuN species will be converted into malonyl-ZhuN by the MAT ($k_{cat} > 3000$ min^{-1}) (*9*).

We also measured the transacylation rate between [^{14}C]-malonyl-ZhuG and ZhuN in the presence of ZhuC. Not surprisingly, ZhuC was also able to transfer the [^{14}C]malonyl group onto ZhuN at a similar rate as shown in Figure 8 (data not shown). On the basis of these results, we ruled out ZhuC as a transacylase facilitating the acyl transfer from the initiation module to the elongation module in vivo.

DISCUSSION

ZhuC and its homologues, such as FrenK from the frenolicin biosynthetic pathway and DpsD from the daunorubicin pathway, are only found in type II PKSs equipped with initiation modules. Our investigation of ZhuC was motivated by its essential role in the biosynthesis of non-acetate-primed polyketides by both the *act* and the *tcm* minimal PKS in *S. coelicolor*. Here we have shown that ZhuC is a potent thioesterase that selectively hydrolyzes acetyl-ACPs. ZhuC is therefore an editing enzyme for removing acetate starter units that might otherwise compete with alkylacyl-ZhuG species for priming the elongation PKS module (Figure 6).

Proofreading enzymes exhibiting similar functions as ZhuC are present in type I PKSs and nonribosomal peptide synthases (*26*). The stand-alone thioesterase II (TEII) enzymes found in the gene clusters of erythromycin (*ery*) (*27*), pikromycin (*pik*) (*28*), and other macrolides catalyze analogous reactions. The *pik* TEII hydrolyzes incorrectly loaded short-chain fatty acids from ACPs during pikromycin biosynthesis (*29*). The *ery* TEII selectively hydrolyzes acetyl groups attached to ACP_L of the loading didomain, while leaving propionyl-ACP_L intact (*27*). The presence of *ery* TEII dramatically increased the ratio of 6-DEB (propionate-primed) to 15-nor-6DEB (acetate-primed) (*27*). Elucidation of the functional role of ZhuC expands this proofreading paradigm to type II PKSs.

The *tcm* KS-CLF can be primed directly with alkylacyl-CoAs ranging from propionyl-CoA to decanoyl-CoA. The priming efficiencies of the acyl-CoA species at the concentration examined (2 mM) relative to acetate priming vary with acyl group chain length. Short-chain-length acyl-CoAs such as propionyl-CoA and butyryl-CoA prime the KS-CLF at a slow rate, hence requiring the addition of ZhuC to suppress the dominant acetate-priming pathway. Medium-chain-length acyl-ACPs such as hexanoyl-ACP and octanoyl-ACP prime the KS-CLF at comparable rates to acetyl-ACP, resulting in the synthesis of comparable quantities alkylacyl- and acetate-primed polyketides. Addition of ZhuC boosts the amount of hexanoyl- and octanoyl-primed compounds to >90% of all polyketides observed. Long-chain substrates such as decanoyl-CoA and lauroyl-CoA bind to the KS-CLF with high affinity, resulting in the complete loss of acetate priming. However, polyketide synthases primed with decanoyl-CoA turn over slowly, whereas those primed with lauroyl-CoA cannot be released by the KS-CLF.

On the basis of our understanding of ZhuC, we can speculate about the properties of its homologues found in other non-acetate-primed polyketide pathways. FrenK, the corresponding thioesterase present in the frenolicin initiation module (*12*), must selectively hydrolyze acetyl-FrenN to facilitate priming of *fren* KS-CLF by butyryl-FrenJ. Similarly, DpsD found in the initiation module of *dnr* PKS may participate in the proofreading of acetyl-DpsG during daunorubicin biosynthesis in *S. peucetius (11)*. However, DpsD was previously determined to be nonessential for propionate priming (*14*). The most likely reason for the dispensable role of DpsD is that propionyl-CoA is present at high concentrations in *S. peucetius*. Additionally, there might be intrinsic differences in propionate specificity between the *tcm* KS-CLF and the *dnr* KS-CLF. We observed that *tcm* KS-CLF is primed poorly by propionyl-CoA (2 mM) in the absence of ZhuC (Figure 7B). It is likely that the *dnr* KS-CLF has a significantly higher affinity for propionyl-CoA. Indeed, in the absence of both DpsC and DpsD, aklanonic acid (propionate primed) and desmethylaklanonic acid (acetate primed) are synthesized at a ratio of 3:2 by the *dnr* PKS (*14*), suggesting that propionyl-CoA is able to directly prime the daunorubicin KS-CLF at comparable levels as acetyl-DpsG.

In a recent study (*30*), the ZhuC homologue EncL found in the *enc* PKS was also shown to be unnecessary for the benzoate priming of *enc* KS-CLF. The *enc* gene cluster has a dedicated set of enzymes that synthesize benzoyl-CoA using two different pathways. Thus the intracellular concentration of benzoyl-CoA is likely to be appreciable. More importantly, the authors showed that the *enc* KS-CLF has no affinity toward acetate starter units (*30*). Heterologous expression of the minimal *enc* PKS without the benzoyl-ACP biosynthetic enzymes did not produce any polyketides. When the benzoate:CoA ligase (EncN) is coexpressed, and benzoate is included in the growth medium, benzoyl-primed polyketides were recovered. These results showed that the *enc* KS-CLF already has an internal mechanism that suppresses acetate priming, hence no longer requiring the service of the acetyl-thioesterase EncL.

The priming properties of the *tcm* KS-CLF suggest that starter units other than those specified by the daunorubicin, frenolicin, and R1128 initiation modules can be incorporated into aromatic polyketides. Although we have only demonstrated the loading of alkylacyl-CoA primers, it is possible that acyl-CoAs containing other functional groups, such as alkenes, dienes, alkynes, halogens, and amines, can prime the KS-CLF with high efficiency. The in vitro assay described here provides a convenient method to monitor the promiscuity of the KS-CLF.

With this study we have identified all gate-keeping enzymes involved in aromatic polyketide biosynthesis: (1) primer unit biosynthesis is largely determined by the KSIII homologue found in initiation modules (*10*); (2) starter unit selection by the elongation module is modulated by the acetyl-thioesterase; (3) extender unit fidelity is maintained by the highly stringent MAT (*15*); and finally (4) chain length control is facilitated by the catalytically inactive chain length factor (*31*). Together, these four enzymes ensure the biosynthesis of a desired nascent polyketide, which can be subjected to modifications by the tailoring enzymes. Protein engineering of these four enzymes will expand the potential of combinatorial biosynthesis of aromatic polyketides.

ACKNOWLEDGMENT

We thank Yinyan Tang for providing ZhuC and Ho Young Lee for providing Sfp. Y.T. thanks Dr. Chris Boddy for helpful discussions.

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BI049157K