Biochemical Evidence for an Editing Role of Thioesterase II in the Biosynthesis of the Polyketide Pikromycin*

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The pikromycin biosynthetic gene cluster contains the pikAV gene encoding a type II thioesterase (TEII). TEII is not responsible for polyketide termination and cyclization, and its biosynthetic role has been unclear. During polyketide biosynthesis, extender units such as methylmalonyl acyl carrier protein (ACP) may prematurely decarboxylate to generate the corresponding acyl-ACP, which cannot be used as a substrate in the condensing reaction by the corresponding ketosynthase domain, rendering the polyketide synthase module inactive. It has been proposed that TEII may serve as an "editing" enzyme to reactivate these modules by removing acyl moieties attached to ACP domains. Using a purified recombinant TEII we have tested this hypothesis by using in vitro enzyme assays and a range of acyl-ACP, malonyl-ACP, and methylmalonyl-ACP substrates derived from either PikAII or the loading didomain of DEBS1 (6-deoxyerythronolide B synthase; ATL-ACP). The pikromycin TEII exhibited high $K_m$ values (>100 μM) with all substrates and no apparent ACP specificity, catalyzing cleavage of methylmalonyl-ACP from both $AT_1$-ACP$_L$ ($k_{cat}/K_m$, 3.3 ± 1.1 (M$^{-1}$s$^{-1}$)) and PikAIII ($k_{cat}/K_m$, 2.9 ± 0.9 μM$^{-1}$s$^{-1}$). The TEII exhibited some acylgroup specificity, catalyzing hydrolysis of propionyl ($k_{cat}/K_m$, 15.8 ± 1.8 s$^{-1}$) and butyryl ($k_{cat}/K_m$, 17.5 ± 2.1 μM$^{-1}$s$^{-1}$) derivatives of $AT_1$-ACP$_L$ faster than acetyl ($k_{cat}/K_m$, 4.9 ± 0.7 μM$^{-1}$s$^{-1}$), malonyl ($k_{cat}/K_m$, 3.9 ± 0.5 μM$^{-1}$s$^{-1}$), or methylmalonyl derivatives. PikAIV containing a TEI domain catalyzed cleavage of propionyl derivative of $AT_1$-ACP$_L$ at a dramatically lower rate than TEII. These results provide the first unequivocal in vitro evidence that TEII can hydrolyze acyl-ACP thioesters and a model for the action of TEII in which the enzyme remains primarily dissociated from the polyketide synthase, preferentially removing aberrant acyl-ACP species with long half-lives. The lack of rigorous substrate specificity for TEII may explain the surprising observation that high level expression of the protein in Streptomyces venezuelae leads to significant (>50%) titre decreases.

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### Table 1: Enzyme Assay Results

| Enzyme | $k_{cat}/K_m$ [μM$^{-1}$s$^{-1}$] |
|--------|---------------------------------|
| Acetyl  | 0.25 |
| Butyryl | 0.5 |
| Propionyl | 1.0 |
| Malonyl | 0.1 |
| Methylmalonyl | 0.05 |

Polyketides are a large and structurally diverse class of natural products that possess a wide range of biological activities (1). These compounds are used throughout medicinal and agricultural fields as antimicrobials, immunosuppressants, antiparasitics, and anticancer agents. Despite their structural diversity, polyketides are assembled by a common mechanism of decarboxylative condensations of simple malonate derivatives by polyketide synthases (PKSs) in a manner very similar to fatty acid biosynthesis (2, 3). Type I PKSs are a family of PKSs that are analogous to vertebrate fatty acid synthase that catalyze the biosynthesis of the polyketide moieties of various secondary metabolites in Streptomyces (4–6). They are gigantic multifunctional modular proteins. Each module is responsible for one cycle of polyketide chain elongation and contains a set of discrete catalytic domains of ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP). Ketoreductase, dehydratase (DH), and enoyl reductase domains may also be present, allowing structural variation in the level of processing of the β-ketoacyl chain (4, 6). The fully extended polyketide chain bound to the PKS as an acyl-ACP thioester is often released and cyclized by a thioesterase domain (TEII) covalently linked to the last extending module of the PKS (7).

In many cases, additional genes encoding a TE have been found within a polyketide biosynthetic gene cluster, for example, the tylosin PKS of Streptomyces fradiae (8), pikromycin PKS of Streptomyces venezuelae (5), rifamycin PKS of Amycolatopsis mediterranei (9), and the erythromycin PKS (DEBS) of Saccharopolyspora erythraea (10). These genes encoding a discrete protein were named as TE to differentiate from the chain releasing TEII domains in modular polyketide synthases. Discrete TEII enzymes are also associated with bacterial nonribosomal peptide synthases, responsible for the production of macrocyclic peptide compounds (11), and animal fatty acid synthases (12). Sequence analysis has revealed that these thioesterases are probably structurally and evolutionarily related (13). They have a common serine esterase motif, GXSGX, ~100 residues from the N terminus and another conserved down-stream motif, GXXH, of which histidine might accept a proton from the hydroxyl group of the active site serine, thereby increasing its nucleophile character (13). Because TEII domains have been shown to be a necessary and sufficient factor for the release and cyclization of the polyketide chain in vivo and in vitro (7, 14–16), the role of the TEII enzyme encoded within many PKS gene clusters has presented an intriguing question.

The pikromycin biosynthetic gene cluster contains the pikAV gene encoding a type II thioesterase (TEII). TEII is not responsible for polyketide termination and cyclization, and its biosynthetic role has been unclear. During polyketide biosynthesis, extender units such as methylmalonyl acyl carrier protein (ACP) may prematurely decarboxylate to generate the corresponding acyl-ACP, which cannot be used as a substrate in the condensing reaction by the corresponding ketosynthase domain, rendering the polyketide synthase module inactive. It has been proposed that TEII may serve as an “editing” enzyme to reactivate these modules by removing acyl moieties attached to ACP domains. Using a purified recombinant TEII we have tested this hypothesis by using in vitro enzyme assays and a range of acyl-ACP, malonyl-ACP, and methylmalonyl-ACP substrates derived from either PikAII or the loading didomain of DEBS1 (6-deoxyerythronolide B synthase; ATL-ACP). The pikromycin TEII exhibited high $K_m$ values (>100 μM) with all substrates and no apparent ACP specificity, catalyzing cleavage of methylmalonyl-ACP from both $AT_1$-ACP$_L$ ($k_{cat}/K_m$, 3.3 ± 1.1 (M$^{-1}$s$^{-1}$)) and PikAIII ($k_{cat}/K_m$, 2.9 ± 0.9 μM$^{-1}$s$^{-1}$). The TEII exhibited some acylgroup specificity, catalyzing hydrolysis of propionyl ($k_{cat}/K_m$, 15.8 ± 1.8 s$^{-1}$) and butyryl ($k_{cat}/K_m$, 17.5 ± 2.1 μM$^{-1}$s$^{-1}$) derivatives of $AT_1$-ACP$_L$ faster than acetyl ($k_{cat}/K_m$, 4.9 ± 0.7 μM$^{-1}$s$^{-1}$), malonyl ($k_{cat}/K_m$, 3.9 ± 0.5 μM$^{-1}$s$^{-1}$), or methylmalonyl derivatives. PikAIV containing a TEI domain catalyzed cleavage of propionyl derivative of $AT_1$-ACP$_L$ at a dramatically lower rate than TEII. These results provide the first unequivocal in vitro evidence that TEII can hydrolyze acyl-ACP thioesters and a model for the action of TEII in which the enzyme remains primarily dissociated from the polyketide synthase, preferentially removing aberrant acyl-ACP species with long half-lives. The lack of rigorous substrate specificity for TEII may explain the surprising observation that high level expression of the protein in Streptomyces venezuelae leads to significant (>50%) titre decreases.

1 The abbreviations used are: PKS, polyketide synthase; PikPKS, pikromycin (Pik) polyketide synthase; DEBS, 6-deoxyerythronolide B synthase; KS, ketosynthase; AT, acyltransferase; ACP, acyl carrier protein; TE, thioesterase; DT, dithiothreitol; HPLC, high performance liquid chromatography.

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By far most of the information about the role of TEII in polyketide biosynthesis comes from gene disruption and complementation studies. When the rifR gene (encoding a TEII) was deleted from the rifamycin PKS, production of the polyketide rifamycin B dropped to 30–60% of the normal yields in A. mediterranei (17). Disruption of the TEII-encoding gene (tylO) in the tylosin producer S. fradiae also resulted in 85% reduction of polyketide production (18). Production in the disrupted strain was restored by complementation with the intact tylO gene. More interestingly, it was reported that heterologous TEIIs (nmbB from S. narbonensis and scoT from Streptomyces coelicolor) could also successfully complement the inactivated TEII gene by restoring polyketide production (18, 19).

In contrast to these observations it has been reported that the activated TEII gene by restoring polyketide production (18, 19). In vivo experiments with overexpression of pikAV in pikromycin-producing cultures of S. venezuelae are shown to be consistent with this observation.

EXPERIMENTAL PROCEDURES

Materials and General Methods—[1-14C]Acetyl-CoA (53 Ci/mmol, 50 μCi/ml), [1-14C]propionyl-CoA (55 mCi/mmol, 50 μCi/ml), [1-14C]butyryl-CoA (53 mCi/mmol, 100 μCi/ml), and [2-14C]malonyl-CoA (55 mCi/mmol, 20 μCi/ml) were purchased from Novasep Biochemicals. DL-2-[14C]methylmalonyl-CoA (60 mCi/mmol, 10 Ci/ml) were purchased from Movarek Biochemicals. [5-2-14C]Methylmalonyl-CoA (60 Ci/mmol, 10 μCi/ml) was purchased from American Radiolabeled Chemicals, Inc. All other chemicals were purchased from Sigma and were of the highest available grade. Standard molecular cloning techniques were employed (26). PCR amplifications were performed using standard PCR strategies with Pfu Turbo DNA polymerase (Stratagene) (27).

Protein Expression and Purification—TEII was obtained by expression of pikAV of S. venezuelae in Escherichia coli. The pikAV gene (GenBank™ accession number AF79138:36899–37634) was amplified from a genomic library of S. venezuelae by PCR using the following primers designed with NdeI and HindIII sites at the 5’ and 3’ ends, respectively (5’-AAGCGGGCATATGACCGACAGACCTCTGAA-3’ and 5’-CGTAGGTCTCGTGGGTTCTGCCACTCT-3’). The PCR product was ligated into the pET21c expression vector to give pBK18, which was maintained in E. coli TG2. E. coli BL21-CodonPlus-RP was used as a host for the expression of PikAV. Overexpression (2–4 mg/liter) was accomplished by induction using 0.8 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 0.5–0.6 and incubation of the culture at 28°C overnight. Cells were harvested by centrifugation at 3,000 × g and resuspended with buffer A (50 mM Tris (pH 8.0), 2.5 mM dithiothreitol (DTT), 10% glycerol). After disruption of the cells using French press at 10,000 p.s.i., the lysate was clarified by centrifugation at 10,000 × g. The supernatant was loaded onto nickel-nitrilotriacetic acid column previously equilibrated with 50 mM Tris (pH 8.0), and washed with 10 mM imidazole in the same buffer. The pikromycin TEII was eluted with buffer A containing 100 mM imidazole. Fractions containing the protein were pooled and concentrated with a Centricron™ (Amicon, Inc.) concentrator and subsequently diluted with buffer A containing 5 mM imidazole. The concentrated protein was further purified using a Hi-Trap chelating high performance column (5 ml, Amersham Biosciences) with a gradient elution from 5 to 250 mM imidazole in buffer A. The purification was monitored spectrophotometrically by absorbance at 280 nm. The pooled peak fractions were concentrated, and buffer was subsequently changed to buffer B (100 mM Na2HPO4, (pH 7.2), 2.5 mM normal condensation reaction. The polyketide biosynthetic process would thus be derailed, resulting in low yields of the fully extended polyketide product (24). TEII-catalyzed cleavage of these ACP-bound acyl residues would allow the activated dicarboxylic acid extender units to be loaded onto the PKS, restoring polyketide production. Consistent with such a role is the observation that the homologous TEII and TEI from an animal fatty acid synthases have substrate specificity for medium length fatty acid chains (C8, C10, and C12) and longer chain fatty acids (C14, C16, and C18), respectively (25). Recent studies on TEII (tylO) of the Ty1PKS show that it could hydrolyze synthetic acyl (acetyl-, propionyl-, and butyryl-)-p-nitrophenyl esters and N-acetyl cysteamine thioesters, providing additional preliminary support for the proposed editing role of TEII (24).

In this study, we have generated recombinant TEII (PikAV) from S. venezuelae and performed the first catalytic study of such an enzyme with physiologically relevant substrates, various acyl- and carboxylated acyl-ACP thioesters. The pikromycin TEII exhibits a very high Km for all ACP substrates and likely is predominantly dissociated from the PKS, minimizing its potential to block the normal biosynthetic process. TEII is active with a wide range of ACP-bound thioesters, consistent with an editing role for all of the modules within a PKS. However, this lack of rigorous substrate specificity allows the TEII to remove acyl-ACP thioesters in the PKS-loading module required to initiate the process or the activated carboxylated acyl-ACP thioesters in the extension modules. Thus, either an excess or lack of TEII may lead to decreases in polyketide production. In vivo experiments with overexpression of pikAV in pikromycin-producing cultures of S. venezuelae are shown to be consistent with this observation.
DTT, 1 mM EDTA, 20% glycerol. The concentration of the resulting pure TEII solution was determined using the doSMETRIC\textsuperscript{TM} assay kit from Bioworld.

The last module of PikPKS, PikAIV, containing a TEII domain was expressed in E. coli. After several steps of PCR and subcloning, the pikAIV gene (GenBank\textsuperscript{TM} accession number AF79138: 32952–36992) was cloned into pET24b as an NdeI-HindIII fragment to give pDH54188, which expresses pikAIV as a C-terminal His-tagged fusion protein. The plasmid pDH54188 was transferred into BL21(DE3) strain harboring the sfp gene (pRSG56) (28) and induced by 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 0.6 absorbance. After overnight culture at 25°C, the cells were harvested and disrupted using a French press at 10,000 psi. The PikAIV-TEII was expressed at level of 8–12 mg/liter and purified as described above for TEII. The protein (25 mg/ml) was kept in 100 mM NaH\textsubscript{2}PO\textsubscript{4} (pH 7.3), 1 mM EDTA, 0.2 mM DTT, 10% glycerol after dialysis in the same buffer. The activity of TEII and TEII was confirmed by hydrolysis assay using the p-nitrophenyl esters of acetate, propionate, and butyrate in sodium phosphate buffer (pH 7.5), 100 mM EDTA as described previously (24). The substrates were dissolved in assay buffer using dimethyl sulfoxide.

To obtain the loading domain (ΔT\textsubscript{AT}–ACP) of DEBS1 with a C-terminal hexahistidine tag, the corresponding gene (GenBank\textsuperscript{TM} accession number 63677) was cloned from an XhoI-HindIII fragment into pET21c (Novagen) overexpression vector to give pBK12. The PCR amplification was from a DEBS1 construct (pBK3) (29) using the primers 5′-GAATTCATGGAAGGCGCGGCCTCCGGCGGCTAGCGTCGCCTTCGAGACG-3′ and 5′-TTCCGTTAAGCTTGCATTGCCTTCGTTG3′. For expression of the holo-ΔAT–ACP, didomain, pBK12 was introduced into E. coli BL21-CodonPlus-RP (Stratagene) containing the plasmid pRSG56, which carries a kanamycin gene and the sfp gene. The plasmid pBK12-CodonPlus-RP/pBK12 was cultured in LB supplemented with 50 mg/ml kanamycin and 100 mg/ml ampicillin at 37°C. When the growth reached A\textsubscript{600} = 0.5–0.6, expression of the didomain (16–18 mg/liter) was induced by 0.8 mM isopropyl-1-thio-β-D-galactopyranoside followed by 12 h of incubation at 30°C. Cells were harvested by centrifugation at 3000 × g and resuspended in 50 mM Tris (pH 8.0). The resuspended cells were lysed by a French press at 10,000 psi and pelleted by centrifugation at 10,000 × g. The published purification procedure was then used to obtain 8.6 mg/ml concentration of holo-ΔAT–ACP (30).

The pikAIII gene (GenBank\textsuperscript{TM} accession number AF79138: 28161–32849) was cloned into pET28b as an NdeI-HindIII fragment after multi-step PCR reactions and subcloning steps. The resulting construct (pDH54405) was used to express PikAIII (8–12 mg/liter) as a fusion protein with an N-terminal His tag in E. coli. Expression and purification were analogous to that described for PikAIV.

Autoradiography Demonstrating Decacylation of Propionyl AT–ACP, by TEII—To generate a radiolabeled acyl-AT–ACP, holo-ΔAT–ACP\textsuperscript{(50 μM)} was incubated at room temperature in a reaction mixture containing 180 μM 14C-propionyl-CoA, 100 mM NaH\textsubscript{2}PO\textsubscript{4} (pH 7.2), 2.5 mM DTT, 1 mM EDTA, and 20% glycerol. After a 45-min incubation, the reaction mixture was quenched by 100 mM EDTA and 1 M TEII or PikAIV-TEI in 50 mM phosphate buffer with 1 M TEII or PikAIV-TEI or PikAIII in 50 mM phosphate buffer at 4000 g for 30 min. The samples were lyophilized and resuspended in 2% SDS, 20 mM NaOH. The protein suspension was mixed with a loading dye and heated to 95°C, and the samples were loaded on a 15% acrylamide gel and run at 70 V. When the marker dye reached the bottom of the gel, it was stained, destained, and dried on filter paper. The autoradiograms were analyzed using a computer software package.

RESULTS

Generation of Recombinant TEII and Acyl-ACP Substrates—The TEII protein encoded by pikAIV was expressed from plasmid pBK18 in E. coli as a fusion protein tagged with a C-terminal hexahistidine and purified to homogeneity using Ni\textsuperscript{2+} affinity column chromatography (Fig. 2). The recombinant protein was stable in 50 mM phosphate buffer with 1 M TEII or PikAIV-TEI or PikAIII in 50 mM phosphate buffer at 4000 g for 30 min. The samples were lyophilized and resuspended in 2% SDS, 20 mM NaOH. The protein suspension was mixed with a loading dye and heated to 95°C, and the samples were loaded on a 15% acrylamide gel and run at 70 V. When the marker dye reached the bottom of the gel, it was stained, destained, and dried on filter paper. The autoradiograms were analyzed using a computer software package.
reaction, the pelleted proteins were redissolved into 20 μl of 12% polyacrylamide gel and electrophoresed at 70 V. A. SDS-PAGE analysis visualized by Coomassie staining shows 59-kDa ATL-ACPL and 32-kDa TEII. First lane, reaction mixture without TEII; second lane, the reaction mixture with TEII. B, autoradiography after the exposure of the SDS-PAGE gel to PhosphorImager for 48 h. First lane, reaction mixture without TEII; second lane, the reaction mixture with TEII.

The thioesterase activity of the TEII was confirmed by autoradiography using a radiolabeled acyl-ACP substrate. The radiolabeled propionyl-ATL-ACPL (20 μM) was incubated with 5 μM TEII in 50 mM NaH₂PO₄ (pH 8.0) at room temperature for 6 h. After quenching the reaction, the pellet proteins were redissolved into 20 μl of protein gel loading buffer and loaded onto a 15%–25% polyacrylamide gel and electrophoresed at 70 V. A. SDS-PAGE analysis visualized by Coomassie staining shows 59-kDa ATL-ACPL and 32-kDa TEII. First lane, reaction mixture without TEII; second lane, the reaction mixture with TEII. B, autoradiography after the exposure of the SDS-PAGE gel to PhosphorImager for 48 h. First lane, reaction mixture without TEII; second lane, the reaction mixture with TEII.

The gene encoding the ATL-ACPL didomain region of DEBS1 was PCR amplified as an NdeI and HindIII fragment and cloned into an expression vector pET21c to give pBK12. The didomain fragment includes the N-terminal 107 extra amino acids that have been shown recently to be essential for activity of this didomain (30). The gene encoding the Sfp (a phosphopantetheine transferase from Bacillus subtilis) was co-expressed to ensure posttranslational modification of the apo-ATL-ACPL to the holo form (28). The holo-ATL-ACPL was expressed as a C-terminal hexahistidine-tagged fusion protein and purified by nickel affinity chromatography to 99% purity.

The ATL-ACPL was incubated with [1-14C]propionyl-CoA, leading to rapid acylation. Other acyl-ATL-ACPL derivatives were also generated in the same reaction mixture using different radiolabeled CoA derivatives (acetyl-, butyryl-, malonyl-, and methylmalonyl-CoA). Prolonged incubations (up to 3 h) ensured that greater than 95% of the protein was converted to thioester, even with substrates such as malonyl-CoA.

**Thioesterase Activity of TEII on Propionyl-ATL-ACPL**—The radiolabeled propionyl-ATL-ACPL was incubated at room temperature with or without TEII for 6 h. After trichloroacetic acid precipitation, the reaction mixture was analyzed by SDS-PAGE and autoradiography. The reaction without TEII showed a clear radioactive protein band at the position of ATL-ACPL, whereas the corresponding band was barely detected in the reaction containing TEII protein (Fig. 3), indicating hydrolysis of the radioactive propionyl residue from the protein. A kinetic analysis of this phenomenon using liquid scintillation counting showed that the loss of radioactivity of propionyl-ATL-ACPL was proportionally correlated with the TEII concentration, indicating the decylation is the result of enzyme reaction by TEII. These results provide the first clear evidence of thioesterase activity of TEII on the acyl thioester of a PKS ACP domain, as might be generated by aberrant decarboxylation of extending units during polyketide biosynthesis.

A comparison of the thioesterase activity of PikAIV TEII was made with that of TEI at the C terminus of PikAIV. The pikromycin PikAIV-TEI was overexpressed in E. coli and purified as a C-terminal His-tag fusion protein (Fig. 2B). Numerous in vivo and in vitro studies have previously shown that an equivalent TEI domain at the end of the erythromycin PKS can catalyze hydrolysis and cyclization reactions with shorter chain acyl-ACPs than the natural length of acyl-ACP (34, 35). A time course experiment comparing catalytic activity of TEII and the PikAIV-TEI multifunctional protein revealed that the propionyl group was removed from the ACP domain more rapidly by the TEII (Fig. 4A). The concentration dependence of the propionyl-ATL-ACPL hydrolysis by TEII and PikAIV-TEI (Fig. 4B) was determined. TEII removed propionyl groups from the ACP domain about 8 times more efficiently than the TEI.

**Substrate Specificity of TEII**—More quantitative informa-
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Fig. 5. Thioesterase activity of TEII on short-chain acyl-ATL-
ACP<sub>3</sub> thioesters. TEII-catalyzed hydrolysis rates were measured at increasing concentrations of various short chain acyl-ACP and carboxylated acyl-ACP groups. A series of concentrations of the radiolabeled acetyl (open circles), propionyl (closed circles), butyryl (open triangles), and malonyl (closed triangles)-ATL-ACP<sub>3</sub> were incubated with 1.4 μM TEII in 50 mM phosphate buffer (pH 8.0, final volume 25 μl) respectively. The reactions were measured for loss of radioactivity by liquid scintillation counting.

Fig. 6. ACP specificity of the pikromycin TEII. Hydrolysis rates of methylmalonyl-ACP thioesters by TEII were determined using the loading domain ACP of DEBS1 (open circles) and the extension module ACP of PikAIII (closed circles). Reaction conditions were as described for Fig. 5.

Effect of TEII Expression on Aglycone Production in Pikromycin PKS—We carried out a complementation study of TEII in S. venezuelae strain SC1022, which has an in-frame deletion of pikAV. In previous studies, this strain has been shown to produce only aglycones, as the deleted region of pikAV contains a transcription unit essential for expression of the des genes responsible for the production of the final glycosylated products (32). The titer of the polyketide products was apparently not affected by deletion of the TEII gene (yields were comparable with that observed for a desVI mutant) (20). Complementation of strain SC1022 with the TEII gene expressed from the pikAI promoter in a low copy number (pSC38) plasmid had no affect upon aglycone levels (10-deoxymethynolide and narbonolide levels of 9 ± 2 and 49 ± 4 mg/liter, respectively) (47). No suppression of aglycone production was observed with SC1022 carrying either pBK56, a pSE34 derivative expressing the S99A TEII mutant, or pSE34.

DISCUSSION

KS enzymes catalyze the elongation step in the biosynthesis of fatty acids and polyketides in a multi-step process involving decarboxylation of the elongation unit (typically malonyl- or methylmalonate-ACP) and attack of the resulting carbonan on an acyl group covalently bound to the active site cysteine residue (36). Evidence indicates that the decarboxylation reaction is most efficient when its active site cysteine is thus modified, thereby minimizing nonproductive decarboxylation of the activated extender units (36). Nonetheless, studies have shown that decarboxylation of malonyl-ACP without prior acylation of the active site, so-called aberrant decarboxylation, could take place, albeit at a lower rate. The KS domain of the yeast fatty acid synthase and the KS components of type II fatty acid synthase system can catalyze malonyl-ACP decarboxylation in the absence of an acyl-enzyme intermediate (37, 38). Examples of extender unit decarboxylation in the absence of the acylated KS have also been seen in modular PKSs. Under normal conditions, DEBS3 from S. erythraea can catalyze the last two extending reactions in 6-deoxyerythronolide B biosynthesis us-

ACP Specificity of TEII—The use of the DEBS PKS AT<sub>3</sub>
ACP<sub>3</sub>-loading domain and its established broad substrate specificity allowed comparison of the relative activities of pikromycin TEII of a range of acyl and carboxylated substrates bound to the same ACP. To investigate the ACP specificity of the pikromycin TEII, the hydrolysis rate of the methylmalonyl thioesters from DEBS AT<sub>3</sub>-ACP<sub>3</sub>-loading domain and PikAIII (a single modular protein of PikPKSs and, thus, a potential physiological substrate for TEII) were compared (Fig. 6). The TEII showed significant and indistinguishable k<sub>cat</sub>/K<sub>m</sub> (3.3 ± 1.1 and 2.9 ± 0.9 m<sup>−1</sup>s<sup>−1</sup>) for methylmalonyl thioesters of AT<sub>3</sub>-ACP<sub>3</sub> and PikAIII, respectively.)
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7. Effect of TEII overexpression on polyketide production in *S. venezuelae*. The aglycones (10-deoxymethynolide and narbonolide) produced by pSC38/SC1022 (solid line) and pSC70/SC1022 (dashed line) were quantified by HPLC analysis. pSC70 is a high copy number TEII (PikAV) expression plasmid derived from pWHM3 containing the ermA* promoter (pSE4). The pSC38 plasmid derived from pDHS702 is based on SCP2* origin of replication with TEII expression under control of the pikAI promoter, which allows regulated expression in *S. venezuelae*. Fermentations of SC1022 carrying pBR56 (expressing an inactive TEII, pSE34 (an empty plasmid control), or pSC38 were indistinguishable. The fermentation and analysis conditions were as described under “Experimental Procedures.”

As an editing enzyme, TEII would need to scan all of the ACP domains within a given PKS and would not be expected to exhibit significant ACP specificity, accounting for the observation that methyl malonate units were removed from ACP domains of DEBS loading domain and PikAIII at nearly identical rates. Such low ACP specificity for TEII may also explain why heterologous expression of the TEII gene (pikAI) from *S. narbonensis* in *S. fradiae* increased tylactone production in a TEII (tylO) mutant from 15% to that observed in the wild type strain (18). A similar observation involving complementation of the *tylO* mutant strain with a TEII (scoT) obtained from a putative type I modular PKS gene cluster of *S. coelicolor* A3 (2) has also been reported (15, 19). In fact it appears that the ACP domain may not contribute significantly to substrate binding and catalysis with TEII. The hydrolysis rates reported for TyLO with propionyl- and butyryl-N-acetyl cysteamine thioesters (12.9 and 6.5 \( \text{m}^{-1}\text{s}^{-1} \), respectively) (24) are comparable with our observations of the PikAV (TEII) with the corresponding thioesters of the DEBS AT1-ACP, loading domain (17.5 ± 2.1 and 15.8 ± 1.8 \( \text{m}^{-1}\text{s}^{-1} \)).

Although TEII is not predicted to have ACP specificity, it might be expected to hydrolyze acyl-ACP units formed by aberrant decarboxylation more efficiently than with the corresponding carboxylated acyl-ACP substrates (required for the extension steps in polyketide biosynthesis). Consistent with this prediction, the pikromycin TEII hydrolyzed propionyl- and butyryl-ACP at a rate higher than for malonyl- and methylmalonyl-ACP derivatives. At odds with the prediction was the observation that acetyl-ACP was cleaved at a rate comparable with malonyl-ACP (the correct substrate for the PikAII-catalyzed second extension step in pikromycin biosynthesis) and significantly slower than butyryl-ACP (the ethylmalonyl-ACP, which would decarboxylate to generate this substrate, is not used in pikromycin biosynthesis). Preferential hydrolysis of propionate- and butyrate-p-nitrophenol esters (data not shown) was also observed with the TEII, suggesting this observation was not an artifact associated with use of the DEBS AT1-ACP, loading domain. In a previous observation, the tylosin TEII was shown to prefer the hydrolysis of an N-acetyl cysteamine thioesters over that observed for either propionate and acetate thioesters despite tylosin being generated using methylmalonyl-, ethylmalonyl-, and malonyl-ACP extender units (24). Thus, there are some small differences in the acyl group specificities of the tylosin and pikromycin TEII, which do not readily correlate with the substrate specificities of the extension modules within the two corresponding PKSs.

The pikromycin TEII exhibited a very high \( K_m \) (>100 \( \mu \text{M} \)) with all thioesters of PikAIII and DEBS AT1-ACP, which have previously been reported for the tylosin TEII using propionyl (37.9 \( \mu \text{M} \)) and butyryl (28.2 \( \mu \text{M} \)) N-acetyl cysteamine thioesters (24). Because concentrations of each of the PKS polypeptides within a cell are unlikely to even approach 100 \( \mu \text{M} \), the pikromycin TEII would appear to operate under non-saturating conditions. The pikromycin TEII would, thus, be predominately dissociated from the PKS and, as such, more able to scan all of the ACP domains within a PKS. A greater capacity of TEII to bind ACP would presumably give rise to a higher affinity for acyl-ACP and a more efficient enzyme. However, a concomitant increase in affinity for the carboxylated acyl-ACP derivatives or even *holo*-ACP domains would be predicted. Not only might this interfere with an ability to scan all of the PKS ACP domains, but a tightly bound TEII could also interfere with the normal processing of the PKS. A low affinity for the PKS and a relatively slow rate of reaction may thus...
ensure that TEII only becomes involved once an aberrant de-carboxylation has occurred and the biosynthetic process has stalled (providing acyl-ACP substrates with significant half-lives). As such, carboxylated acyl-ACP units, although substrates for TEII, should be processed readily by the PKS and not readily hydrolyzed in vivo. The kcat of 5 ± 1 min⁻¹ for triketide production by DEBS1-TE (7) is much faster than the observed rate of 0.15 min⁻¹ for propionyl-ACP (150 µmol) hydrolysis observed for TEII under nonsaturating conditions. The editing function of TEII might, thus, be dependent not only upon its substrate specificity but also a low affinity for ACP thioester substrates and a significant difference in the reaction catalyzed with that of the normal functioning PKS.

An editing role for TEII is consistent with previous observations that deletion of this gene generally leads to loss of production of polyketide or polypeptide. This is not the case for the pikromycin PKS, where deletion of the gene in strain SC1022 has no previously been reported to have no effect upon polyketide yields. No increase in aglycone production in SC1022 was observed with the TEII complementation plasmid consistent with this previous observation. The reason why the TEII encoded by pikAV are not required for maximal polyketide production in SC1022 or unclear but may be due to the presence of another enzyme within S. venezuelae with similar activity. Such a possibility is quite reasonable given that putative TEII genes are often associated with modular PKS gene clusters, and many streptomycetes (41) including S. venezuelae contain several distinct enzyme clusters. An editing role for TEII suggests that manipulation of its expression level or properties might have significant benefits for commercially important fermentation processes (24). It has recently been shown that expression of the TEII for S. erythraea leads to an 80% increase in production of the erythromycin aglycone, 6-deoxyerythronolide B, in cultures of recombinant E. coli (42). It has also been suggested that in genetically engineered PKSs, aberrant decarboxylation may be more prevalent, and TEII may help boost polyketide production (although levels of polyketides produced by hybrid pikromycin PKSs in S. venezuelae are very low despite the presence of the pikromycin TEII and possibly an additional TEII (29, 43)). However, the observation that TEII removes an acyl group from a loading domain ACP (the DEBS ATL-ACPL) and indications that it is unable to rigorously differentiate between an acyl-ACP substrate and its carboxylated derivative on an extension module suggest high level expression of TEII could suppress, not enhance, polyketide production in vivo. The decreased yields of aglycone production with plasmid-based high level expression of the pikromycin TEII in strain SC1022 are consistent with this prediction. For this reason these decreases are unknown. However, the observation that high level expression of an inactivating TEII does not suppress titers suggests that it is a result of the catalytic activity of TEII rather than simply binding to the PKS. Thus, it would appear that in addition to substrate specificity and kinetic properties, control of the timing and level of TEII expression are important for the enzyme to function appropriately as an editing enzyme. Within this context we note that recent S1 nuclease mapping studies of the TEII (sco7) promoter region in S. coelicolor have shown that transcription is dependent on growth phase and is only detectable during the late transition period. A regulatory system presumably prevents expression of the gene throughout most of the growth cycle and coordinates expression with the associated modular PKS gene cluster (19). Indeed, expression profiling of the entire pik gene cluster will determine the coordinated regulation of all pikA biosynthetic genes, including pikAV.

Note-Added in Proof—An elegant biochemical characterization of two TEIIIs associated with nonribosomal peptide synthases was published (44) after acceptance of this manuscript.