An Unexpected Interaction between the Modular Polyketide Synthases, Erythromycin DEBS1 and Pikromycin PikAIV, Leads to Efficient Triketide Lactone Synthesis†

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ABSTRACT: An unusual feature of the 6-module pikromycin polyketide synthase (PikPKS, PikAI−PikAIV) of *S. venezuelae* is the ability to generate both 12- and 14-membered ring macrolides. The PikAIV component containing the last extension module and a thioesterase domain is responsible for generating both of these products. In the case of the 12-membered ring macrolide, an acyl-enzyme intermediate on PikAIII is able to efficiently "skip" the last extension step and is cyclized by the TE domain of PikAIV, presumably as a result of a PikAIII−PikAIV interaction. Herein we report that plasmid-based expression (pBK3) of DEBS1, which comprises the loading domain and the first two modules of the *Saccharopolyspora erythrea* 6-deoxyerythronolide B synthase, in *S. venezuelae* leads to efficient 15 ± 3 mg/L production of triketide lactone products (TKLs). Comparable levels of TKLs were observed with a plasmid (pBK1) which expressed DEBS1 fused to a TE domain (DEBS1-TE). These results are in stark contrast to previous in vivo and in vitro analyses, where only DEBS1-TE efficiently produces TKLs. Levels of TKLs decreased dramatically with expression of DEBS1 in both *pikAIV* and *pikAIII-pikAIV* deletion hosts (0.5 mg/L), but not DEBS1-TE, and could be partially restored by addition of a PikAIV complementation plasmid. These data suggest that PikAIV is able to efficiently catalyze formation of 6-membered lactone ring products from acyl-bound intermediates on DEBS1 in a manner analogous to that observed for 12-membered macrolide products from PikAIII. Significant sequence similarity and length of the C-terminal linker region of PikAIII and DEBS1 suggest that this region may be responsible for the interaction with PikAIV. A replacement of this linker region of DEBS1 with the corresponding region of PikAI led to a 95% decrease in TKL levels in *S. venezuelae*, consistent with this hypothesis.

Polyketides are a class of structurally diverse natural products important for their wide-ranging biological activities, which have been used in medicinal and agricultural fields as antimicrobials, immunosuppressants, antiparasitics, and anticancer agents (*1*). They are synthesized on polyketide synthases (PKSs)[1] using simple precursors such as propionyl-CoA and methylmalonyl-CoA in a manner similar to fatty acid biosynthesis (*2*, *3*). Type I PKSs are large, multifunctional enzymes that consist of several discrete modules, each responsible for one round of acyl chain elongation. A typical module is comprised of acyltransferase (AT), acyl carrier protein (ACP), and ketosynthase (KS) domains catalyzing each decarboxylative condensation. Modules may also contain ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains which determine the level of processing of the β-keto group after each extension step (*4*). The C-terminus of the last module typically contains a thioesterase (TE) domain that catalyzes acyl chain release and lactonization (*5*). Although polyketides are assembled by a common mechanism, their structural diversity can be generated by the number of condensation reactions, the type of starter and extender units, and the extent and stereochemistry of reduction. The domain-based structural variations and modular organization of type I PKSs have made it possible to produce novel polyketides through combinatorial engineering (*6*, *7*).

The first modular polyketide synthase to be characterized was 6-deoxyerythronolide B synthase (DEBS) of *Saccharopolyspora erythraea*, which produces the erythromycin aglycon 6-deoxyerythronolide B (Figure 1B) (*4*, *8*). DEBS consists of a loading module and six extender modules housed in three large polypeptides. In addition to the core modules, each subunit has short N- and C-terminal segments of amino acids termed "inter-polypeptide linker regions".

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[1] Abbreviations: PKS, polyketide synthase; PikPKS, pikromycin polyketide synthase; DEBS, 6-deoxyerythronolide B synthase; KS, ketosynthase; AT, acyltransferase; ACP, acyl carrier protein; TE, thioesterase; TKL, triketide lactone; 10-DMD, 10-deoxymethynolide; ND, narbonolide.

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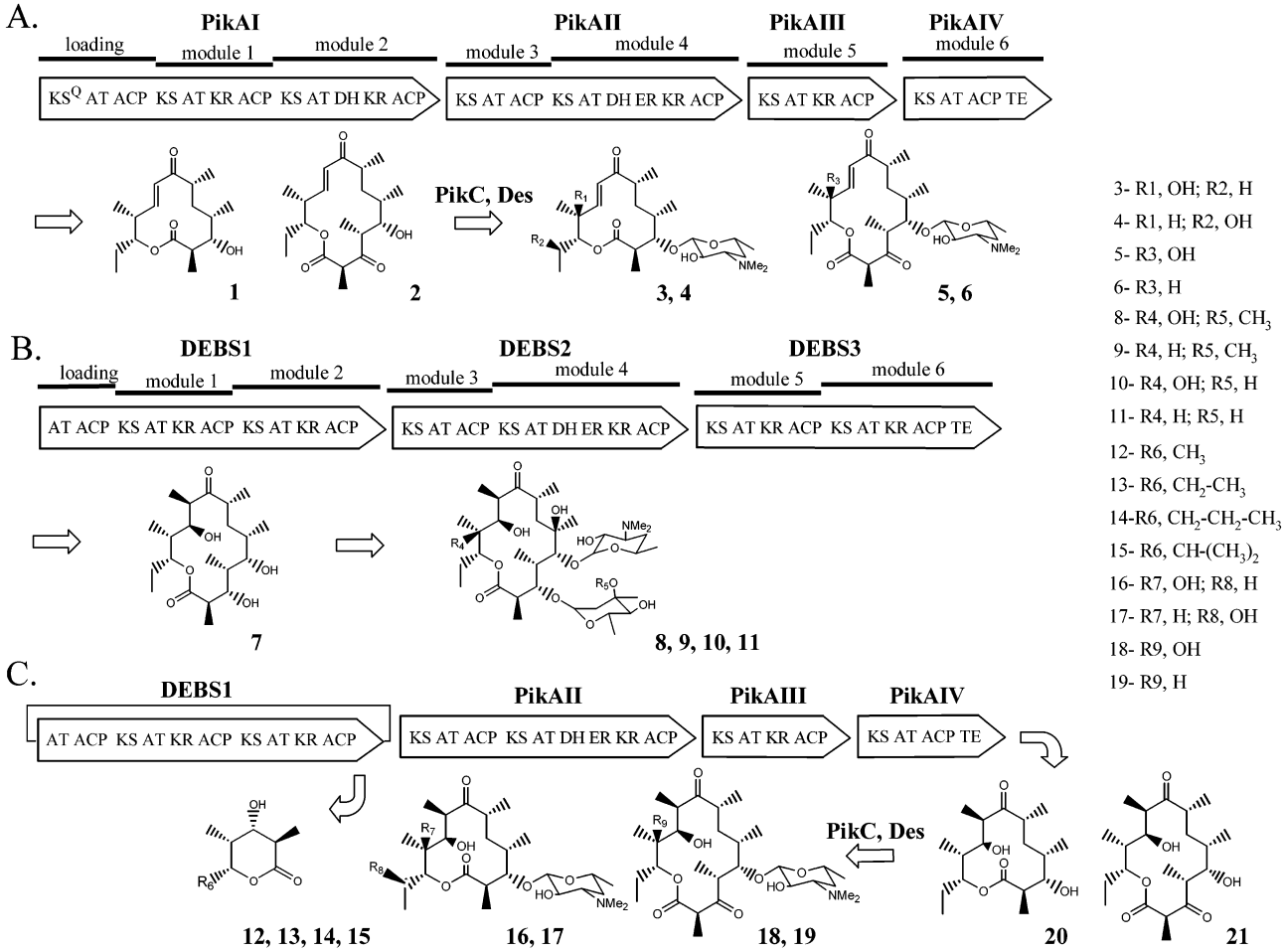

FIGURE 1: Modular arrangement and the products of (A) pikromycin PKS (PikPKS), (B) 6-deoxyerythronolide B synthase (DEBS), and (C) a heterologous expression system of DEBS1 in *pikAI* deletion mutant (BB138/pBK3). PikPKS has six modules housed in four discrete polypeptides. PikPKS has a unique feature of a single modular PKS to produce two different sizes of aglycon structures, 12-membered 10-deoxymethynolide (10-DMD) **1** and 14-membered narbonolide (ND) **2**, which are further processed by glycosylase (DesVII) and hydroxylase (PikC) to generate methymycin **3**, neomethymycin **4**, pikromycin **5**, and narbomycin **6**. PikPKS is closely related to DEBS, which has a very similar 14-membered aglycon structure, **7**, which is further elaborated to **8**−**11**. The heterologous expression system of DEBS1 in *S. venezuelae pikAI* deletion mutant generates novel methylated and hydroxylated 10-DMD **20** and ND **21**, which were further processed by Des and PikC to produce novel pikromycin analogues **16**, **17**, **18**, and **19**. In addition, substantial amounts of triketide lactones **12**, **13**, **14**, and **15** were produced by premature release from DEBS1.

These segments have been reported to be important for the correct inter-polypeptide communication or recognition between PKS subunits (*9*−*11*). DEBS is known to be amenable to genetic modification such as inactivation, substitution, or addition of catalytic domains or modules (*12*−*17*). In many of these cases, the engineered DEBS polypeptides are catalytically active, generating acyl-enzyme intermediates with the predicted structural changes and elongating them to produce novel polyketide products. In many cases, the productivity of the hybrid PKS is compromised (*6*). To avoid decreased catalytic efficiencies that may result from the generation of a hybrid protein, an alternative combinatorial biosynthetic approach has been used in which entire PKS polypeptides from different biosynthetic pathways are exchanged (*7*, *10*, *18*, *19*). In such an approach, proper communication between the polypeptides would appear to be an important factor for successful production of hybrid polyketides.

The pikromycin PKS cluster (PikPKS) in *Streptomyces venezuelae* (Figure 1A) (*20*) is similar to DEBS in that it also contains a loading module and six extension modules. In this case, however, the modules are housed in four rather than three discrete polypeptides. The pikromycin PKS is unusual in that it produces two different sizes of aglycon structures, 12-membered 10-deoxymethynolide (10-DMD) **1** and 14-membered narbonolide (ND) **2**. These aglycons are further processed by a glycosyltransferase (DesVII) and P450 hydroxylase (PikC) to generate methymycin **3**, neomethymycin **4**, pikromycin **5,** and narbomycin **6**. It has been shown that the TE domain at the C-terminus of the last extension module PikAIV is required for formation of both the 12- and 14-membered macrolides (*21*). This TE domain can release and cyclize the fully extended polyketide chain attached to the ACP_6 domain of PikAIV, as well as the acyl chain intermediate attached to ACP_5 of PikAIII. In the latter case, it remains to be determined if TE-catalyzed cyclization occurs directly from ACP_5 of PikAIII, or after a transfer or "skipping step" (occurring without polyketide chain elongation) to ACP_6 of PikAIV. In either case, it is clear that the pikromycin PKS has a novel alternative chain termination mechanism governed by a protein−protein interaction between the PikAIII and PikAIV.

The 14-membered aglycon **2** generated by the PikPKS is closely related to 6-deoxyerythronolide B **7**, but contains a

C3 keto group and a C-10,11 double bond rather than a C10 methyl group and C11 hydroxyl group (Figure 1A). These differences originate from the malonyl-CoA-specific AT domain and the DH domain of PikAI module 2, and the absence of a KR domain in PikAIV module 6 (Figure 1A). To generate novel methylated and hydroxylated ND or 10-DMD analogues, we have constructed and introduced a plasmid expression system of DEBS1 in a *S. venezuelae pikAI* deletion mutant (*19*). The resulting heterologous expression system produced low levels of both the predicted 12- and 14-membered novel polyketides (Figure 1C). These novel aglycons were further processed by DesVII and PikC, and yielded compounds with antimicrobial activity against *Bacillus subtilis* (*19*).

Herein we report that in addition to these novel ketolides, strains containing DEBS1 and PikAIV produced substantial quantities of triketide lactones **12**−**15 (**Figure 1C). In earlier studies, triketide products **12**−**14** have been produced by repositioning the terminal thioesterase domain (TE) of DEBS3 to the carboxyl terminus of DEBS1 (DEBS1-TE) in *S. erythraea* and *Streptomyces coelicolor* (*13, 22*). In the absence of a covalently linked TE domain, however, the triketide production was greatly reduced (*23*). In contrast, we observe significant and indistinguishable levels of triketide lactones using both DEBS1 and DEBS1-TE in the *S. venezuelae pikAI* deletion strain. The yields of the triketide products generated by DEBS1 decrease dramatically either if the linker region of DEBS1 is exchanged for that of PikAI, or if PikAIV is absent. These in vivo studies thus reveal an unexpected functional protein−protein interaction between DEBS1 and PikAIV and demonstrate that the unique ability of the TE domain of PikAIV to efficiently catalyze cyclization of polyketide chain intermediates through such interactions extends beyond that observed for PikAIII.

MATERIALS AND METHODS

Strains and DNA Manipulations. E. coli TG2 strain was used as the standard cloning host for the plasmid constructions. *E. coli* transformants were selected and cultured following standard procedures. Polymerase chain reactions (PCRs) were carried out using Pfu polymerase (Stratagene) following the procedures suggested by the manufacturer. *Streptomyces venezuelae* ATCC 15439 was used for construction of several pikromycin PKS disruption mutants. Three pikPKS deletion mutants used for heterologous expression of DEBS1 constructs, *pikAI* deletion mutant BB138, *pikAIV* deletion mutant HK954, and *pikAIII-IV* deletion mutant YJ004, have been described previously (*19*).

S. venezuelae strains were transformed by the standard procedures. The resulting transformants were selected on R2YE agar plates overlaid with appropriate antibiotics (*24*), and grown on SPA agar with the same antibiotics (12.5 mg/L thiostrepton, 50 mg/L kanamycin, and 25 mg/L apramycin). SGGP liquid medium was used for propagation of *S. venezuelae* strains (*20*).

Construction of Plasmids. All plasmids were derivatives of pSE34, a multicopy shuttle plasmid provided by Pfizer (Groton, CT) containing the *ermE** promoter (*25*). Plasmid pBK3 containing the whole *eryAI* gene of *S. erythraea* under the control *ermE** (a *Xba*I and *Hin*dIII fragment containing the entire *eryAI*) was constructed by several steps of subcloning from a cosmid clone, pAIEN22 (provided by Abbott Laboratories). Plasmid pBK1 producing DEBS1-TE fusion protein is a derivative of pBK3 where the thioesterase domain of DEBS3 was fused using a natural *Sac*I site (*22*). The TE fragment was obtained as a *Sac*I−*Eco*RI fragment by PCR using the primers 5′-TTC ACC **GAG CTC** GGC TTC GAC TCG CTG-3′ and 5′-AGC CCG GC**G AAT TCG** GTC GTG GTC ATG-3′. Plasmid pBK7 is a derivative of pBK3 producing a hybrid DEBS1 protein of which the C-terminal linker region downstream of ACP_2 was replaced with that of PikAI. The C-terminal linker region of PikAI was obtained as an *Eco*RI−*Hin*dIII fragment by PCR using the primers 5′-TGC C**GA ATT C**CG CCT CGG ACC AGG ACG GAG-3′ and 5′-AGG CGC GCA TG**A AGC TT**T TCG AGT CGA GGT-3′. This fragment was cloned into *eryAI* and created an *Eco*RI site downstream from the region encoding ACP_2 of DEBS1. The PikAIV expression vector (pDHS4162) used for complementation of *S. venezuelae pikAIV* deletion mutants has been described previously (*19*).

Isolation, Purification, and Characterization of Propionate-Derived Triketide Lactone. The *S. venezuelae* BB138/pBK1 strain was cultured in 1 L of SCM medium (*20*) for 3 days at 30 °C. After centrifugation at 7000*g*, the culture broth was extracted twice with chloroform and evaporated under reduced pressure to dryness, and then dissolved in a small volume of chloroform. The culture extract was loaded onto an open column filled with silica gel (63−220 mesh, Selecto Scientific, GA). The column was eluted with stepwise gradients of chloroform and ethyl acetate. Triketides were found in the fractions of eluate of ethyl acetate/chloroform (25:75). The pooled fractions containing triketides were further purified by preparative HPLC with a C18 reversed-phase column (250 × 4.6 mm, 5 μm, Phenomenex, CA). The chromatography was performed at a flow rate 0.85 mL/min using a linear gradient solvent system from 20% acetonitrile to 80% acetonitrile in water during 50 min. The eluate was collected by monitoring at 220 nm with an UV detector. The propionate-derived triketide was eluted at 11.8 min. The ^{1}H NMR spectrum of the purified triketide in $CDCl_3$ was recorded on a Varian 300 NMR spectrometer (Varian Inc., CA) and was in good agreement with that previously reported (*23*). The purified triketide was used as a standard for qualitative and quantitative analysis using GC-MS.

GC-MS Quantitation of Triketide Lactone Production. Each strain was grown in SCM medium for 3 days at 30 °C. The clarified broth was extracted with an equal volume of chloroform and dried. The extracts were treated with Trisil-Z for 30 min at 60 °C, quenched with water, extracted with $CHCl_3$, and analyzed by GC-MS (HP6890 GC/HP5973 MS, Hewlett-Packard, CA). Triketides were identified by characteristic fragments at *m/e* 73, 115, 130, and M^+, depending on the starter unit. Purified triketide lactone was similarly derivatized and used to generate a standard curve for quantitation of triketide lactone production.

Feeding studies to distinguish between butyrate and isobutyrate-derived triketide products involved addition of 5 mM perdeuterated butyrate (Cambridge Isotope Laboratories, Inc., Andover, MA) after 24 h fermentation. The corresponding butyrate-derived triketide was identified by the presence of an M^++7 mass ion peak. Alteration of the

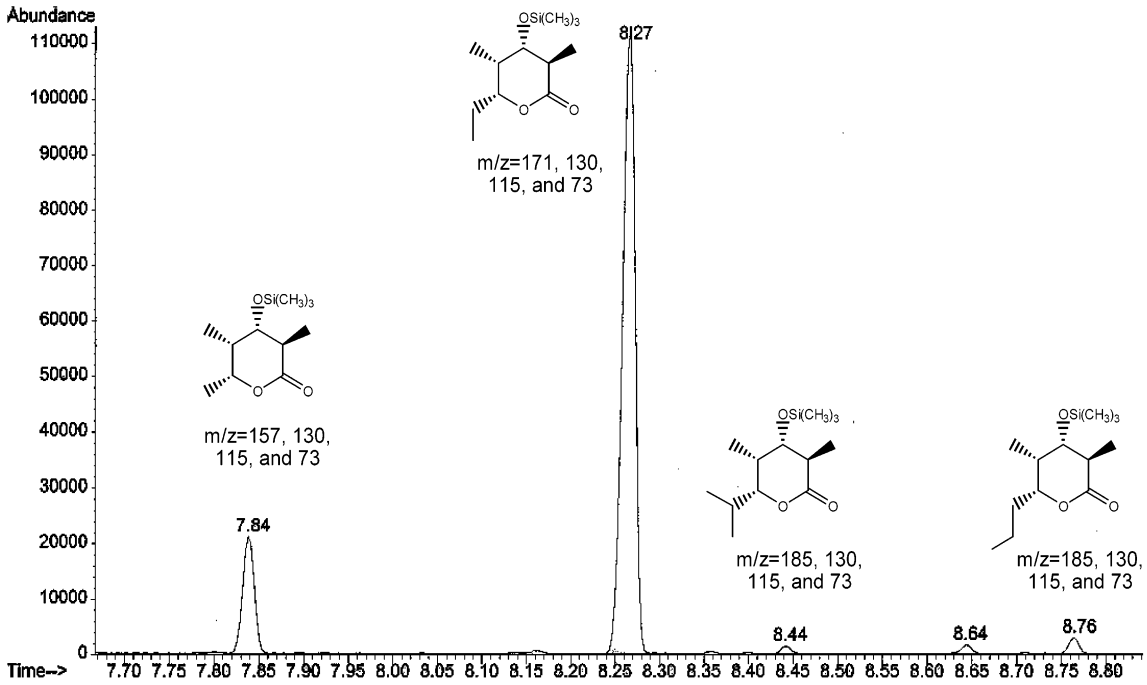

FIGURE 2: GC chromatograph of triketide lactones produced by DEBS1 expressed in *S. venezuelae* PikAI deletion mutant (BB138/pBK3).

relative levels of triketide products was accomplished by addition of valine (100 mM) after 24 h of fermentation.

RESULTS AND DISCUSSION

DEBS1 Produces Significant Levels of Structurally Related Triketide Lactone Products in S. venezuelae. To investigate the possibility of generating new polyketides through interactions of erythromycin and pikromycin PKS proteins, we generated a DEBS1 expression plasmid (pBK3) and introduced this into a *pikAI* deletion mutant of *S. venezuelae* (BB138). In the resulting BB138/pBK3 transformants, substitution of the loading domain and first two extension modules of PikAI by the corresponding catalytic domains of DEBS1 leads to production of 8-methyl-9-hydroxy-10-deoxymethynolide **20** and 10-methyl-11-hydroxynarbonolide **21** (Figure 1C). LC-MS analyses revealed that these aglycons were indeed produced and processed by PikC and DesVII to produce four novel pikromycin analogues: 8-methyl-9-hydroxy-methymycin **16**, 8-methyl-9-hydroxy-neomethymycin **17**, 10-methyl-11-hydroxy-pikromycin **18**, and 10-methyl-11-hydroxy-narbomycin **19** (*19*). The yields of these compounds (μg/L) were orders of magnitude lower than the yields of pikromycin compounds that could be obtained by addition of a PikAI complementation plasmid to the BB138 strain, and not readily detectable by TLC analyses (*19*).

The TLC analyses of culture extracts of BB138/pBK3, however, did indicate significant quantities of triketide lactones, which stain blue when plates are developed with vanillin−sulfuric acid. The most abundant triketide was purified by consecutive flash column chromatography and reverse-phase HPLC. The triketide structure was confirmed as **13**, (3*S*,5*R*)-dihydroxy-(2*R*,4*R*)-dimethyl-*n*-heptanoic acid lactone, by ^{1}H NMR. This triketide is derived from a propionyl-CoA starter unit that has been previously reported. (*22*, *23*).

For quantitative analysis of triketides, the culture broth of BB138/pBK3 strain was extracted with chloroform after 3 days cultivation, and the triketides were converted to their trimethyl silyl esters and analyzed by GC-MS (Figure 2). These analyses demonstrated significant yields (10.9 mg/L) of the most abundant propionate-derived triketide, **13**, in fermentations of BB138/pBK3. Several other triketide analogues identified by GC-MS analysis included those derived from acetate (**12**), and butyrate starter units (**14** and **15**) (Figure 1C and Figure 2). When fermentations of BB138/pBK3 were carried out in the presence of perdeuterated butyrate, the derivatized triketide lactone at the later retention time (**14**, 8.76 min) exhibited an *m*/*z* of 192 (185 + 7 deuteriums), consistent with this being derived from an *n*-butyryl-CoA starter unit. Approximately 89% of this triketide was derived from the perdeuterated butyrate starter unit. The peak with the earlier retention time (8.44 min) was not labeled to any significant extent (<0.1%), indicating this triketide lactone was derived from an isobutyryl-CoA starter unit (**15**). Consistent with this assignment was the observation that addition of 100 mM valine, which is efficiently degraded to isobutyryl-CoA in *Streptomycetes* (*26*), led to a 37% increase in the levels of **15**, relative to the major propionate-derived triketide lactone **13**. Trace amounts of derivatized methylbutyrate-derived triketides were also observed, with later retention times than the peak for **14**.

Previous in vitro studies with DEBS1 and DEBS1-TE (designed to produce triketide only) and more recently of AT_L-ACP_L have demonstrated the relaxed substrate specificity of the loading domain of DEBS1 (*16*, *27*). While the triketide analogues **12**, **13**, **14**, and **15** have been synthesized by DEBS1 in vitro, it is clear that propionyl-CoA is the preferred starter unit (*28*, *29*). However, in vivo production by DEBS1 or DEBS1-TE of butyryl-CoA- (**14**), isobutyryl-CoA- (**15**), and methylbutyryl-CoA-derived triketides has not been reported (*13*, *23*). Triketide lactones generated from branched-chain acyl-CoA starter units have only previously been reported for a hybrid DEBS1-TE containing the loading domain of the avermectin PKS (*16*). Our analyses clearly

demonstrate that the native DEBS1 protein can utilize branched-chain as well as straight-chain acyl-CoA precursors in vivo, so long as there is sufficient substrate availability, and a sensitive enough assay to resolve and detect the resulting product. Results from recent feeding studies on erythromycin production in *Saccharopolyspora erythraea* are consistent with this conclusion (*30*).

DEBS1 and DEBS1-TE Produce Comparable Levels of Triketide Lactone Products in S. venezuelae. Triketide lactone has been made by repositioning of the thioesterase domain from the carboxyl terminus of DEBS3 to DEBS1 (DEBS1-TE), which catalyzes the first two rounds of elongation and lactonization (*22*). The TE domain is known to be required for efficient acyl chain termination and cyclization. When DEBS1 was fused with a nonfunctional TE, triketide production decreased to 1% of that observed with DEBS1-TE. In the case of heterologous expression in *S. coelicolor*, DEBS1 alone can manage to produce small quantities of triketide **13** (*23*); however, fusion of the TE domain to the C-terminus of DEBS1 significantly enhanced the production level in the same heterologous expression system (*13*).

When we expressed DEBS1 from pBK3 in the *S. venezuelae pikAI* deletion mutant (BB138), significant levels of triketide lactones were produced without a covalently linked TE domain (Figure 1C and Figure 2), comparable to levels previously reported for DEBS1-TE (*13*, *22*). To determine if triketide production in *S. venezuelae* by DEBS1 is as efficient as DEBS1-TE, we constructed a derivative of pBK3 (pBK1) and introduced this to the *S. venezuelae* BB138 strain to make a comparable heterologous expression system. The mutant, which expressed DEBS1-TE, produced all the triketide analogues at similar levels to the *S. venezuelae* mutant harboring DEBS1 alone (BB138/pBK3) (Figure 3A).

The levels of triketide production by DEBS1-TE and DEBS1 in the *S. venezuelae pikAI* deletion mutant were comparable to that observed by DEBS1-TE in other expression systems such as *S. erythraea* (8.9 mg/L) and *S. coelicolor* (30 mg/L) (*13*, *22*), and are orders of magnitude higher than the levels of new 12- and 14-membered macrolides. This observation suggested that the majority of the acyl intermediates bound to the C-terminal ACP domain of DEBS1 were cyclized to generate triketide lactones rather than being elongated by the PikPKS, and might contribute to the poor production of the expected pikromycin analogues.

PikAIV Containing a TE Domain Is Required for Efficient Production of Triketide Lactones by DEBS1 in S. venezuelae. The efficiency of the premature release of triketide intermediate from DEBS1 suggests a specific enzyme activity rather than spontaneous termination and lactonization. The TE domain of PikAIV in *S. venezuelae*, which is able to catalyze efficient premature cyclization of polyketide chain elongation intermediates from PikAIII, might similarly be responsible for the chain termination and lactonization of triketide from DEBS1. To investigate the role of PikAIV in triketide production, the DEBS1 expression plasmid (pBK3) was introduced into a *S. venezuelae pikAIV* deletion mutant (HYK954) (*19*). Cultures of HYK954/pBK3 produced about 2% of the levels of triketide products observed for BB138/pBK3 grown under the same conditions (Figure 3B). The levels of triketide products generated by pBK1 (expressing DEBS1-TE) were not reduced in the *pikAIV* deletion strain, and were comparable to those observed for both DEBS1 and DEBS1-TE in the *pikAI* deletion strain (Figure 3B). These observations clearly support the role of PikAIV in efficient cleavage of triketide lactone product from DEBS1, but not DEBS1-TE. Efficient triketide production by DEBS1 and PikAIV contrasts recent in vitro experiments in DEBS1 and module 3 of DEBS2 with a fused TE domain (containing the same catalytic domains as PikAIV) where the tetraketide lactone product was preferentially formed (*5*, *10*).

There are several examples where polyketide synthase intermediates can "skip" a module without undergoing the normal extension and β-ketoprocessing steps. Different hypotheses have been advanced for there observations (*5*, *10*, *20*, *21*, *31*). In the case of PikAIV evidence, it has been suggested that an N-terminal truncated PikAIV, present under certain fermentation conditions, might be responsible for this skipping step (*21*). While the data described herein implicate PikAIV as a major factor in efficient triketide production by DEBS1 in *S. venezuelae*, they do not indicate whether this process utilizes a truncated PikAIV or some other mechanism.

Confirmation of the role of PikAIV in catalyzing the premature release of triketide intermediates from DEBS1 was obtained by introduction of a PikAIV complementation plasmid to the HYK954/pBK3 strain. Plasmid-based complementation of PikAIV in the HYK954 strain has been shown to restore production of 12- and 14-membered macrolides to wild-type levels (*19*). Expression of *pikAIV* from pDHS4162 in the HYK954/pBK3 strain resulted in partial restoration of triketide production to a level 3−7 times more than the amount produced by DEBS1 alone in the *pikAIV* deletion mutant, supporting the importance of the role of PikAIV in this process. The reason triketide levels were not fully recovered by the complementation of PikAIV is likely the result of the culture conditions used; two antibiotic selection markers are required to maintain both the pBK3 DEBS1 (thiostrepton) and the pDHS4162 PikAIV (apramycin) expression plasmids. Consistent with this hypothesis, a 70% reduction of triketide production by DEBS1-TE (pBK1) in the wild-type strain was observed when pDHS4162 was introduced and maintained using apramycin.

PikAIII Is Not Required for Efficient Production of Triketide Lactones by DEBS1 in S. venezuelae. A similar series of experiments were carried out to both confirm the importance of PikAIV and determine any role of PikAIII, in efficient production of triketide lactone by DEBS1 in *S. venezuelae*. In this case, introduction of pBK3 expressing DEBS1 into a *pikAIII-pikAIV* mutant strain (YJ004) led to the same 98% decrease in the levels of triketides as observed for the *pikAIV* deletion strain (HYK954). Once again, levels of triketides made by pBK1 expressing DEBS1-TE were unaltered. Thus, DEBS1-TE consistently produced the same high yields of TKLs in all strains, while DEBS1 produced the same high levels of TKLs only in strains carrying PikAIV.

A partial restoration of triketides was obtained by introduction of PikAIV expression plasmid (pDHS4162) to YJ004/pBK3, comparable to that observed in the analogous complementation experiment in HYK954/pBK3 (Figure 3C). This observation indicated that efficient release of the triketide lactone from DEBS1 does not involve PikAIII, and most likely is a result of a direct interaction with PikAIV.

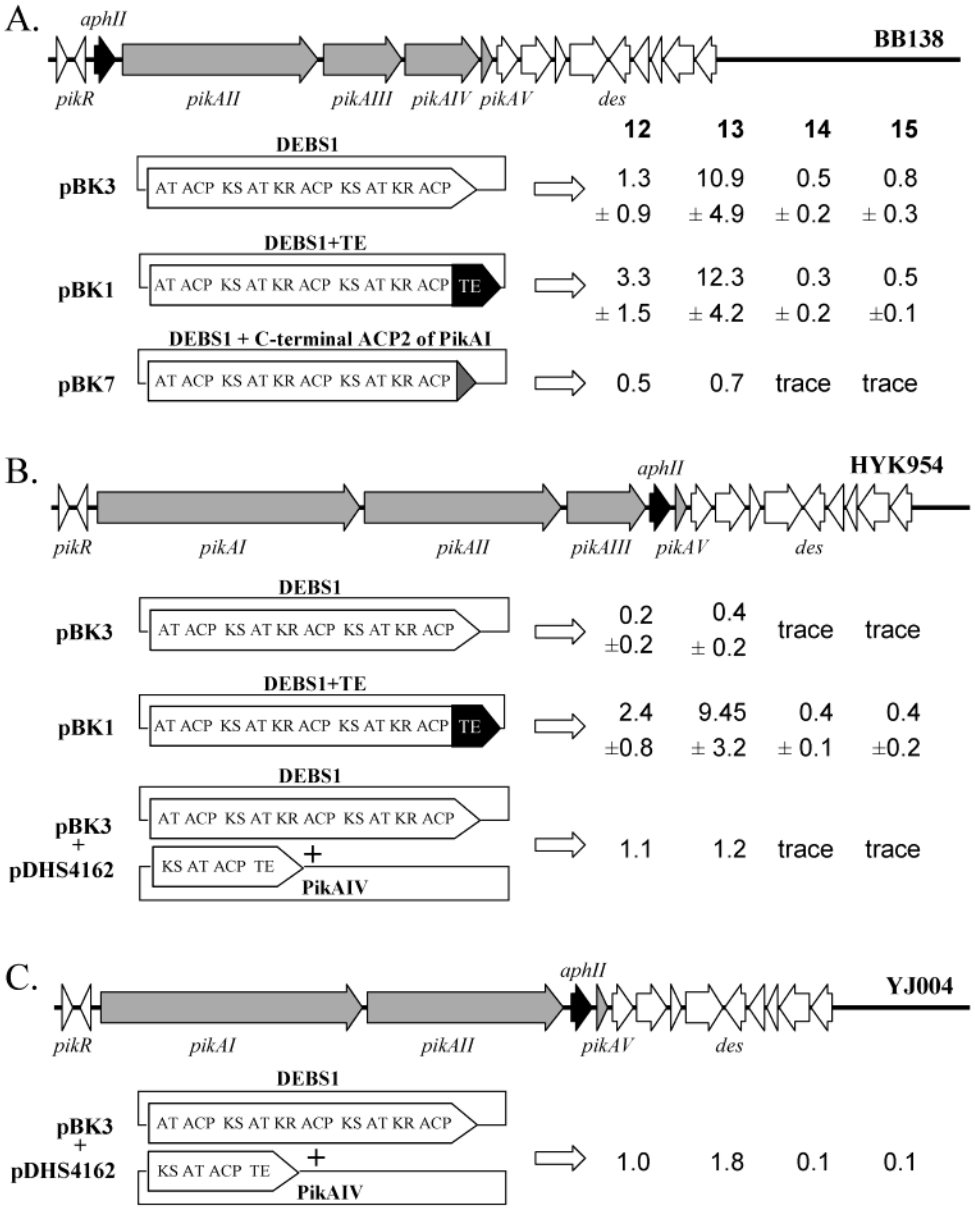

FIGURE 3: Triketide production from different combinations of engineered DEBS1 and *S. venezuelae* mutants. (A) *S. venezuelae pikAI* deletion mutant was used as a host for expression of DEBS1 (pBK3), DEBS1-TE (pBK1), and DEBS1 with a C-terminal PikAI linker region (pBK7). (B) The *pikAIV* deletion mutant was used for expression of DEBS1 and coexpression of DEBS1 and PikAIV (pDHS4162). (C) The *pikAIII-pikAIV* deletion mutant was used for coexpression of DEBS1 and PikAIV. The yields of triketide analogues **12**, **13**, **14**, and **15** by each combination are reported in mg/L and are reported from analyses of triplicate fermentations. Yields with complementation experiments with pDHS4162 were significantly more variable and are reported from a single analysis.

The C-Terminal Linker Region of DEBS1 Is Important for Interaction with PikAIV. Premature release of acyl intermediates from an extending module by a noncovalently linked TE domain of PikAIV occurs naturally during pikromycin biosynthesis in *S. venezuelae* (*20*). The pikromycin PKS contains a single TE domain in PikAIV and generates the 14-membered macrolide narbonolide **2** from a heptaketide intermediate which has been fully processed through all 6 extension modules (*21*). This TE domain of the PikAIV also catalyzes formation of 12-membered 10-DMD from a hexaketide which "skips" the last extension step. In this case, a protein interaction between PikAIII, which contains this hexaketide intermediate, and PikAIV, with the TE domain, allows for the efficient generation (under certain growth conditions) of 10-DMD (*20*). We surmised a similar protein interaction between DEBS1 and PikAIV where the TE domain might contribute to the highly efficient premature release of triketide lactones from DEBS1 in *S. venezuelae*.

Linker regions play an important role in facilitating functional interactions between different proteins of a modular PKS (*10*). We compared the C-terminal region, which extends beyond the highly conserved residues at the boundaries of the last ACP domain, of DEBS1, PikAIII, and PikAI (Figure 4). The lengths of the C-terminal linker sequence (extending beyond the conserved region of the last ACP) of DEBS1 (84 amino acids) and PikAIII (84 amino acids) were also identical, with some low degree of sequence similarity. In contrast, the PikAI linker had comparable levels of sequence similarity in certain regions, but was substantially longer (131 amino acids) than either PikAIII or DEBS1. Presumably, the difference in the PikAI and PikAIII linker regions is important for PikAI−PikAII and PikAIII−PikAIV

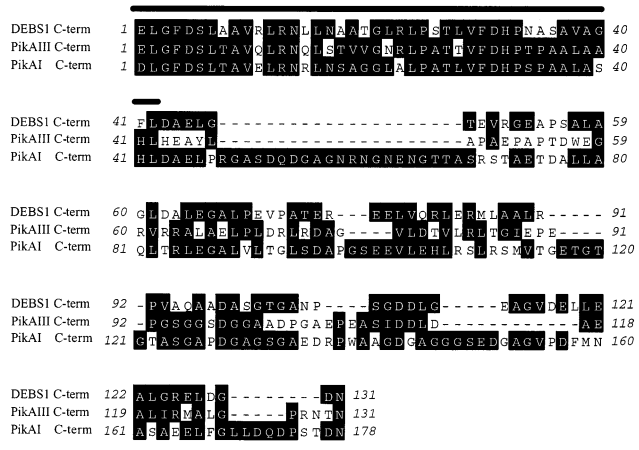

FIGURE 4: Sequence alignment of the C-termini of DEBS1, PikAIII, and PikAI. The extent of ACP domain is indicated by the black bar above the sequences.

interactions, and the correct organization of PikPKS components for generating 12- and 14-membered macrolide ring products. Conversely, the similarity of the DEBS1 and PikAIII linker regions is consistent with the data that suggest DEBS1 forms a more effective interaction with PikAIV (generating triketide lactone products) than PikAII (required for novel macrolide formation).

The hypothesis that the C-terminal linker region of DEBS1 is an important factor contributing to the surprising interaction with PikAIV was tested by generating a DEBS1 hybrid containing the C-terminal linker region of PikAI. We anticipated that a poorer interaction between DEBS1 and PikAIV as a result of this linker region switch would lead to a decrease in the levels of triketide production. A more efficient interaction with PikAII might also facilitate passage of the acyl chain intermediates from DEBS1 and lead to an increase in the titer of novel ketolides through a more efficient shift of the acyl chain intermediate from DEBS1 to PikAII. As expected, the triketide production of BB138/pBK7 decreased substantially to 5% of the yield observed with native DEBS1 (Figure 3A). However, LC-MS analysis suggested a less than 2-fold increase in the levels of hybrid ketolides in BB138/pBK7 as compared to BB138/pBK3. These observations indicate that low yields of novel pikromycins in these systems neither are the result of PikAII and PikAIV competing for interaction with DEBS1, nor are the result of catalytic inefficiency of DEBS1. Low-level production of the novel macrolides must be the result of some other factor, such as substrate discrimination on the part of one or more catalytic domains of PikAII and/or PikAIII.

Conclusions. The TE domain of PikAIV is known to have relaxed substrate specificity and to be essential for catalyzing formation of 12- and 14-membered macrolide ring products. The experiments described herein clearly establish that PikAIV containing this TE domain is also required for efficient formation of a 6-membered ring product from DEBS1. In the case of the 6- and 12-membered ring products, the efficient cyclization of polyketide chain elongation intermediates by PikAIV might be the result of an interaction of the C-terminal linker regions of corresponding PKS proteins (DEBS1 and PikAIII). In both cases, the unique properties of PikAIV allow the chain elongation intermediate to be efficiently processed by the TE domain without the corresponding elongation step.

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