Identification of Domains within Megalomicin and Erythromycin Polyketide Synthase Modules Responsible for Differences in Polyketide Production Levels in *Escherichia coli*

Sumati Murli,* Misty Piagentini, Robert McDaniel, and C. Richard Hutchinson

Kosan Biosciences, Inc., 3832 Bay Center Place, Hayward, California 94545

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ABSTRACT: The megalomicin and erythromycin polyketide synthases (PKSs) produce the same aglycon product, 6-deoxyerythronolide B (6-dEB). Both PKSs were examined in an *Escherichia coli* strain metabolically engineered to support complex polyketide biosynthesis. Production of 6-dEB in shake flask fermentations was undetectable by mass spectrometry in the strain expressing the megalomicin (Meg) PKS genes, whereas 31 mg/L 6-dEB was produced by the strain with the erythromycin (DEBS) PKS. The genes for each of the three subunits comprising the PKSs were expressed in different combinations from three compatible expression vectors (e.g., DEBS1, DEBS2, and MegA3) to identify two Meg PKS subunits, MegA1 and MegA3, which conferred lower 6-dEB titers than their DEBS counterparts. Comparison of protein expression levels and 6-dEB titers by engineered hybrid DEBS/Meg PKS genes further defined regions within modules 2 and 6 of MegA1 and MegA3, respectively, which limit protein expression and 6-dEB production in *E. coli*. Meg module 2 + TE (M2 + TE) and a hybrid DEBS M2/Meg M2 + TE protein were engineered and purified for in vitro comparisons with DEBS M2 + TE. The specific activity of the hybrid M2 + TE was approximately 16-fold lower than DEBS M2 + TE and only twice as high as the Meg M2 + TE enzyme in diketide elongation assays. Since the hybrid M2 worked comparably to DEBS M2 in vivo, this suggests that boosting subunit concentration could serve as a useful approach to overcome enzyme deficiencies in heterologous polyketide production.

Megalomicin and erythromycin are derived from the same polyketide aglycon, 6-deoxyerythronolide B (6-dEB),[1] by condensations between a propionyl-CoA starter unit and six methylmalonyl-CoA extension units. The megalomicin (Meg) and erythromycin (6-deoxyerythronolide B synthase, DEBS) modular polyketide synthases (PKSs) that produce 6-dEB are structurally and mechanistically very similar (*1*) (Figure 1). Each PKS consists of three protein subunits, with the first containing the loading module and two extension modules and the next two subunits containing two modules each. Each module contains a set of domains responsible for a single round of polyketide chain elongation and β-ketone modification. In the case of Meg PKS and DEBS, each of the corresponding modules of the PKSs contains the same set of domains that perform the same sequence of reactions with the same specificities. Thus, the Meg PKS and DEBS present a useful model system for attempting to understand the structural basis for differences in the activities of PKS enzymes.

The titers of erythromycin from the wild-type erythromycin producer, *Saccharopolyspora erythraea*, are significantly higher than the titers of megalomicin in the wild-type megalomicin producer, *Micromonospora megalomicea* (>100 mg/L vs ~10 mg/L). The reasons for the lower productivity of the megalomicin producer could include reduced catalytic efficiency, reduced expression of the PKS, or reduced precursor availability. The production of these polyketides in two different hosts makes it difficult to directly compare the relative efficiencies of the PKSs. Although both PKSs led to similar production levels of 6-dEB (30−40 mg/L) in the heterologous host *Streptomyces lividans*, the relative amounts of PKS proteins present were not determined in that study (*1*). As reported here, attempts to produce 6-dEB in a recently developed *Escherichia coli* polyketide production system (*2*−*4*) with Meg PKS did not afford detectable levels of the polyketide. We have therefore used *E. coli* to compare expression profiles of and polyketide production by a series of DEBS and Meg PKS hybrid subunits to identify potential modules and domains with different levels of activity in these two related PKSs. Subunit complementation and construction of hybrid modules isolated those modules and domains of the Meg PKS that are likely responsible for lower protein levels and activity. A recently developed procedure for efficient purification of PKS modules (*5*) made it possible to obtain poorly expressed modules in sufficient quantity for in vitro characterization and validation of these findings.

MATERIALS AND METHODS

Strains and Plasmids. E. coli strain K207-3, a derivative of BL21(DE3) engineered to support polyketide production

* To whom correspondence should be addressed. Phone: (510) 732-8400. Fax: (510) 732-8401. E-mail: murli@kosan.com.

[1] Abbreviations: PKS, polyketide synthase; 6-dEB, 6-deoxyerythronolide B; DEBS, 6-deoxyerythronolide B polyketide synthase; Meg, megalomicin; M2, polyketide synthase module 2; M6, polyketide synthase module 6; KS, ketosynthase; AT, acyltransferase; KR, ketoreductase; DH, dehydratase; ER, enoylreductase; ACP, acyl carrier protein; TE, thioesterase; TKL, triketide lactone.

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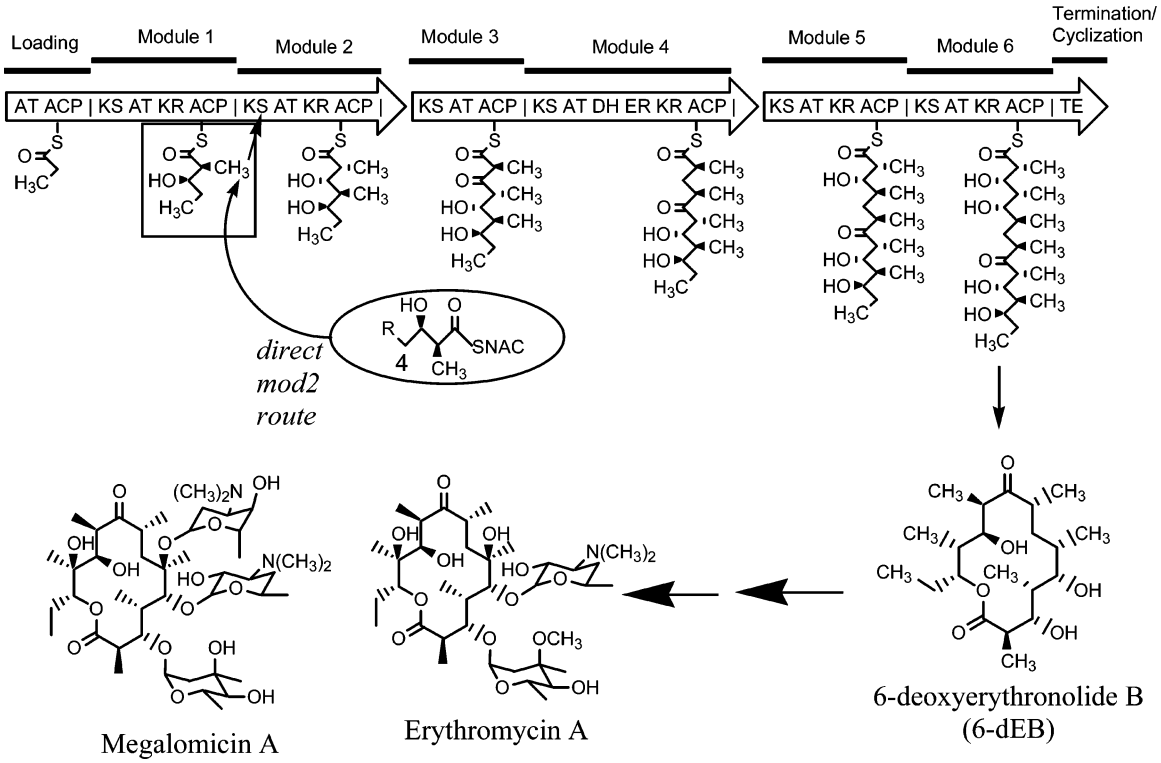

FIGURE 1: Modular organization of DEBS and Meg PKS. Each PKS consists of six modules encoded on three polypeptides (DEBS1, DEBS2, and DEBS3 or MegA1, MegA2, and MegA3). Each module (KS, AT, ACP) is responsible for one cycle of polyketide chain elongation using methylmalonyl-CoA as a substrate and various degrees of β-keto modification (KR, DH, ER). The first polypeptide in each PKS also encodes a loading domain with specificity for propionyl-CoA, and the last polypeptide encodes a TE domain responsible for cyclization and release of 6-dEB. Two sugars, mycarose and desosamine, are added to 6-dEB to produce erythromycin A. The attachment of an additional deoxy sugar, megosamine, in the post-PKS modification pathway distinguishes the final product megalomicin from erythromycin.

Table 1: Plasmids Used in This Study

plasmid	PKS genes	marker	origin of rep
pKOS173-158	DEBS1	Kan	pMB1
pKOS207-004	DEBS2	Tet	p15A
pKOS173-176	DEBS3	Strep/Spec	colD(RSF1010)
pKOS285-134a	DEBS M2	Kan	pMB1
pKOS207-126	MegA1	Kan	pMB1
pKOS207-127	MegA2	Tet	p15A
pKOS207-128	MegA3	Strep/Spec	colD(RSF1010)
pKOS207-180	Meg M2	Kan	pMB1
pKOS207-169	DEBS1/MegA1 hybrid 1	Kan	pMB1
pKOS207-168	MegA1/DEBS1 hybrid 2	Kan	pMB1
pKOS207-183	MegA1/DEBS1 hybrid 3	Kan	pMB1
pKOS285-018	MegA3/DEBS3 hybrid 4	Strep/Spec	colD(RSF1010)
pKOS285-003	Meg M2/DEBS M2 hybrid 5	Kan	pMB1
pKOS285-004	Meg M2/DEBS M2 hybrid 6	Kan	pMB1
pKOS285-005	DEBS M2/Meg M2 hybrid 7	Kan	pMB1
pKOS196-037	DEBS M2 + TE	Carb	pMB1
pKOS285-106	Meg M2 + TE	Carb	pMB1
pKOS367-026	DEBS M2/ Meg M2 + TE (hybrid 7 + TE)	Carb	pMB1

due to integration of T7 promoter regulated *sfp*/*prpE*/*pccB*/*accA1*, has been previously described (*4*). Relevant plasmids discussed in this report are listed in Table 1.

All of the *E. coli* PKS expression plasmids used in this study were based on plasmids pKOS173-158, pKOS207-004, and pKOS173-176 (Table 1), which have been previously described (*4*). The DEBS gene on each of these plasmids was replaced with a corresponding Meg PKS gene, M2 gene, or hybrid PKS gene as follows. Plasmid pKOS285-134a (Table 1) was constructed by cloning the DEBS M2 fragment from pKOS207-142a (*6*) into pKOS173-158 using *Nde*I and *Eco*RI, replacing DEBS1. The three Meg PKS subunits, MegA1, MegA2, and MegA3, were each subcloned from plasmid pKOS108-06 (*1*) into pALTER (Promega Biosciences) using PCR and standard molecular biological techniques. A unique *Nde*I site at the start codon and a unique *Eco*RI site immediately after the stop codon were introduced in each subunit by PCR, and the resulting plasmids, which were verified by sequencing, were pKOS136-1241 (MegA1), pKOS136-1351 (MegA2), and pKOS112-17030 (MegA3) (L. Peck, unpublished results). The MegA1 fragment from pKOS136-1241 was used to replace DEBS1 in pKOS173-158 using *Nde*I and *Eco*RI, generating pKOS207-126 (Table 1). DEBS2 in pKOS207-004 was replaced with the MegA2 containing *Nde*I/*Eco*RI fragment from pKOS136-1351 to generate pKOS207-127 (Table 1). The MegA3 fragment from pKOS112-17030 was used to replace DEBS3 in pKOS173-176 using *Nde*I and *Eco*RI, generating pKOS207-128 (Table 1). To construct a Meg M2 expression vector, the *Nde*I/*Eco*RI DEBS M2 fragment in pKOS285-134a, which has the DEBS M5 linker fused to DEBS M2 (*7*), was cloned into pUC18 (New England Biolabs) digested with *Nde*I/*Eco*RI, generating pKOS207-170. Next, the DEBS M2 containing *Bsa*BI/*Eco*RI fragment from pKOS207-170 was replaced with the Meg M2 *Bsa*BI/*Eco*RI fragment from

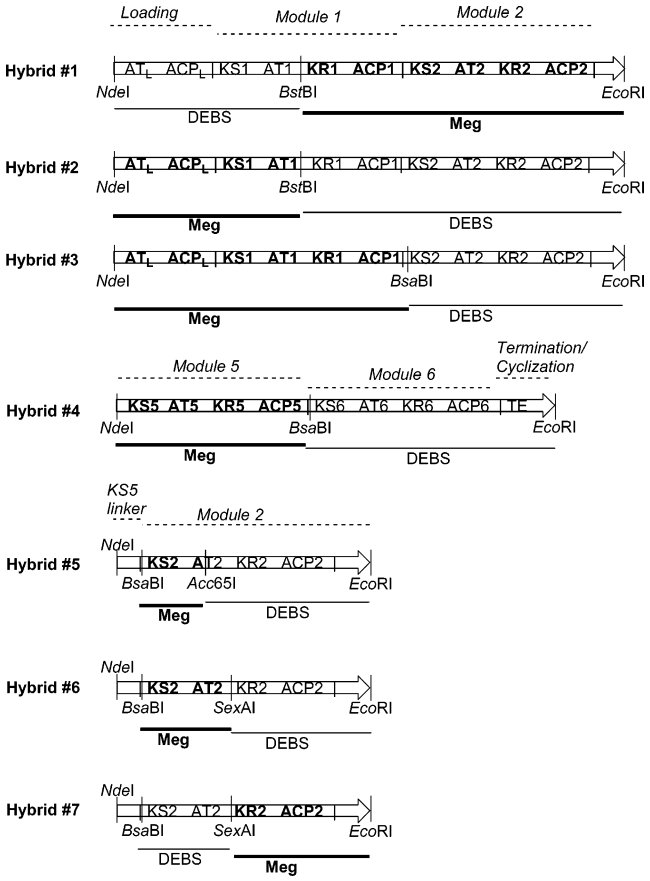

FIGURE 2: Schematic representation of the seven hybrid Meg PKS and DEBS proteins analyzed in this study. The region of the protein from the Meg PKS is indicated in bold type.

pKOS207-126, generating pKOS207-179 which has the DEBS M5 linker fused to Meg M2 (the parallel construction to pKOS285-134a) as a *Nde*I/*Eco*RI insert in pUC18. This fragment with the DEBS M5 linker fused to Meg M2 was moved using *Nde*I and *Eco*RI into pKOS173-158, replacing DEBS1 and generating pKOS207-180 (Table 1).

All of the DEBS/Meg hybrid constructs described below are schematically represented in Figure 2. For hybrid 1, the ~5.9 kb *Bst*BI/*Eco*RI fragment from pKOS207-126, containing the region from the beginning of Meg KR1 to the end of Meg M2, was ligated with the backbone fragment of pKOS173-158 digested with *Bst*BI/*Eco*RI, generating pKOS207-169 (Table 1). For hybrid 2, the ~4.6 kb *Mfe*I/*Bst*BI fragment from pKOS207-126, with the loading, KS1 and AT1 domains from MegA1, was ligated with the backbone fragment of pKOS173-158 digested with *Mfe*I/*Bst*BI, generating pKOS207-168 (Table 1). For hybrid 3, first the *Nde*I/*Eco*RI fragment containing DEBS1 was cloned into pUC18 and then the *Mfe*I/*Bsa*BI fragment from pKOS207-126, containing the Meg loading and M1 domains, was used to replace the corresponding DEBS domains generating pKOS207-178. Next, the *Nde*I/*Spe*I fragment from pKOS207-178 was ligated with the backbone fragment of pKOS173-158 digested with *Nde*I/*Spe*I, generating pKOS207-183 (Table 1) with the Meg loading and M1 domains fused to DEBS M2. For hybrid 4, first the *Nde*I/*Eco*RI fragment containing MegA3 from pKOS207-128 was cloned into pUC18 generating pKOS285-009. Next, the *Bsa*BI/*Eco*RI fragment containing DEBS M6 plus TE was cloned from pKOS173-176 into pKOS285-009 replacing the corresponding Meg domains and generating pKOS285-016. Finally, the *Nde*I/*Eco*RI fragment from pKOS285-016 containing Meg M5 fused to DEBS M6 plus TE was ligated with the backbone fragment from pKOS173-176 digested with *Nde*I/*Eco*RI, generating pKOS285-018 (Table 1).

For hybrid 5, the ~2.2 kb *Acc*65I/*Eco*RI fragment from pKOS207-180 containing the region from the middle of Meg AT2 to the end of Meg M2 was replaced with the corresponding ~2.2 kb *Acc*65I/*Eco*RI DEBS fragment from pKOS207-170 (described above), generating pKOS285-003 (Table 1). For hybrid 6, the ~1.4 kb *Sex*AI/*Eco*RI fragment from pKOS207-180 containing the region from the beginning of Meg KR2 until the end of Meg M2 was replaced with the corresponding ~1.4 kb *Sex*AI/*Eco*RI DEBS fragment from pKOS207-170, generating pKOS285-004 (Table 1). For hybrid 7, the ~3.3 kb *Nde*I/*Sex*AI fragment from pKOS207-180 containing the region from the beginning of Meg M2 until the start of Meg KR2 was replaced with the corresponding ~3.3 kb *Nde*I/*Sex*AI DEBS fragment from pKOS207-170, generating pKOS285-005 (Table 1).

Plasmid pKOS196-037 (Table 1) was made by replacing the *Nde*I/*Eco*RI DEBS M6 containing fragment in pKOS196-123 (*5*) with the *Nde*I/*Eco*RI DEBS M2 containing fragment from pRSG64 (*7*), generating an expression plasmid for DEBS M2 plus DEBS TE with C-terminal biotin and 6× His tags. To fuse the DEBS TE to Meg M2, a *Spe*I site was introduced by site-directed mutagenesis into the corresponding site after the ACP of Meg M2 as in DEBS M2 + TE (*8*), generating pKOS235-109 (A. Yeliseev, unpublished results). The *Bsa*BI/*Spe*I fragment from pKOS235-109 containing the region from the beginning of Meg M2 to the introduced *Spe*I site was used to replace the corresponding *Bsa*BI/*Spe*I DEBS M2 fragment in pKOS207-170 (described above), generating pKOS285-103. Finally, the *Nde*I/*Spe*I fragment from pKOS285-103 was used to replace the *Nde*I/*Spe*I DEBS M2 fragment in pKOS196-037, generating pKOS285-106 (Table 1), an expression plasmid for Meg M2 plus the DEBS TE with C-terminal biotin and 6× His tags. The ~3.3 kb *Nde*I/*Sex*AI fragment from pKOS285-106 containing the region from the beginning of Meg M2 to the beginning of Meg KR2 was replaced with the corresponding ~3.3 kb *Nde*I/*Sex*AI DEBS fragment (Figure 2) from pKOS207-170, generating pKOS367-026 (Table 1), an expression plasmid for hybrid 7 fused to the DEBS TE with C-terminal biotin and 6× His tags.

6-dEB and 15-Methyl-6-dEB (15-Me-6-dEB) Shake Flask Fermentations in E. coli. The protocol followed has been described previously (*2*−*4*). Briefly, *E. coli* strain K207-3 (*4*) with the indicated plasmids introduced was grown overnight in LB medium with streptomycin (strep), kanamycin (kan), and tetracycline (tet) in a 37 °C shaker. The next morning, cultures were diluted 1:50 into 25 mL of LB in a 250 mL flask with tet only. The three PKS subunits were expressed from three compatible plasmids with pMB1, colD, and p15A origins of replication. pMB1 and colD plasmids are very stable whereas p15A plasmids are lost at a modest frequency when antibiotic selection is not maintained; hence, tet is the only antibiotic added during the fermentation (*4*). The cultures were grown in a 37 °C shaker to an OD_{600} of ~0.4−0.5. Cultures were subsequently cooled to room temperature before the following were added: 0.5 mM IPTG, 5 mM sodium propionate, 50 mM sodium

glutamate, 50 mM succinic acid (adjusted to pH 7 with NaOH), and 0.5 mg/mL 3-hydroxy-2-methylhexanoic acid *N*-propionylcysteamine thioester (propyl diketide-SNPC) when diketide feeding was required. The cultures were incubated at 22 °C with shaking for 48 h before being harvested by centrifugation. The OD_{600} of the cultures were determined at the time of harvest. To extract 6-dEB, 5 mL of culture supernatant was mixed with 5 mL of ethyl acetate, shaken for 20 min at room temperature, and centrifuged for 10 min at 2400*g*. The organic layer was removed, and the sample was dried under vacuum. The residue was resuspended in 250 μL of methanol and analyzed by LC/MS as previously described (*3*, *4*). The 6-dEB was quantified using evaporative light scattering detection (ELSD) and comparing the peak areas to a standard curve of peak areas generated from authentic samples. The cell pellet at the end of the fermentation was processed for SDS−PAGE analyses to determine protein levels (see below). Polyketide titers are reported with standard errors from duplicate or triplicate samples derived from independent colonies of the strain analyzed.

SDS−PAGE Analysis of PKS Proteins. The cell pellets from the shake flask fermentations were resuspended at a concentration of 3 OD_{600} units/mL in 20 mM Tris, pH 7.5, and 150 mM NaCl in the presence of Complete protease inhibitor cocktail (Roche Applied Science, used following the manufacturer's protocol). The cells were lysed by sonication at power level 4 for 10 s, 50% power with a Fisher Scientific 550 Sonic Dismembrator. The lysate was spun at room temperature at 12 000*g* for 5 min. The supernatant was collected and analyzed by Bradford assay (Bio-Rad Laboratories) to equalize the total amount of protein loaded on the SDS−PAGE gel. The PKS proteins were separated on a Novex 3−8% Tris−acetate gel (Invitrogen Life Technologies) and visualized by staining with Coomassie blue. The relative levels of the PKS subunits were determined by comparing bands on an SDS−PAGE gel.

Purification of M2 + TE Proteins. Three plasmid constructs, pKOS196-37, pKOS285-106, and pKOS367-26, encoding C-terminal biotin tagged M2 + TE proteins (Table 1) were introduced into *E. coli* BAP1 + pBirAcm (*2*, *5*). Transformed cells were grown at 37 °C to an OD_{600} of 0.4, and the temperature was reduced to 22 °C for 30 min before gene expression was induced with 1 mM IPTG and 20 μM biotin was added. Cells were further incubated with shaking at 22 °C for 18 h. Cells were harvested by centrifugation and stored at −80 °C until needed. The C-terminal biotin tags on each protein allowed rapid purification with a Nuetravidin bead capture procedure described previously (*5*). However, because of poor expression of Meg M2 + TE, the amount of cells used was scaled up. Purified enzyme was quantified by SDS−PAGE, comparing bands to purified DEBS M6 + TE standards of known concentration. Approximately 60 μg of Meg M2 + TE was recovered from cells grown in 2 L of LB, whereas 120 μg of DEBS M2 + TE and 160 μg of DEBS M2/Meg M2 + TE, respectively, were obtained from cells grown in 0.5 L of LB. Assays with the purified protein bound to Nuetravidin beads were carried out by incubating 1 μg of enzyme with 0.5 mM ^{14}C-labeled methylmalonyl-CoA, 4 mM NADPH, and 20 mM 2-methyl-3-hydroxyhexanoic acid *N*-propionylcysteamine thioester (2*S*,3*R* and 2*R*,3*S* racemic mixture) in reaction buffer [100 mM NaH_2PO_4 (pH 7.1), 1 mM EDTA, 2.5 mM DTT, 20% glycerol] at 30 °C for 1 h in a total volume of 50 μL. The radiolabeled triketide lactone (TKL) products were extracted from the assay using 3 × 200 μL of ethyl acetate:1% acetic acid, concentrated, separated by TLC, and visualized with a Typhoon imager (Amersham Biosciences) in phosphorimaging mode.

Table 2: Characterization of DEBS and Meg PKS Subunits in *E. coli*

PKS subunit 1	PKS subunit 2	PKS subunit 3	6-dEB titer (mg/L)
DEBS1	DEBS2	DEBS3	31 ± 1
MegA1	MegA2	MegA3	undetectable
MegA1	DEBS2	DEBS3	trace (<0.1)
DEBS1	MegA2	DEBS3	19 ± 1
DEBS1	DEBS2	MegA3	0.59 ± 0.04
DEBS1	MegA2	MegA3	0.39 ± 0.04

RESULTS

Comparison of DEBS and Meg PKS Subunits in E. coli. The megalomicin and erythromycin PKSs were examined in *E. coli* K207-3, a strain metabolically engineered to support complex polyketide biosynthesis, adapted from the system described by Pfeifer et al. (*2*). Each of the three subunits of the DEBS PKS or the Meg PKS was expressed in different combinations from three compatible plasmids under the control of T7 promoters (*4*). The parallel construction of the DEBS and Meg PKS genes on these plasmids permitted both the examination of the activities of cognate PKS subunits and the determination of the ability of heterologous subunits from the DEBS and Meg PKS to interact functionally to make 6-dEB. Thus, the various permutations between DEBS1 or MegA1, DEBS2 or MegA2, and DEBS3 or MegA3 were assessed (Table 2).

The complete set of DEBS subunits produced 31 mg/L 6-dEB in *E. coli*. However, the three subunits of the Meg PKS did not produce detectable amounts of 6-dEB in this system. The MegA2 subunit supported 19 mg/L 6-dEB production when combined with DEBS1 and DEBS3 and, therefore, is not the likely source of poor production by the Meg PKS in *E. coli*. However, when either MegA1 was combined with DEBS2 and DEBS3 or MegA3 was combined with DEBS1 and DEBS2, less than 1 mg/L 6-dEB was observed. These results indicate that all three Meg PKS subunits are functional in *E. coli* but that MegA1 and MegA3 appear to support very low production levels. MegA3 was also combined with DEBS1 and MegA2 to determine if suboptimal interactions between DEBS2 and MegA3 were responsible for the lower production levels of the DEBS1/DEBS2/MegA3 hybrid PKS (adequate interaction between DEBS1 and MegA2 was demonstrated by the DEBS1/MegA2/DEBS3 hybrid). Again, less than 1 mg/L 6-dEB was produced, indicating an inherent problem in MegA3 rather than a reduced ability of MegA3 to functionally interact with DEBS2.

The 6-dEB production results are consistent with expression levels of the Meg PKS and DEBS proteins observed in *E. coli*. Whereas all three DEBS subunits and MegA2 are easily detectable by SDS−PAGE analysis, neither MegA1 nor MegA3 subunits are evident (Figure 3A). This is in contrast to expression in *S. lividans*, where the Meg and

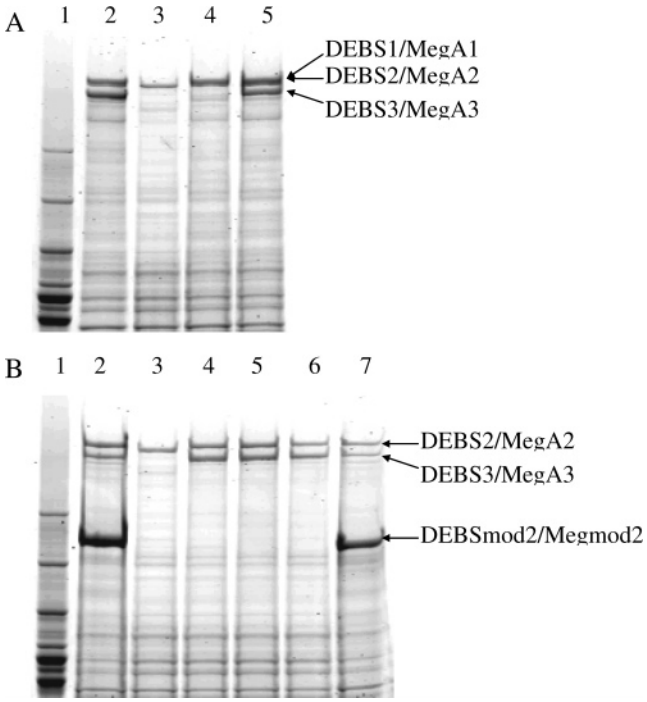

FIGURE 3: SDS−PAGE analyses of PKS proteins. Cell pellets were collected and processed at the end of the fermentations as described in Materials and Methods. Samples were equalized for total protein loaded by Bradford assay and analyzed by SDS−PAGE (see Materials and Methods). (A) Lane 1, 10 kDa ladder; lane 2, DEBS1/DEBS2/DEBS3; lane 3, MegA1/MegA2/MegA3; lane 4, DEBS1/DEBS2/MegA3; lane 5, DEBS1/DEBS2/hybrid 4. (B) Lane 1, 10 kDa ladder; lane 2, DEBS M2/DEBS2/DEBS3; lane 3, Meg M2/MegA2/MegA3; lane 4, Meg M2/DEBS2/DEBS3; lane 5, hybrid 5/DEBS2/DEBS3; lane 6, hybrid 6/DEBS2/DEBS3; lane 7, hybrid 7/DEBS2/DEBS3.

Table 3: Characterization of MegA1/DEBS1 Hybrid Subunits and M2 Subunits in *E. coli*

PKS subunit 1	PKS subunit 2	PKS subunit 3	6-dEB (mg/L)
MegA1	DEBS2	DEBS3	trace (<0.1)
DEBS1/MegA1 hybrid 1	DEBS2	DEBS3	undetectable
MegA1/DEBS1 hybrid 2	DEBS2	DEBS3	23 ± 3
MegA1/DEBS1 hybrid 3	DEBS2	DEBS3	27 ± 2
DEBS M2	DEBS2	DEBS3	6 ± 2[a]
Meg M2	DEBS2	DEBS3	1.6 ± 0.3[a]
Meg M2	MegA2	MegA3	0.14 ± 0.04[a]

[a] 15-Me-6-dEB.

DEBS PKSs yield similar 6-dEB titers (*1*) and the Meg PKS subunits can be detected in protein extracts on SDS−PAGE gels (see Supporting Information, Figure S1). Thus, the presence of significantly lower amounts of the MegA1 and MegA3 subunits in *E. coli* is the likely cause of poor production by the Meg PKS.

MegA1 Module 2 Is Associated with Low Expression and Polyketide Production. To identify regions of MegA1 potentially responsible for poor expression and/or activity, three MegA1/DEBS1 hybrids were constructed using restriction sites that are naturally present in both sequences (Figure 2). The MegA1/DEBS1 hybrids were combined with DEBS2 and DEBS3 subunits to assess polyketide production levels in *E. coli* (Table 3). Comparison of DEBS1/MegA1 hybrid 1, which produces little or no 6-dEB (Figure 2 and Table 3), to MegA1/DEBS1 hybrids 2 and 3, which both produce >20 mg/L 6-dEB (Figure 2 and Table 3), suggests that module 2 of MegA1 is largely responsible for low polyketide titers. Next, Meg M2 and DEBS M2 expression vectors were constructed to directly compare the activities of these two modules. Cultures expressing the M2 genes, along with DEBS2 and DEBS3, were fed a synthetic diketide, 3-hydroxy-2-methylhexanoic acid *N*-propionylcysteamine thioester, which is utilized by module 2 to initiate polyketide biosynthesis in the absence of the loading domain and module 1, and elongated to produce 15-Me-6-dEB (*6*, *9*). Consistent with the data presented above implicating module 2 in the lower titers associated with MegA1, the Meg M2 subunit supported reduced 15-Me-6-dEB titers compared to DEBS M2 with diketide feeding (Table 3). SDS−PAGE analyses of these strains revealed that those subunits comprised of Meg M2 were present at very low or undetectable levels (Figure 3 and data not shown), consistent with the low titers.

Table 4: Characterization of MegA3/DEBS3 Hybrid Subunits in *E. coli*

PKS subunit 1	PKS subunit 2	PKS subunit 3	6-dEB (mg/L)
DEBS1	DEBS2	MegA3	0.59 ± 0.04
DEBS1	DEBS2	MegA3/DEBS3 hybrid 4	31 ± 2
MegA1/DEBS1 hybrid 3	DEBS2	MegA3/DEBS3 hybrid 4	30 ± 1
MegA1/DEBS1 hybrid 3	MegA2	MegA3/DEBS3 hybrid 4	9 ± 1

MegA3 Module 6 Is Associated with Low Expression and Polyketide Production. Modules 2 and 6 of Meg PKS contain an exact DNA duplication extending from within the KS domain to the linker region between the KS and AT domains (*10*). To examine whether Meg M6 contributed to the low productivity of MegA3 observed above, a fusion between Meg M5 and DEBS M6, hybrid 4, was constructed (Figure 2). The junction between these modules was at the naturally occurring *Bsa*BI site located at the beginning of Meg KS6 and DEBS KS6. When combined with either DEBS1 and DEBS2, or MegA1/DEBS1 hybrid 3 and DEBS2, the MegA3/DEBS3 hybrid was able to fully complement production to the levels obtained with DEBS3 (30 mg/L; Table 4). These data support the hypothesis that Meg M6 is responsible for the low production associated with MegA3. By substituting Meg M2 and M6 with DEBS M2 and M6 (MegA1/DEBS1 hybrid 3, MegA2 and MegA3/DEBS3 hybrid 4), titers of 6-dEB can be raised from <0.1 mg/L to 9 mg/L (Table 4). SDS−PAGE analyses show a high level of the hybrid 4 subunit (Figure 3A, lane 5) relative to MegA3 (Figure 3A, lanes 3 and 4), which likely explains, at least partly, the reason for the improved production by the hybrid subunit.

Further Dissection of the Region within Meg Module 2 Associated with Poor Expression/Activity. To more narrowly define regions or domains of Meg M2 that confer reduced activity, three hybrids between Meg M2 and DEBS M2 were constructed (Figure 2) and analyzed by diketide feeding in *E. coli* with DEBS2 and DEBS3. Meg M2/DEBS M2 hybrids 5 and 6 contain the amino-terminal 2.4 kb and 3.3 kb, respectively, of Meg M2 fused to the corresponding remainder of DEBS M2 (Figure 2). Hybrids 5 and 6 include the KS and AT regions (KS domain and a portion of the AT

Table 5: Characterization of Meg Module 2/DEBS Module 2 Hybrid Subunits in *E. coli*

PKS subunit 1	PKS subunit 2	PKS subunit 3	15-Me-6-dEB (mg/L)
Meg M2	DEBS2	DEBS3	1.6 ± 0.3
Meg M2/DEBS M2 hybrid 5	DEBS2	DEBS3	0.65 ± 0.02
Meg M2/DEBS M2 hybrid 6	DEBS2	DEBS3	0.20 ± 0.02
DEBS M2/Meg M2 hybrid 7	DEBS2	DEBS3	4 ± 1
DEBS M2/Meg M2 hybrid 7	MegA2	DEBS3	1.6 ± 0.1

domain in hybrid 5; KS and AT domains in hybrid 6) from Meg M2, which includes the region repeated exactly in Meg M6 (*10*). Both hybrids displayed very low levels of protein (see Figure 3B, lanes 5 and 6), detectable only by Western blot (data not shown). When combined with DEBS2 and DEBS3, both hybrids 5 and 6 produced less 15-Me-6-dEB than the complete Meg M2 (Table 5). Conversely, DEBS M2/Meg M2 hybrid 7 (Figure 2), which contains 3.3 kb from the beginning of DEBS M2 (KS and AT domains) fused to the remainder of Meg M2 (KR and ACP domains), displayed high levels of protein, clearly detectable by Coomassie staining (Figure 3B, lane 7). When combined with DEBS2 and DEBS3, titers were comparable to those produced by DEBS M2 (see Tables 3 and 5). These results suggest that poor expression associated with the KS and/or AT domains of Meg M2 may be responsible wholly or in part for the low titers associated with MegA1.

The high protein levels and 15-Me-6-dEB titers of DEBS M2/Meg M2 hybrid 7 allowed us to test whether the linker at the end of MegA1 (i.e., at the end of Meg M2) discriminates between DEBS2 and MegA2. Hybrid 7 yields lower titers when combined with MegA2 and DEBS3 (~1.6 mg/L) than with DEBS2 and DEBS3 (~4 mg/L) (Table 5), suggesting that the C-terminal linker of MegA1 has similar, if not better, affinity for DEBS2 as it does for MegA2.

Relative Activities of DEBS M2 + TE, Meg M2 + TE, and Hybrid DEBS M2/Meg M2 + TE in Vitro. To determine whether the lower titers associated with Meg M2 were due to diminished catalytic activity, in addition to the reduced levels of these proteins in *E. coli*, two M2 + TE fusion proteins, Meg M2 fused with DEBS TE (Meg M2 + TE) and DEBS M2/Meg M2 hybrid 7 fused with DEBS TE (DEBS M2/Meg M2 + TE), were engineered and compared to DEBS M2 + TE (*7*, *11*). Each protein was expressed in *E. coli* with C-terminal biotinylation to facilitate single-step purification and immobilization onto Nuetravidin-coated microbeads (*5*). This procedure was particularly helpful in the purification of Meg M2 + TE, which was produced at levels approximately 8-fold lower than DEBS M2 + TE (see Materials and Methods for yields). The relative protein expression of the three TE fusions in *E. coli* was similar to the relative amounts of the M2 subunits observed in the complementation studies above (DEBS M2 + TE ~ DEBS M2/Meg M2 + TE > MegM2 + TE).

Chain elongation assays using diketide substrate were performed with each of the enzymes immobilized on Nuetravidin beads as described in Materials and Methods. All three enzymes produced TKL as detected by TLC autoradiography. However, under the conditions used, the activity of Meg M2 + TE and DEBS M2/Meg M2 + TE was too low to make reliable kinetic measurements. Therefore, an estimation of relative activities was made with a single end point assay in which the product, TKL, could be quantified. It was estimated that the activity of DEBS M2 + TE was ~16 times higher than DEBS M2/Meg M2 + TE, which was ~2 times more active than Meg M2 + TE (Figure 4). This was surprising given that DEBS M2/Meg M2 hybrid 7 was capable of providing titers similar to DEBS M2 in vivo (Tables 3 and 5; see Discussion below). Thus, it appears that the increase in titers associated with the hybrid DEBS M2/Meg M2 that was observed above is largely due to increases in the expression of the PKS module rather than an improvement in enzyme activity. Since both the hybrid DEBS M2/Meg M2 and DEBS M2 proteins contain the same KS, the reduced activity is most likely due to impaired condensation or processing activities rather than different affinities for the diketide substrate. This is consistent with studies performed on other hybrid modules in which the impaired activity was attributed to attenuated condensation activity in the module (*12*).

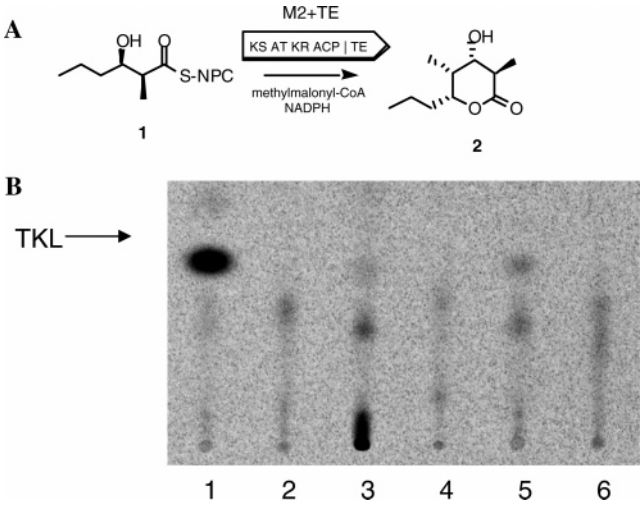

FIGURE 4: In vitro analyses of M2 + TE constructs. Conversion of diketide SNPC (**1**) to triketide lactone (**2**) by purified DEBS M2 + TE (lane 1), Meg M2 + TE (lane 3), and DEBS M2/Meg M2 hybrid 7 + TE (lane 5). See Materials and Methods for reaction conditions. Lanes 2, 4, and 6 are control reactions without added diketide SNPC (**1**).

DISCUSSION

Despite possessing redundant protein architectures with highly homologous domains and catalyzing exactly the same sequence of reactions, DEBS and Meg PKS have very different polyketide production properties in *E. coli*. Comparison of the two PKSs in *E. coli*, encoding each of the subunits on separate expression plasmids, facilitated the identification of modules and domains that give rise to the disparity in 6-dEB production. Both MegA1 and MegA3 subunits appear to be associated with poor 6-dEB titers whereas MegA2 functions as well as DEBS2. Analyses of hybrids between MegA1 and DEBS1 suggest that Meg M2 limits the yield by the MegA1 subunit and, likewise, the MegA3 and DEBS3 hybrid implicates Meg M6 as the limiting module in MegA3. In any of the natural or engineered hybrid subunits containing either Meg M2 or Meg M6, the intracellular concentration of the protein subunit is

greatly diminished or undetectable and can account for most, if not all, of the diminished 6-dEB production in each case. Comparison of the codon usage of Meg M2 and M6 with their cognate DEBS modules shows that, when the critical frequency is set to account for the rarest codons in *E. coli* (i.e., a critical frequency of 2.5 per 1000), Meg M2 and M6 actually have fewer of the rarest codons than DEBS M2 and M6 (5 in each of the Meg modules while DEBS M2 has 9 and DEBS M6 has 6), and none of these modules have two rare codons in a row. This suggests that rare codon usage is unlikely to account for the different protein levels. Protein and mRNA stability are critical contributors to the regulation of protein levels, and further work will be required to determine the role(s) of these factors in the reduced protein levels associated with Meg M2 and M6 relative to DEBS M2 and M6.

Meg M2 and Meg M6 contain an exact DNA duplication (encoding 204 amino acids) that extends from within the KS domain to the linker region between the KS and AT domains (*10*). DEBS M2 and DEBS M6 do not contain any significant stretches of identity. Replacing the KS and AT domains of Meg M2 with the corresponding region of DEBS M2 resulted in a hybrid module which is expressed at significantly higher levels and is able to provide titers comparable to DEBS M2. Therefore, it is likely that this duplicate region in Meg M6 also contributes to the low protein yield.

Analysis of the M2 + TE fusion proteins in vitro suggests that, in addition to its poor expression, the activity of Meg M2 is significantly lower than DEBS M2. It is unknown which of these deficiencies is the primary cause of low production by the Meg PKS. However, replacing the KS and AT domains of Meg M2 with the KS and AT domains of DEBS M2 (DEBS M2/Meg M2 hybrid 7) resulted in a module that was expressed well and was able to support nearly the same level of 15-Me-6-dEB production as DEBS M2 even though the in vitro activity of this module was ~16-fold lower than DEBS M2.

We have shown here that, in an engineered or heterologous system, suboptimal production can be attributed to the poor expression level of individual modules or subunits relative to the rest of the PKS. Therefore, identifying problematic modules or domains and replacing them with functionally equivalent ones with better expression profiles may provide a general strategy for improved production by natural and engineered PKSs. Furthermore, in the case of hybrid modules that are engineered with new but poor activities, overexpression of the hybrid module relative to the remaining ones may overcome a common problem associated with the engineered biosynthesis of polyketide natural products.

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