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Redesign, synthesis and functional expression of the 6-deoxyerythronolide B polyketide synthase gene cluster

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Abstract A generic design of Type I polyketide synthase genes has been reported in which modules, and domains within modules, are flanked by sets of unique restriction sites that are repeated in every module [1]. Using the universal design, we synthesized the six-module DEBS gene cluster optimized for codon usage in *E. coli*, and cloned the three open reading frames into three compatible expression vectors. With one correctable exception, the amino acid substitutions required for restriction site placements were compatible with polyketide production. When expressed in *E. coli* the codon-optimized synthetic gene cluster produced significantly more protein than did the wild-type sequence. Indeed, for optimal polyketide production, PKS expression had to be down-regulated by promoter attenuation to achieve balance with expression of the accessory proteins needed to support polyketide biosynthesis.

Keywords Polyketide synthase · Heterologous expression · 6-Deoxyerythronolide B · Gene synthesis · Synthetic biology

Introduction

Type I modular polyketide synthase (PKS) genes of the actinomycete and myxobacterial groups of prokaryotes determine the biosynthesis of valuable polyketide natural products such as erythromycin, epothilone, tacrolimus and many others [2]. These genes encode giant enzymes consisting of sets (modules) of active sites (domains). The domains in each module catalyze the assembly of a specific 2-carbon unit component of the polyketide, and the successive modules form a protein 'assembly line' that builds the carbon chain of the final product in a step-wise fashion. The paradigm Type I PKS, 6-deoxyerythronolide B synthase (DEBS, Fig. 1), which builds the aglycone of erythromycin, 6-deoxyerythronolide B (6dEB), consists of three large polypeptide chains that contain six modules–two in each polypeptide–flanked by a loading domain on the N-terminus and a thioesterase on the C-terminus [3].

A primary objective of this laboratory is the development of technology to enable facile genetic engineering of Type I PKS genes to facilitate combinatorial biosynthesis and generate "unnatural natural products." One difficulty in achieving this is that organisms that naturally produce polyketides are often not amenable to current genetic methodologies. Introduction of DNA and genetic exchanges are frequently problematic and time consuming, many target organisms are uncultured, and there are few systems that can be used in such organisms to regulate expression of PKSs and their accessory genes [4]. To circumvent such problems, *E. coli* was engineered to serve as a host for polyketide biosynthesis [5] and shown capable of supporting production of large amounts of product [6]. Clearly, since molecular biology tools for *E. coli* are so advanced and experiments can be performed so quickly, this organism is an ideal host for combinatorial biosynthesis of polyketides.

A second difficulty in combinatorial biosynthesis is that natural PKS genes are difficult to manipulate; they are extraordinarily long (35 to >200 kb), about 70% G+C, highly repetitive, and rarely contain conserved restriction sites that facilitate exchange of domains or modules [4]. To circumvent these, we sought to recreate PKS genes by total gene synthesis such that codon composition could be controlled and unique restriction sites could be introduced to permit facile mobilization of DNA fragments. We recently reported enabling technology for the synthesis of ~5 kb sequences of DNA [7]—the average size of Type I PKS modules—as well as a generic design of PKS genes such that modules and domains can be flanked by unique restriction sites repeated in every

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module [1]. With this design, PKS modules and other components can be exchanged as cassettes, providing a truly modular system to facilitate experiments in combinatorial biosynthesis.

A proof of principle of our PKS gene redesign, re-synthesis approach was recently demonstrated by the synthesis of a contiguous 32 kb DEBS gene cluster and its expression in *E. coli* [7]. However, production of polyketide was extremely low precluding the practical use of this tool for combinatorial biosynthesis. In the present work we report high expression of the redesigned PKS genes using a three-plasmid system, and improved polyketide production in *E. coli*. Effects of sequence changes due to introduced restriction sites and the use of different promoters to drive PKS expression were explored.

Materials and methods

Oligonucleotides were purchased from Operon. DNA manipulations were performed using standard methods [8]. *E. coli* DH5α was used for routine plasmid DNA preparation. Restriction enzymes were purchased from New England Biolabs, and other reagents were the highest quality commercially available. Polyclonal rabbit anti-pccB was a gift from H. Gramajo and monoclonal anti-biotin was purchased from Roche. The *E. coli* polyketide production strain, K207-3 [BL21 *D prpBCD::T7prom prpE, T7prom accA-pccB, T7prom sfp*] has been described [9].

Gene synthesis

Components of DEBS ORFs were synthesized as previously described [7].

Plasmids

pKOS392-97, pKOS455-016, pKOS346-155b, pKOS346-155c and pKOS346-155d were obtained from S. Mutka and J. Kennedy (unpublished work). pKOS392-97 was derived from P_{BAD}/Myc-HisA (Invitrogen) as follows: (i) the *Nde*I site at nucleotide 2846 was removed by cleavage, fill-in and blunt end ligation; (ii) an *Nde*I site was engineered into the start codon. The *Bgl*II/*Nde*I fragment from pKOS392-97 containing the P_{BAD} promoter was cloned into the same sites of pKOS207-4 to generate pKOS285-101 and into pCDF1b (Novagen) to generate pKOS455-016. pKOS346-155b (50% T7), pKOS346-155c (25% T7) and pKOS346-155d (10% T7) expression vectors were obtained by insertion of synthetic DNAs containing the corresponding point mutations in the T7 promoter [10] into the *Bgl*II/*Xba*I sites of pKOS173-158 to replace the wild-type promoter in this plasmid. pKOS422-108-2 was created by cloning a 250 bp synthetic DNA containing, from 5' to 3', a *Bgl*II site, *lac*UV5 promoter, *lac* operator, RBS and *Eco*RI site into the *Bgl*II/*Eco*RI sites of pKOS207-4. All the ORFs were inserted into the indicated vectors using *Nde*I/*Eco*RI restriction sites. The DEBS expression plasmids used are listed in Table 1.

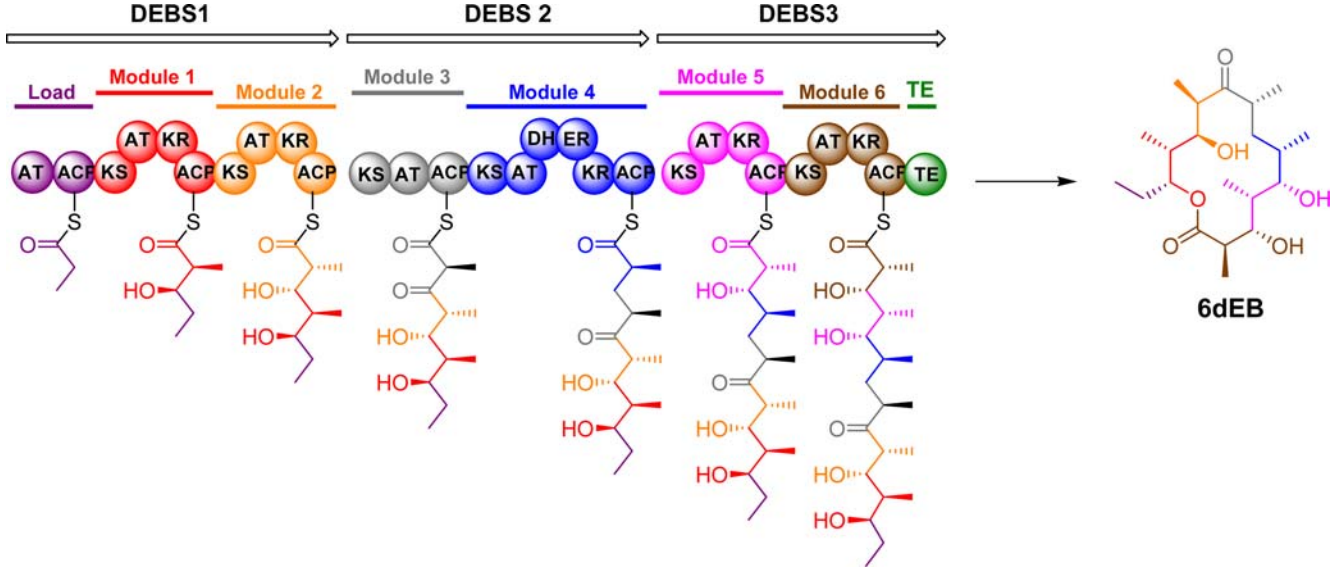

Fig. 1 Biosynthesis of 6dEB on a protein 'assembly line' consisting of the three proteins DEBS1, DEBS2 and DEBS3. The six modules are connected by three intra-polypeptide linkers (Mod1/2, 3/4 and 5/6) and two inter-polypeptide linkers (Mod 2/3 and 4/5). *Load* loading module; *KS* ketosynthase; *AT* acyl transferase; *DH* dehydratase; *ER* enoyl reductase; *KR* ketoreductase; *ACP* acyl carrier protein and *TE* thioesterase domain

Table 1 Expression plasmids used in this work

Plasmid	ORF	Parent	Replicon	Promoter	Resistance	Reference
pKOS207-142a	wtMod2	RSF1010	RSF1010	T7	Str	[11]
pKOS285-140a	wtMod2	RSF1010	RSF1010	10% T7	Str	This work
pKOS285-140b	wtMod2	RSF1010	RSF1010	25% T7	Str	This work
pKOS285-140c	wtMod2	RSF1010	RSF1010	50% T7	Str	This work
pBP130	wtDEBS2/wtDEBS3	pET26b	ColE1	T7	Kan	[5]
pKOS173-158	wt DEBS 1	pET26b	ColE1	T7	Kan	[9]
pKOS207-4	wt DEBS 2	pACYC184	P15A	T7	Tet	[9]
pKOS173-176	wt DEBS 3	RSF1010	RSF1010	T7	Str	[9]
pKOS422-33-1	sy DEBS 1	pET26b	ColE1	T7	Kan	This work
pKOS422-51-1	sy DEBS 2	pACYC184	P15A	T7	Tet	This work
pKOS422-31-2	sy DEBS 3	RSF1010	RSF1010	T7	Str	This work
pKOS422-57-2	sy DEBS 1	pKOS346-155b	ColE1	50% T7	Kan	This work
pKOS422-57-3	sy DEBS 1	pKOS346-155c	ColE1	25% T7	Kan	This work
pKOS422-57-4	sy DEBS 1	pKOS346-155d	ColE1	10% T7	Kan	This work
pKOS422-79-1	wt DEBS 1	pKOS392-97	ColE1	P_{BAD}	Kan	This work
pKOS285-101	wt DEBS 2	pACYC 184	P15A	P_{BAD}	Tet	S. Murli, unpublished
pKOS422-83-1	wt DEBS 3	pKOS455-016	RSF1010	P_{BAD}	Str	This work
pKOS422-74-2	sy DEBS 1	pKOS392-97	ColE1	P_{BAD}	Kan	This work
pKOS422-77-3	sy DEBS 2	pKOS285-101	P15A	P_{BAD}	Tet	This work
pKOS422-83-2	sy DEBS 3	pKOS455-016	RSF1010	P_{BAD}	Str	This work
pKOS422-108-3	wt DEBS 2	pKOS422-108-2	P15A	*lac*UV5	Tet	This work
pKOS422-108-4	sy DEBS 2	pKOS422-108-2	P15A	*lac*UV5	Tet	This work

sy synthetic ORF; *wt* wild-type ORF; *Kan* kanamycin; *Tet* tetracycline; *Str* streptomycin

Expression and 6dEB detection

Erlenmeyer flasks containing LB and appropriate antibiotics were grown at 37°C to mid-log phase (0.4–0.6 OD_{600}). Gene expression was induced with 0.5 mM IPTG and/or 2 mg/mL L-arabinose (final concentration), as appropriate, and 5 mM propionate, 50 mM succinate, and 50 mM glutamate (final concentration) were added. Cultures were further incubated for 48 h at 22°C. Samples (1 mL) of each culture were centrifuged at 14,000*g* for 3 min, resuspended in 1 mL 20 mM Tris, 150 mM NaCl, pH7.5, and lysed by sonication. After 10 min of centrifugation at 14,000*g*, pellets were resuspended in 1 mL lysis buffer, and soluble and insoluble fractions equivalent to 10 ul cell suspension were analyzed on NuPAGE Novex 3–8% Tris-Acetate gels (Invitrogen) with Sypro-Red Staining (Molecular Probes).

Cell-free supernatants (1 mL) were extracted twice with an equal volume of ethyl acetate. The extracts were dried in vacuo and dissolved in 1 mL of methanol. 6dEB was analyzed by LC-MS and quantified by ELSD using concentration standards as previously described [9].

PCCB and ACCA detection by western blot

Soluble extracts (4 ug total protein) were separated by SDS-PAGE and transferred to a PVDF (Bio-Rad) membrane following manufacturer's recommendations. Polyclonal rabbit anti-pccB and monoclonal anti-biotin were used as primary antibodies and Alexa 647 anti-mouse and Alexa 488 anti-rabbit as secondary antibodies (Molecular Probes). The images were captured using a Molecular Dynamics Typhoon 2D imager.

Results

Figure 2 shows the universal design of PKS components that enables facile construction or mobilization of PKS genes [1]. As shown, the components consist of: (i) N-terminal components (loading module or N-terminal linkers) with a 5′ *Nde*I site at the start codon and a 3′ *Xba*I site; (ii) core extender modules containing three to six domains having a 5′ *Spe*I site and a *Xba*I site at the 3′ end of the ACP domain; and (iii) C-terminal components (TE and C-terminal linkers) possessing a 5′ *Spe*I site and a 3′ *Eco*RI site. The design is such that the 3′ *Xba*I overhang of any N-terminal or internal module can be joined with any 5′ *Spe*I overhang of an adjacent downstream module, linker or TE with concomitant destruction of both sites, allowing their repetitive use to create multi-module ORFs.

In addition to these cloning sites, redesigned modules contain a standard set of restriction sites flanking domains (Fig. 2c), some of which demand amino acid changes to be accommodated. The restriction sites *Mfe*I and *Pst*I used in redesigned PKS modules can result in changes in the amino acid sequence, and the *Spe*I/*Xba*I fusions always do. In the final redesign of the 31-kb DEBS ORFs, 15 of the restriction sites introduced resulted in 20 amino acid substitutions in the encoded PKS. The PIA tripeptide needed for the *Mfe*I site is naturally found in five of the six DEBS modules, and Mod1 has a conserved PVA. The LQ dipeptide required for the *Pst*I site is found naturally only in DEBS Mod3; four modules have L followed by E (Mod1), L (Mod2), P (Mod5) or A (Mod6), and Mod4 has an unusual PR that is found in only 2% of PKS modules. The *Spe*I/*Xba*I fusion used to connect all redesigned modules

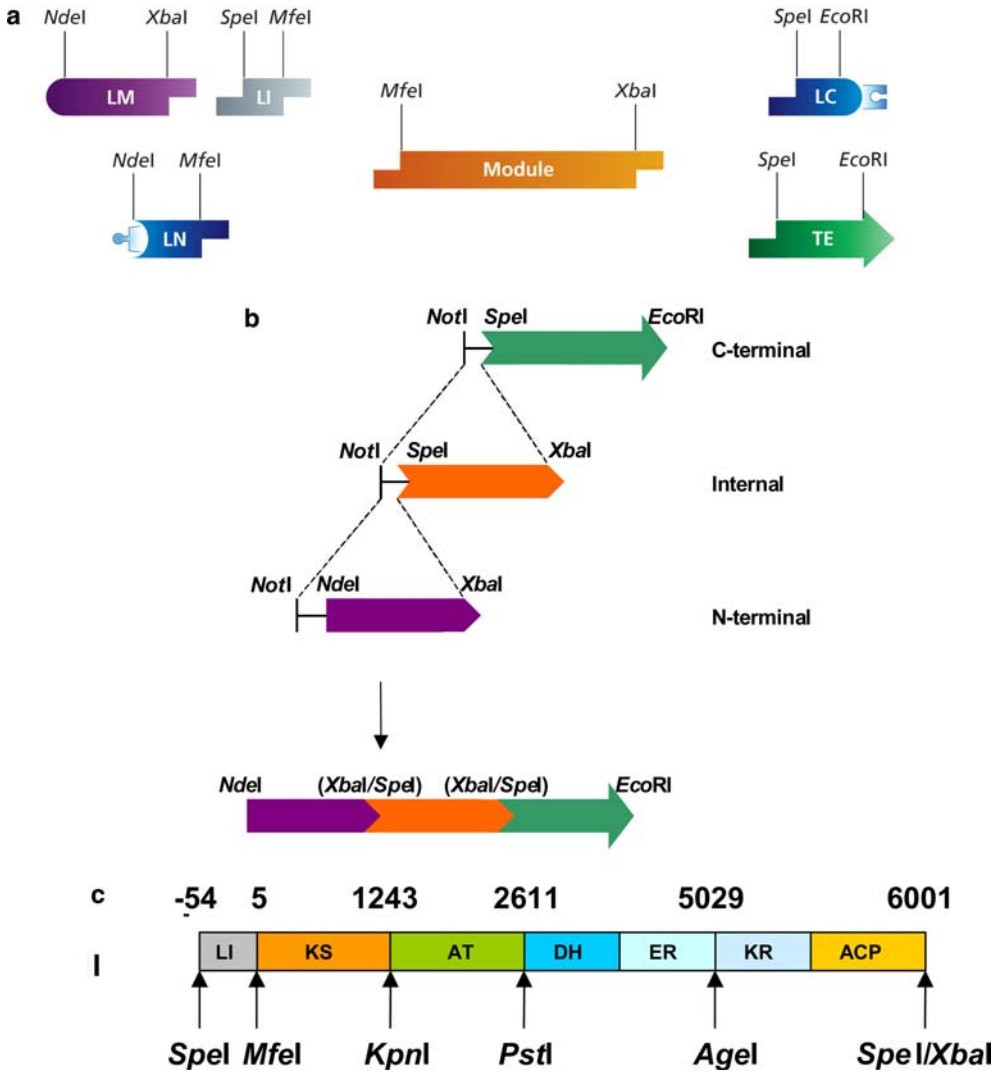

Fig. 2 **a** Building blocks for combinatorial assembly of PKS genes, comprising *LM* loading module; *LI* intrapeptide linker; *LN* N-terminal interpeptide linker; extender module; *LC* C-terminal interpeptide linker and *TE* thioesterase. **b** Construction of a typical three module PKS gene. **c** Generic PKS module design. Restriction site positions are referenced to module 4 of DEBS. Numbering begins at the first residue of the motif EPIAIV on the N-terminal edge of the KS domain

results in a SS dipeptide that is unnatural to all modules, although TS in the same position is tolerated in some [12]. Overall, 13 of 20 amino acid changes in the final redesigned DEBS are to accommodate the *Spe*I/*Xba*I junction, one to accommodate the *Mfe*I of Mod1and six to accommodate the *Pst*I sites in Mod1, 2, 4, 5 and 6 (Table 2). An *Eco*RI was added for cloning convenience at the 3′ edge of every ORF.

To determine if these amino acid changes affected function, we compared expression and 6dEB production of the synthetic DEBS ORFs and gene cluster with the native sequences. The synthetic components of DEBS ORFs were assembled as large *Nde*I- *Eco*RI fragments in pUC vectors (Fig. 2b) [7]. Each of the synthetic and wild-type ORFs [9] were moved into one of three compatible vectors (DEBS1 in ColE1; DEBS2 in P15A; DEBS3 in RSF1010), all under control of a ϕ10 T7 promoter. When individually expressed in the engineered *E. coli* strain K207-3 [9], each codon-optimized ORF produced about fivefold more protein than its native counterpart, as did the complete set of three coexpressed synthetic ORFs (data not shown). However, no product could be detected from the three coexpressed synthetic ORFs, while the wild-type PKS gene set produced 20 mg/L of 6dEB (Table 3, experiments 1 and 2).

Table 2 Amino acids changes introduced to accommodate restriction sites in DEBS

DEBS1	DEBS2	DEBS3
A537S[a]	V1473S[a]	P900Q[a]
A538S[a]	G1474S[a]	V1466S[a]
V560I[b]	P2361L[c]	G1467S[a]
E1436Q[c]	R2362Q[c]	A2357Q[c]
G2015S[a]	F3491S[a]	D2893S[a]
G2016S[a]	A3492S[a]	
L2908Q[c]		
G3461S[a]		
T3462S[a]		

[a] Changes introduced to accommodate the *Xba*I/*Spe*I sites
[b] Change introduced to accommodate the *Mfe*I site
[c] Changes introduced to accommodate the *Pst*I site

Table 3 6-dEB production from combinations of the three DEBS subunits expressed from synthetic and wild-type genes in *E. coli* K207-3

Experiment	ORF combination	6dEB titer (mg/L)[a]
1	*N1N2N3*	19.8 ± 1.8
2	*S1S2S3*	Not detected
3	*S1N2N3*	8.5 ± 1.1
4	*N1S2N3*	Not detected
5	*N1N2S3*	16.3 ± 2.1
6	*N1S2*N3*	12.0 ± 2.4
7	*S1S2*N3*	< 0.1
8	*S1N2S3*	3.1 ± 0.8
9	*N1S2*S3*	3.8 ± 1.3
10	*S1S2*S3*	< 0.1
11	*S150S2*S3*	0.6 ± 0.2
12	*S125S2*S3*	2.1 ± 0.3
13	*S110S2*S3*	1.8 ± 0.1
14	**N1N2N3**	Not detected
15	**S1S2*S3**	8.9 ± 0.9

N wild-type ORFs; *S* synthetic ORFs. *1,2* and *3* DEBS1, DEBS2 and DEBS3 ORFs, respectively. S2* DEBS2 without the *Pst*I site. *italic* T7 promoter; *bold* P_{BAD} promoter; *underline* *lac* UV5 promoter. 50, 25 and 10: mutant weaker T7 promoters

[a]Mean and standard error of three independent fermentations

To pinpoint which module(s) was defective, chimeras of wild-type and synthetic ORFs were constructed by cotransformation of combinations using the three-plasmid system, and of polyketide production was analyzed (Table 3, experiments 3–5). Of the three possible chimeras containing one synthetic and two natural ORFs, only the combination containing synthetic DEBS2 produced no 6dEB. Since the synthetic DEBS2 contained the non-conserved PR-to-LQ mutation to accommodate the Mod4 *Pst*I site, we converted the sequence back to PR. With this change, synthetic DEBS2 combined with the natural DEBS1 and 3 produced high levels of 6dEB (Table 3, experiment 6).

Although substitution of one of the three wild-type ORFs by a synthetic ORF reduced polyketide production by only 15 to 55%, substitution of two of the three ORFs by synthetic sequences significantly reduced the production of 6dEB and when all three ORFs were synthetic, production of 6dEB was meager (Table 3, experiments 7–10). It was imperative to identify the reasons for the poor production and circumvent them.

Expression of some accessory genes is required to produce polyketides in *E. coli*. Strain K207-3 contains *B. subtilis sfp*, encoding a phosphopantetheinyl transferase to attach the prosthetic group to ACP domains; *prpE*, for producing propionyl(Pr)-CoA from exogenous propionate; and *S. coelicolor pccB*/*accA*, encoding the Pr-CoA carboxylase (PCC) complex needed to produce methylmalonyl(MeMal)-CoA from Pr-CoA. All the accessory genes are integrated into the chromosome under the control of T7 promoters [9].

In studies of wild-type DEBS genes in *E. coli* K-207-3, we observed that expression from wild-type T7 promoters lowered the expression of at least two of the accessory genes—*accA* and *pccB*—from the same promoter; conversely, use of promoters weaker than wild-type T7 attenuated expression of DEBS genes and resulted in increased expression of the two accessory genes. Mod2 is a truncated form of DEBS1 that is co-expressed with DEBS 2 and 3 in strain K-207-3 to produce 6dEB analogs via chemo-biosynthesis [11, 13]. Fig. 3a shows the SDS-PAGE of Mod2-DEBS2-DEBS3 and Fig. 3b shows the western blot detection of the two subunits of PCC, ACCA and PCCB. When the expression of Mod2 was driven by mutant T7 promoters of lower strength [10], the production of Mod2 decreased with a concomitant increase of the PCC subunits required for precursor supply (Fig. 3c). We surmised that the high levels of DEBS expressed using the strong T7 promoters caused a decrease in production of the accessory proteins.

T7 promoter-driven expression of the codon-optimized synthetic DEBS is fivefold higher than that of the wild-type genes, and is accompanied by a 10-fold decrease in ACCA and PCCB compared to wild-type genes (data not shown); we did not test for T7-driven Sfp or PrpE production which could likewise be lowered. To test whether the low polyketide production in the face of high PKS expression could be due to excessive DEBS production, we cloned the synthetic DEBS1 ORF downstream of weaker variants of the T7 promoter, and coexpressed these with DEBS2 and 3 under control of the wild-type T7 promoter. The constructs containing DEBS1 with attenuated T7 promoters produced significantly less DEBS, more ACCA, PCCB and 6dEB (Fig. 4 and Table 3, experiments 11–13) than did those driven by the wild-type promoter.

Based on the above results, we expressed each of the three synthetic and natural DEBS ORFs using weaker promoters. When individually expressed from the L-arabinose inducible P_{BAD} promoter, each DEBS ORF provided three to fourfold less PKS protein than when expressed under control of the T7 promoter. For unknown reasons, cotransformation of P_{BAD}-DEBS2 with either or both of the other reading frames resulted in poor cell growth. The wild-type and synthetic versions of DEBS2 ORF were therefore cloned under the control of the even weaker *lac*UV5 promoter. When expressed alone, the codon-optimized *lac*UV5-DEBS2 ORF produced >10-fold less PKS protein than obtained using the T7 promoter. However, when the synthetic *lac*UV5-DEBS2 ORF was coexpressed with the P_{BAD} expression plasmids containing synthetic DEBS1 and DEBS3 ORFs, cells grew normally and produced 9 mg/L of 6dEB. In contrast, 6dEB was undetectable when the *lac*UV5-driven wild-type DEBS2 ORF was coexpressed with the P_{BAD} vectors containing DEBS1 and DEBS3 ORFs (Table 3, experiments 14 and 15). Thus, it seems that weaker promoters are needed to express the codon-optimized genes in order to maintain the balance of expression of the accessory proteins required for polyketide production, while stronger promoters may be necessary to achieve balance when the high-GC native genes are used.

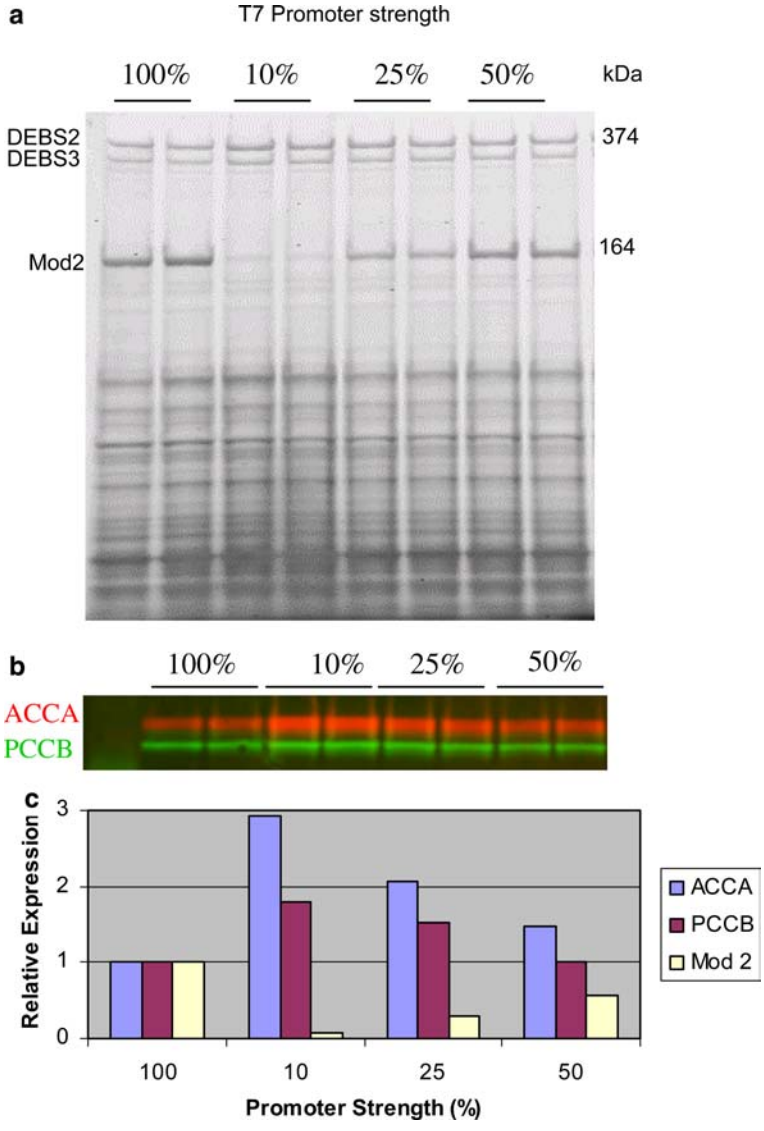

Fig. 3 Modulation of expression of ancillary pathway genes. The wild-type DEBS system (module 2 of DEBS 1, DEBS 2, and DEBS 3) was expressed in *E. coli* K-207-3, as described [9, 11]. DEBS module 2 was expressed from T7 promoter mutants with strengths ranging from 10 to 100% (wild-type). Samples were obtained from two independent cultures of primary transformants. **a** SDS-PAGE stained with Sypro Red. DEBS proteins are indicated. **b** Western blot of SDS-Gel shown in "A." PCC proteins (ACCA, *red* and PCCB, *green*) are indicated. **c** Plot of relative (average) expression of *accA* and *pccB* as a function of T7 promoter strength driving Mod2 (the data were normalized to PCC expression in the 100% strain)

Discussion

We recently reported an enabling technology for high-throughput synthesis of long genes with codons optimized for expression in any given organism [7], as well as a universal design of PKS modules to make rapid experiments in combinatorial biosynthesis [1]. Each redesigned PKS module is flanked by the same pair of restriction sites such that 3′ and 5′ ends can be ligated; likewise, corresponding domains within all modules are flanked by the same two unique restriction sites (Fig. 2). Thus, modules may be connected at will to make long PKS genes, and modules or domains may be readily interchanged as cassettes. The redesigned genes require, at most, three amino acid changes per PKS module, and these are usually conservative with respect to the native sequence.

Using this approach, the six extender modules as well as the loading and thioesterase domains of 6dEB were synthesized using codons optimized for *E. coli*, and assembled as three ORFs into a single 32 kb sequence [7]. However, production of 6dEB was meager. It has been previously established that coexpression of the three natural ORFs on three separate plasmids in *E. coli* K207-3 produces 6dEB [9]. In the present work, each of the three synthetic ORFs of the DEBS gene was likewise placed under control of a T7 promoter in three separate compatible plasmids. Expression of the synthetic ORFs in *E. coli* led to readily visualized protein on SDS-PAGE but initially no polyketide was detected by LC-MS. Then, by comparing 6dEB production by chimeras containing one synthetic and two natural ORFs, we located the defect to DEBS2, where a non-conserved mutation of PR to LQ in Mod4 was uniquely necessary to introduce a desired *Pst*I site; chimeras lacking synthetic DEBS2 all produced considerable amounts of 6dEB. Conversion of the LQ sequence back to the natural PR sequence resulted in substantial 6dEB production, thus validating the catalytic activity of all the synthetic modules.

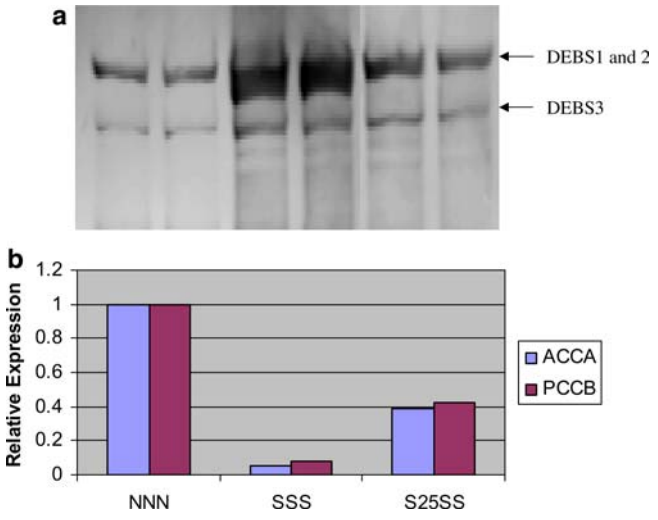

Fig. 4 Modulation of the production of the accessory proteins ACCA and PCCB by the expression DEBS genes in *E. coli* K-207-3. **a** SDS-PAGE stained with Sypro Red. DEBS proteins are indicated. **b** Plot of relative (average) production of ACCA and PCCB in *E. coli* expressing wild-type and synthetic DEBS genes. *NNN* combination of wild-type DEBS genes. *SSS* combination of synthetic DEBS genes. *S25* synthetic DEBS1 expressed from a 25% mutant T7 promoter

A dramatic reduction in 6dEB production was found when two or three synthetic ORFs were coexpressed. When the expression of redesigned genes was attenuated by weaker promoters; production of the polyketide product increased, as did expression of required accessory proteins. Thus, it appears that the very high expression of codon-optimized PKS genes achieved with the T7 promoter causes a decrease in the production of other proteins needed for 6dEB production.

Utility of the generic design of PKS modules is well illustrated by the facile production of constructs made and utilized in this study. Codon optimization of the naturally high G+C sequences of PKS genes provide significant increases in protein expression in *E. coli* and, with only one exception thus far—the PR to LQ mutation needed for a *Pst*I site in DEBS Mod4—the amino acid changes needed to incorporate restriction sites did not greatly effect polyketide production. An important finding was that overexpression of PKS genes promoted by the codon-optimized sequences in fact decreased 6dEB production, confirming that "more is not always better" when expressing a catalyst to create a new metabolic pathway in a cell [14]. In the present case, the problem was surmounted by attenuating transcription of the PKS genes, but at some point will need to be resolved by development of selective tuning systems for the expression of the numerous foreign proteins required for polyketide production.

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