Heterologous expression of diverse propionyl-CoA carboxylases affects polyketide production in Escherichia coli

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INTRODUCTION

Polyketide synthase (PKS) enzymes catalyze the biosynthesis of polyketide natural products.1 One class of PKS enzymes, called multi-modular or assembly-line PKSs, are organized into multiple modules, where each module covalently adds a ketide unit derived from an acyl-CoA to the growing natural product. PKSs of this class produce the polyketide backbone of antibacterials (erythromycin), antifungals (amphotericin), immunosuppressive agents (rapamycin), and anticancer compounds (epothilone). In microbial genomes, PKS genes are often co-localized with all other genes required for the production of a given compound in a biosynthetic gene cluster. With the increasing ease of DNA sequencing, hundreds of novel assembly-line PKS gene clusters have been identified in recent years.2,3

Heterologous expression of natural product genes in model hosts has been applied to access novel natural products including polyketides.4 Escherichia coli is an attractive host for many reasons: it is easy to culture with a well-developed genetic toolbox, the primary metabolism is well understood, and because it is not an endogenous producer of polyketides, potential interference of native proteins with heterologously expressed PKS pathways may be limited.5 Despite these advantages, attempts at heterologous production of polyketides in E. coli have met with limited success. Examples of assembly-line PKS pathways expressed in E. coli include those encoding epothilone6 and 6-deoxyerythronolide B (6-dEB).7 Titers for 6-dEB have ranged from 1 to 20 μg l−1, whereas titers for epothilone were significantly lower (0.001 μg l−1). These low titers have limited the utility of E. coli as a host for production of assembly-line polyketides, and have spurred efforts to improve general characteristics of this host to produce this class of compounds.7

These limitations point to a need for systematic optimization of heterologously expressed pathways. One approach to improving E. coli as a host has been to increase availability of the substrates of PKS enzymes.8 For many assembly-line PKS pathways, the extender unit for the polyketide chain is (2S)-methylmalonyl-CoA (mmCoA), a metabolite not naturally produced by E. coli.9 Murli et al.10 compared three routes of heterologous mmCoA production in E. coli: (1) carboxylation of propionyl-CoA by the propionyl-CoA carboxylase (PCC) from Streptomyces coelicolor, (2) conversion of succinyl-CoA to (2R)-mmCoA and then to (2S)-mmCoA by the mmCoA mutase/epimerase from Propionibacterium shermanii and (3) synthesis of (2S)-mmCoA from methylmalonate by the methylmalonyl-CoA ligase from S. coelicolor. The 6-dEB titers varied depending on the means of mmCoA production, with the PCC system consistently proving the most productive. These results suggested that, despite the low turnover rate of the 6-deoxyerythronolide B synthase (DEBS),11 the choice of pathway for heterologous mmCoA production has an effect on downstream polyketide titers. We hypothesized that further optimization of this upstream step, particularly of the promising PCC pathway, might further increase titers.

PCC is a biotin-dependent enzyme that catalyzes the carboxylation of propionyl-CoA to (2S)-mmCoA. In actinomycetes, such as S. coelicolor, the core catalytic PCC complex consists of α and β-subunits, each encoded by a distinct gene. The α-subunit consists of biotin carboxyl carrier protein and biotin carboxylase domains while the β-subunit has carboxyltransferase activity. Structural studies in S. coelicolor demonstrated that the β-subunits are homohexamers and implied an α6β6 PCC complex architecture.12

In this study, we examined the effect of PCC expression and identity on polyketide production in E. coli by both optimizing the expression of S. coelicolor PCC complex and screening of 13 homologous PCCs from diverse species.

RESULTS AND DISCUSSION

Our study of the effect of the PCC complex on heterologous polyketide production in E. coli began with a previously reported
did not result in higher polyketide titers, we turned our attention to E. coli in (Figure 1c), suggesting that production, when expressed in conjunction with the DEBS PKS genes the optimized PCC complex did not lead to increased polyketide (Supplementary Figure S1). Notably, increased protein expression of whereas no biotinylation was detected on the wild-type streptavidin showed that the optimized wild-type α-subunit expression (Figure 1b). Western blotting with α-subunit was biotinylated, because the PCC β-subunit was highly expressed and PCC with the two genes comprising the S. coelicolor PCC constructs in the soluble cell lysate fraction. (Figure 2) Table of genetic design differences, PCC subunits expression, and 6-deB production from the wild type and optimized constructs. 5′UTR, 5′ untranslated region; 6-deB, 6-deoxyerythronolide B; AmpR, ampicillin resistance gene; CoE1 ori, colicin E1 origin of replication; His6 tag, 6x polyhistidine tag; PCC, propionyl-CoA carboxylase.

Figure 1 Propionyl-CoA carboxylase (PCC) protein expression and 6-deB production of strains harboring wild type and optimized S. coelicolor PCC complexes. (a) Scheme representation of the PCC complex-harboring vectors. (b) Coomassie-stained denaturing protein gel of α and β-subunits of the wild type and computationally optimized S. coelicolor PCC constructs in the soluble cell lysate fraction. (c) Table of genetic design differences, PCC subunits expression, and 6-deB production from the wild type and optimized constructs. 5′UTR, 5′ untranslated region; 6-deB, 6-deoxyerythronolide B; AmpR, ampicillin resistance gene; CoE1 ori, colicin E1 origin of replication; His6 tag, 6x polyhistidine tag; PCC, propionyl-CoA carboxylase.

system in which heterologous expression of the DEBS genes along with the two genes comprising the S. coelicolor PCC complex (PCC α and PCC β-subunit) led to production of 6-deB, the aglycone precursor of erythromycin. In this system, we observed poor expression of the S. coelicolor PCC α-subunit (Figure 1). Because a previous study showed that reduced expression of PCC subunits was correlated with reduced 6-deB production, we first sought to improve PCC α-subunit protein expression. We applied three strategies to increase protein expression. First, we substituted the wild-type ribosome-binding site (RBS) sequence with the RBS sequence upstream of the PCC β-subunit, because the PCC β-subunit was highly expressed (Figure 1b, construct #1). Second, we optimized the first 20 codons to match the codon frequencies of E. coli, since rare codons in the 5′ end of the gene have been shown to reduce expression levels. Third, we applied a computational tool developed by Salis et al. to optimize this gene’s 5′ untranslated region (5′UTR) pre-RBS sequence upstream of the newly substituted RBS. This method correctly predicted low expression of the wild-type PCC α-subunit. Combining these three changes resulted in an optimized construct (Figure 1a, construct #2) that led to ~25-fold increase in α-subunit expression relative to the wild-type α-subunit expression (Figure 1b). Western blotting with streptavidin showed that the optimized α-subunit was biotinylated, whereas no biotinylation was detected on the wild-type α-subunit (Supplementary Figure S1). Notably, increased protein expression of the optimized PCC complex did not lead to increased polyketide production, when expressed in conjunction with the DEBS PKS genes (Figure 1c), suggesting that α-subunit expression was not limiting the ability of the S. coelicolor PCC complex to facilitate 6-deB production in E. coli.

Since higher protein expression of the S. coelicolor PCC α-subunit did not result in higher polyketide titers, we turned our attention to other avenues for polyketide production improvement via PCC complexes. With the increasing availability of DNA sequence and synthesis, one strategy employed in metabolic engineering is comparison of homologous enzymes from diverse organisms. We hypothesized that homologous PCC complexes from nature could lead to higher polyketide titers than the single tested PCC complex from S. coelicolor, for example through higher specific PCC activity in E. coli, improved protein folding in E. coli, interaction with host factors necessary for enzymatic activity, or effects on the PKS itself. We selected genes encoding 13 PCC complexes from bacteria and eukaryotes, nine of which had experimentally verified PCC activity in the literature, and four of which were uncharacterized but shared sequence similarity with a verified PCC (Materials and Methods, Supplementary Figure S2, Supplementary Table S2).

To design PCC expression constructs, we began with the previously published PCC construct discussed above, referred to hereafter as the wild-type PCC from S. coelicolor. In this design, one T7 promoter drives expression of the two pcc genes in E. coli. The β-subunit harbors an N-terminal polyhistidine tag (His6 tag). To facilitate standardized DNA synthesis and to assay protein expression for all PCC complex homologs, we made three major changes from the initial wild-type design. First, each gene was flanked by an N-terminal His6 tag and a C-terminal Flag tag (Figure 2). Second, each α and β-subunit was preceded by a constant 5′UTR (named 5′UTR-A and 5′UTR-B, respectively), each harboring a distinct RBS to drive protein translation. Finally, as these genes come from diverse species, all genes, including those from S. coelicolor, were codon-optimized to match the codon frequencies of highly expressed E. coli genes through synonymous mutations.

| Experiment | PCC homolog | Codon usage | α-subunit N-terminal tag | α-subunit C-terminal tag | β-subunit 5′UTR | β-subunit 5′UTR expression | α-subunit expression | 6-deB (mg/l) |
|------------|-------------|-------------|-------------------------|-------------------------|----------------|---------------------------|---------------------|-------------|
| 1          | S. coelicolor | wild-type   | 60b His6 tag | 48b His6 tag | 5′UTR-B | High | Low | 8 ± 2 |
| 2          | S. coelicolor | wild-type   | 60b His6 tag | 48b His6 tag | 5′UTR-Salis | High | High | 8 ± 2 |

Table S2: Table of genetic design differences, PCC subunits expression, and 6-deB production from the wild type and optimized constructs.
We generated strains of E. coli that harbored an engineered PCC complex (two genes) on one plasmid (Figure 2), and the 6-dEB PKS pathway (three genes) on a second plasmid. Despite the relatively low maximum turnover rate of the complete hexamodular DEBS system in vitro (1.1 min⁻¹; ref. 11), titers of 6-dEB ranged from 0.2 to 6 mg l⁻¹ (Table 1) demonstrating that the identity of PCC significantly affects 6-dEB titers. Possible explanations include (1) in vivo mmCoA concentrations are below the saturation level of the DEBS system, and these concentrations are dependent on PCC identity, possibly through expression level, solubility, α-subunit biotinylation or activity, or (2) PCC identity affects DEBS protein expression. Measuring mmCoA concentrations and DEBS protein expression may clarify among these and other possible explanations.

The strain harboring a PCC homolog from Myxococcus fulvus produced the highest titer of 6-dEB among the 13 strains tested, despite low levels of PCC protein expression (Supplementary Figure S3). The strain harboring the engineered PCC homolog from S. coelicolor (the wild-type version of which had been previously studied) produced the third-highest titer. Four PCC pathways have been previously characterized in terms of kinetic parameters, but there was no observable trend between Km and 6-dEB production (Table S5).

We compared the E. coli strains harboring the 13 engineered PCCs to a strain harboring the previously tested wild-type S. coelicolor PCC (Supplementary Figure S4). To our surprise, the strain harboring the wild-type S. coelicolor PCC resulted in significantly higher titers (20 mg l⁻¹) than all 13 strains harboring engineered PCC homologous complexes, including that of the engineered S. coelicolor PCC (3 mg l⁻¹) and even the best-performing engineered homolog from M. fulvus (6 mg l⁻¹, Table 2, experiments 1, 3 and 5).

This large difference in titer between the wild-type S. coelicolor PCC construct and all engineered PCC constructs prompted us to investigate the genetic differences that could be causing the titer differences. As described above, the engineered PCC constructs were modified from the wild-type constructs in three ways: (1) epitope tags were added to the engineered PCC subunits, (2) the 5'UTR upstream of each PCC subunit was modified, and (3) synonymous mutations of the pcc genes were engineered to match E. coli codon usage.

Since the PCC complex has an αβαβarchitecture in several organisms, including species of Streptomyces21 and Myxococcus,22 we first considered the possibility that epitope tags could influence the protein complex in terms of stability or activity. To test the hypothesis that epitope tags on PCC proteins reduced polyketide titers, we built several new strains harboring engineered S. coelicolor and M. fulvus PCC complexes with presence and absence of N- and C-terminal epitope tags (Table 2). Eliminating two of the four epitope tags from the two PCC proteins dramatically increased polyketide production in strains expressing codon-optimized PCCs from both S. coelicolor (20 mg l⁻¹ vs 3 mg l⁻¹) and M. fulvus (10.5 mg l⁻¹ vs 6 mg l⁻¹, Table 2, experiments 3–6). Similarly, addition of a C-terminal His₆ tag to the α-subunit of the wild-type S. coelicolor PCC significantly reduced polyketide titers (8 mg l⁻¹ with both N- and C-terminal tags vs 20 mg l⁻¹ with only the N-terminal tag, Table 2, experiments 2 and 1). When two of the four tags were eliminated, the engineered S. coelicolor homolog outperformed the engineered M. fulvus homolog (20 mg l⁻¹ vs 10.5 mg l⁻¹, Table 2, experiments 4 and 6) and matched the wild-type S. coelicolor PCC (20 mg l⁻¹ vs 20 mg l⁻¹, Table 2, experiments 4 and 1, respectively). These results demonstrate that certain epitope tags on PCC subunits substantially decrease polyketide production in E. coli.

The PCC complex is homologous to the acetyl-CoA carboxylase (ACC) complex, which exists natively in all bacteria, including E. coli. ACC catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, which is the first committed step of fatty-acid biosynthesis. Similar to the PCC from S. coelicolor, the E. coli ACC is a multi-subunit complex, encoded by a biotin carboxylase, a biotin carboxyl carrier protein and a two-subunit carboxyltransferase.23 In S. coelicolor, the PCC and ACC complexes share a single α-subunit.24 In other species, the carboxylation of propionyl-CoA and acetyl-CoA are catalyzed by a single enzyme.25–28 Since increased protein expression of the S. coelicolor α-subunit did not result in increased polyketide production (Figure 1), it is possible that either the S. coelicolor α-subunit is not rate-limiting, or the heterologous α-subunit is not required for 6-dEB production because the E. coli biotin carboxyl carrier protein subunit of ACC collaborates with the S. coelicolor β-subunit of PCC.

We tested this hypothesis by measuring polyketide production in E. coli strains harboring the engineered S. coelicolor α and β-subunit.
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of 6-dEB of the highest levels of heterologous polyketide production. The molar yield systems tested, the PCC complex from S. coelicolor production. We characterized the best-performing PCC homologs in outperformed all engineered PCC complexes in terms of polyketide strains lacking both subunits (2.2 mg l\textsuperscript{-1}) which produced the highest 6-dEB titer, is 1.2%, which is in good effect on DEBS protein expression. Future work quantifying mmCoA tion in E. coli logous PCC complexes from different species on polyketide produc-

CONFLICT OF INTEREST

The authors declare no conflict of interest.

Table 2 6-dEB production in wild-type S. coelicolor PCC and engineered S. coelicolor and M. fulvus PCC strains.

| Experiment | PCC homolog present | α-subunit present | Codon usage | 5′ UTR | N-terminal tag | C-terminal tag | 6-dEB (mg l\textsuperscript{-1}) |
|------------|---------------------|------------------|-------------|--------|---------------|---------------|-----------------|
| 1          | S. coelicolor       | Yes              | wild-type   | 5′UTR-B | N1          | —             | 20 ± 3          |
| 2          | S. coelicolor       | Yes              | wild-type   | 5′UTR-B | N1          | —             | 8 ± 2          |
| 3          | S. coelicolor       | Yes              | engineered  | 5′UTR-B | N2          | C1            | 3.0 ± 0.4       |
| 4          | S. coelicolor       | Yes              | engineered  | 5′UTR-B | N2          | 5′UTR-A       | C3            |
| 5          | M. fulvus           | Yes              | engineered  | 5′UTR-B | N2          | C1            | 6 ± 1          |
| 6          | M. fulvus           | Yes              | engineered  | 5′UTR-B | N2          | 5′UTR-A       | C3            | 10.5 ± 0.2     |
| 7          | S. coelicolor       | No               | No engineered | —       | —           | 5′UTR-A       | C3            | 2.2 ± 0.7      |
| 8          | S. coelicolor       | No               | No engineered | —       | —           | C1            | 4 ± 1          |
| 9          | No PCC              | No               | No engineered | —       | —           | —             | 0.5 ± 0.3      |

Abbreviations: PCC, propionyl-CoA carboxylase; 5′UTR, 5′ untranslated region.
N1-60b His\textsubscript{16} tag, N2-42b His\textsubscript{16} tag, C1-42b Flag tag, N3-42b Flag tag, C2-48b His\textsubscript{16} tag, C3-42b Histag. Mean of three biological replicates ± s.e.

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References

1. Cane, D. E., Walsh, C. T. & Khosla, C. Harnessing the biosynthetic code: combinations, permutations, and mutations. Science 282, 63–68 (1998).
2. O’Brien, R. V., Davis, R. W., Khosla, C. & Hillenmeyer, M. E. Computational identification and analysis of orphan assembly-line polyketide synthases. J. Antibiot. 67, 89–97 (2014).
3. Hillenmeyer, M. E., Vandonia, G. A., Berlew, E. E. & Charleston, L. K. Evolution of chemical diversity by coordinated gene swaps in type II polyketide gene clusters. Proc. Natl Acad. Sci. USA 112, 13952–13957 (2015).
4. Pfeifer, B. A. & Khosla, C. Biosynthesis of polyketides in heterologous hosts. Microbiol. Mol. Biol. Rev. 65, 106–118 (2001).
5. Gao, X., Wang, P. & Tang, Y. Engineered polyketide biosynthesis and biocatalysis in Escherichia coli. Appl. Microbiol. Biotechnol. 88, 1233–1242 (2010).
6. Mulka, S. C., Carney, J. R., Liu, Y. & Kennedy, J. Heterologous production of epothilone C and D in Escherichia coli. Biochemistry 45, 1321–1330 (2006).
7. Pfeifer, B. A., Admiraal, S. J., Gremer, H., Carle, D. E. & Khosla, C. Biosynthesis of complex polyketides in a metabolically engineered strain of E. coli. Science 291, 1790–1792 (2001).
8. Pickens, L. B., Tang, Y. & Chooi, Y. H. Metabolic engineering for the production of natural products. Annu. Rev. Chem. Biomol. Eng. 2, 21–236 (2011).
9. Zhang, H., Baghipan, B. A. & Pfeifer, B. A. Investigating the role of native propionyl-CoA and methylmalonyl-CoA metabolism in heterologous polyketide production in Escherichia coli. Biotechnol. Bioeng. 105, 567–573 (2010).
10. Murli, S., Kennedy, J., Dywern, L. C., Carney, J. R. & Kealey, J. T. Metabolic engineering of Escherichia coli for improved 6-deoxyerythronolide B production. J. Ind. Microbiol. Biotechnol. 30, 500–509 (2003).
11. Lowy, B. et al. In vitro reconstitution and analysis of the 6-deoxyerythronolide B synthase. J. Am. Chem. Soc. 135, 16809–16812 (2013).
12. Diakovitch, L. et al. Crystal structure of the β-subunit of acetyl-CoA carboxylase: structure-based engineering of substrate specificity. Biochemistry 43, 14027–14034 (2004).
13. Menzel, H. G. et al. Redesign, synthesis and functional expression of the 6-deoxyerythronolide B polyketide synthase gene cluster. J. Ind. Microbiol. Biotechnol. 33, 22–28 (2006).
14. Welch, M., Villalobos, A., Gustafsson, C. & Minshull, J. Designing genes for successful protein expression. Methods Enzymol. 498, 43–66 (2011).
15. Salis, H. M., Minsky, E. A. & Voigt, C. A. Automated design of synthetic ribosome binding sites to control protein expression. Nat. Biotechnol. 27, 946–950 (2009).
16. Bayer, T. S. et al. Synthesis of methyl halides from biomass using engineered microbes. J. Am. Chem. Soc. 131, 6508–6515 (2009).
17. Jendresen, C. B. et al. Highly active and specific tyrosine ammonia-lyases from diverse origins enable enhanced production of aromatic compounds in bacteria and Saccharomyces cerevisiae. Appl. Environ. Microbiol. 81, 4458–4476 (2015).
18. Narcross, L., Bourgeois, L., Fossati, E., Burton, E. & Martin, V. J. Mining enzyme diversity of transcriptome libraries through DNA synthesis for benzylisoquinoline alkaloid pathway optimization in yeast. ACS Synth. Biol. 5, 1505–1518 (2016).
19. Lee, H. Y. & Khosla, C. Bioassay-guided evolution of glycosylated macrolide antibiotics in Escherichia coli. PLoS Biol. 5, e45 (2007).
20 Zhang, H., Wang, Y., Wu, J., Skalina, K. & Pfeifer, B. A. Complete biosynthesis of erythromycin A and designed analogs using E. coli as a heterologous host. Chem. Biol. 17, 1232–1240 (2010).
21 Huang, C. S. et al. Crystal structure of the α6β6 holoenzyme of propionyl-coenzyme A carboxylase. Nature 466, 1001–1005 (2010).
22 Kimura, Y., Kojyo, T., Kimura, I. & Sato, M. Propionyl-CoA carboxylase of Myxococcus xanthus: catalytic properties and function in developing cells. Arch. Microbiol. 170, 179–184 (1998).
23 Li, S. J. & Cronan, J. E. Jr. The genes encoding the two carboxyltransferase subunits of Escherichia coli acetyl-CoA carboxylase. J. Biol. Chem. 267, 16841–16847 (1992).
24 Diacovich, L. et al. Kinetic and structural analysis of a new group of acyl-CoA carboxylases found in Streptomyces coelicolor A3(2). J. Biol. Chem. 277, 31228–31236 (2002).
25 Erfle, J. D. Acetyl-CoA and propionyl-CoA carboxylation by Mycobacterium phlei partial purification and some properties of the enzyme. Biochim. Biophys. Acta 316, 143–155 (1973).
26 Hasse, F. C., Henrikson, K. P., Treble, D. H. & Allen, S. H. The subunit structure and function of the propionyl coenzyme A carboxylase of Mycobacterium smegmatis. J. Biol. Chem. 257, 11994–11999 (1982).
27 Hunaiti, A. R. & Kolattukudy, P. E. Isolation and characterization of an acyl-coenzyme A carboxylase from an erythromycin-producing Streptomyces erythreus. Arch. Biochem. Biophys. 216, 362–371 (1982).

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