Brief report

Probing the heterologous metabolism supporting 6-deoxyerythronolide B biosynthesis in Escherichia coli

Haoran Zhang, Yong Wang, Brett Boghigian and Blaine A. Pfeifer*
Department of Chemical and Biological Engineering, Tufts University, Medford, MA 02155, USA.

Summary

Heterologous biosynthesis offers a new way to capture the medicinal properties presented by complex natural products. In this study, production of 6-deoxyerythronolide B (6dEB), the polyketide precursor to the antibiotic erythromycin, was used to probe the heterologous pathways needed for Escherichia coli-derived biosynthesis. More specifically, the heterologous proteins responsible for 6dEB production were varied by adjusting their respective gene dosage levels. In this way, heterologous components required for posttranslational modification, 6dEB biosynthesis, and substrate provision were adjusted in expression levels to observe the relative effect each has on final heterologous biosynthesis. The results indicate that both the biosynthetic and substrate provision heterologous proteins impact 6dEB formation to a greater extent when compared with posttranslational modification and suggest these components for future protein and metabolic engineering.

Introduction

Complex natural products, such as polyketides, have a well-documented range of medicinal values (Sanchez et al., 2008). The therapeutic value associated with natural products has then placed an emphasis on finding methods to isolate and over-produce such compounds, a task made difficult, in many cases, by the fastidious nature of the native microbial hosts responsible for the bulk of natural compounds. The challenges in producing therapeutic natural compounds from their original host organisms spurred the field of heterologous natural product biosynthesis where a natural product genetic sequence is transferred from the original producer to a new host system with capabilities that include improved growth speed, genetic and metabolic engineering potential, and improved prospects for process scale-up and development (Zhang et al., 2008).

Heterologous hosts include well-characterized microbial organisms such as Escherichia coli, Bacillus subtilis and Saccharomyces cerevisiae. Each has been used in the context of heterologous complex natural product biosynthesis, and in each case, the genetic or molecular biology tools associated with the surrogate host greatly aided the heterologous transfer of the natural product pathways (Eppelmann et al., 2001; Pfeifer et al., 2001; Mutka et al., 2006). In addition, the basic growth requirements and rapid growth speeds of the cited heterologous hosts further simplify efforts to reconstitute foreign natural product pathways. In this setting, production at even the lowest product titres signifies success of the heterologous metabolic engineering effort. Once a natural product pathway has been established, even more tools are then available to probe and further improve heterologous production with two ultimate goals: (i) to maximize production of a specific natural product and (ii) to better characterize and understand the interaction between native and heterologous metabolism such that the resulting information will facilitate future metabolic engineering efforts.

To provide an example relevant to the study conducted here, the complex polyketide compound 6-deoxyerythronolide B (6dEB, the macrocyclic core of the antibiotic erythromycin) was produced through E. coli by engineering intracellular pathways for substrate provision and polyketide biosynthesis (Pfeifer et al., 2001). The native E. coli operon for propionate catabolism was interrupted such that the only gene remaining was prpE (encoding a propionyl-CoA synthetase). In addition, a non-native propionyl-CoA carboxylase (PCC, originally from Streptomyces coelicolor) was introduced to provide a metabolic pathway capable of converting exogenously fed...
propionate to propionyl-CoA and (2S)-methylmalonyl-CoA, the two substrates needed by the three deoxyerythronolide B synthase (DEBS) biosynthetic enzymes responsible for 6dEB biosynthesis. However, prior to catalysing 6dEB formation, the DEBS enzymes require a posttranslational modification step catalysed by a phosphopantetheinytransferase. Native to B. subtilis, a phosphopantetheinytransferase gene termed sfp was transferred to the E. coli chromosome in the same step used to disrupt the prp operon.

Although the cellular design described for E. coli-derived 6dEB resulted in positive production from the new host (with initial titres between 5 and 30 mg l\(^{-1}\)), a simple derived 6dEB resulted in positive production from the new host (with initial titres between 5 and 30 mg l\(^{-1}\)), a simple derived 6dEB resulted in positive production from the new host (with initial titres between 5 and 30 mg l\(^{-1}\)). The experimental approach to do so involved altering the gene copy number associated with Sfp (posttranslational modification), DEBS (6dEB biosynthesis) and PCC (substrate provision) (Table 1). For DEBS, strain YW9, which contains the DEBS genes in the chromosome of E. coli, is compared with BAP1(pBP130/pBP144), which has the DEBS genes within medium copy pET plasmids. Similar strains were used to test the effects of Sfp and PCC on 6dEB biosynthesis (Table 2). In this manner, final titre levels (mg l\(^{-1}\) of 6dEB) would be correlated to gene dosage and expression of the necessary heterologous enzymes needed for 6dEB biosynthesis.

To verify gene expression design, the strains used to vary heterologous gene dosage were first analysed by

### Table 1. Strains and plasmids.

| Description                  | Reference |
|------------------------------|-----------|
| **E. coli strain**           | Pfeifer et al. (2001) |
| BAP1                         | Wang and Pfeifer (2008) |
| YW9                         | This study |
| **Plasmid**                  |           |
| pBP13                       | Pfeifer et al. (2001) |
| pBP144                      | Pfeifer et al. (2001) |
| pYW1200                     | This study |
| pYW7317                     | This study |
| pACYC-sfp                   | This study |

T7 promoters and terminators have been abbreviated as T7prom and T7term, respectively. T7 promoter regions also include a lac operator and a ribosomal binding sequence (rbs). Restriction enzymes and Phusion High-Fidelity PCR Master Mix were purchased from New England Biolabs; PCR primers were synthesized by Operon. Restriction enzyme digestions, plasmid transformations, SDS-PAGE and other standard molecular biology techniques were carried out as described by Sambrook and colleagues (1989). Plasmids pBP130 and pBP144 were constructed as described previously (Pfeifer et al., 2001). Plasmid pYW7317 was constructed by digesting pBP144 with Ndel, which liberated the PCC genes, followed by vector self-ligation. The B. subtilis sfp gene was isolated from pBP80 (a plasmid containing sfp, B. A. Pfeifer and C. Khosla, unpublished) as an Ndel/Xhol fragment and inserted into pACYCDuet-1 (Novagen), resulting in pACYC-sfp. *Escherichia coli* strains BAP1 and YW9 have been described in previous work (Pfeifer et al., 2001; Wang and Pfeifer, 2008). To construct YW1, the method described by Hamilton and colleagues (1989) was used for chromosomal gene replacement. The PCC genes were first cloned from pBP144 into pET21c (pYW1200). The expression cassette containing the PCC subunit genes (T7prom-accA1-rbs-pccB-T7term) was then amplified by PCR with the following primers: 5′-GGCCGCCGCGGCTGACCGCCTGACCTGAAGATTCGATCCCC (forward) and 5′-GCCGCCGCTGACCTGGTACCGATTCC (reverse). The PCR fragment was inserted after the sfp gene in pMAK705 (Pfeifer et al., 2001), and the resulting temperature-sensitive plasmid was used for gene replacement in the chromosome of B21(DE3).

**Results and discussion**

The DEBS, PCC and Sfp enzymes are all heterologous with respect to native *E. coli* metabolism and are all required for eventual 6dEB biosynthesis (Pfeifer *et al.*, 2001). However, though it is known that each enzyme aids 6dEB biosynthesis, the relative impact of each is unknown. Moreover, future efforts to improve heterologous 6dEB biosynthesis will depend on identifying components that most influence and/or present bottlenecks to production.

Therefore, we varied the levels of these important heterologous enzymes with the goal of determining which had the most impact on 6dEB biosynthesis. The experimental approach to do so involved altering the gene copy number associated with Sfp (posttranslational modification), DEBS (6dEB biosynthesis) and PCC (substrate provision) (Table 1). For DEBS, strain YW9, which contains the DEBS genes in the chromosome of *E. coli*, is compared with BAP1(pBP130/pBP144), which has the DEBS genes within medium copy pET plasmids. Similar strains were used to test the effects of Sfp and PCC on 6dEB biosynthesis (Table 2). In this manner, final titre levels (mg l\(^{-1}\) of 6dEB) would be correlated to gene dosage and expression of the necessary heterologous enzymes needed for 6dEB biosynthesis.

To verify gene expression design, the strains used to vary heterologous gene dosage were first analysed by

© 2009 The Authors
Journal compilation © 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Microbial Biotechnology*, **2**, 390–394
Table 2. Strain comparison for PCC, DEBS and Sfp.

| Strain pair                      | Chromosome expression                  | Plasmid expression | Heterologous component |
|----------------------------------|----------------------------------------|--------------------|------------------------|
| BAP1(pBP130/pBP144/pACYCDuet-1) | BAP1(pBP130/pBP144/pACYC-sfp)          | Sfp                |
| YW1(pBP130/pYW7317)             | BAP1(pBP130/pBP144)                    | PCC                |
| YW9(pYW1200)                   | BAP1(pBP130/pBP144)                    | DEBS               |

Variation in heterologous gene dosage was accomplished by coupling strains and plasmids. Final strain pairings are presented between different heterologous components. The ‘Chromosome’ or ‘Plasmid’ heading above each individual strain indicates either chromosomal- or plasmid-borne gene expression for the heterologous component; plasmid-borne expression provides a 10- to 50-fold increase in gene copy. For Sfp variation, BAP1(pBP130/pBP144) was compared with BAP1(pBP130/pBP144/pACYC-sfp). The PCC and DEBS components were similarly compared. For the DEBS case, strain YW9(pYW1200/pET28) could not be used for comparison purposes because of the overlapping kanamycin-resistance marker between strain YW9 and pET28.

SDS-PAGE. As seen in Fig. 1, protein levels correlate with gene dosage design. For example, the DEBS genes are observed when expressed from the pET expression plasmids but are not observed when expressed from low copy number within the chromosome. Figure 1 shows the same trend for the PCC and Sfp enzyme levels. Densitometry analysis was further used to assess and confirm expression differences within the SDS-PAGE data.

Each strain was then tested for 6dEB biosynthesis with results presented in Fig. 2. The 6dEB production levels were evaluated as raw and cell-density-normalized final culture concentrations. Production mirrored the expression results from Fig. 1. Comparing the Sfp, PCC and DEBS heterologous components indicates that between reduced and increased copy numbers, the PCC and DEBS genes had the largest impact on final biosynthesis. The impact caused by the reduction in PCC levels is in contrast to results seen previously from different combinations of E. coli strains and plasmids. The size and location of the Sfp, DEBS and PCC enzymes have been denoted by black-filled dots within the molecular weight marker notations. The Sfp, PCC and DEBS labels above the indicated lanes represent samples exhibiting enhanced protein levels as a result of enhanced gene dosage. Culture conditions were identical to those described for 6dEB product formation (Fig. 2). Post culture, cells were washed and resuspended in TE buffer. Cell densities were equalized between samples and sonication was performed with a Fisher Scientific Sonic Dismembrator Model 100 at maximum setting for a tip probe for three 10 s intervals. Lysates were then centrifuged and the whole cell lysate and soluble fractions were analysed by SDS-PAGE. Only the soluble fraction is presented. The reagents and chemicals used in this study were purchased from Fisher Scientific and Sigma. Below the gel is a semi-quantitative densitometry analysis of protein level differences assessed using ImageJ software version 1.40g (http://rsb.info.nih.gov/ij/). The analysis provides the ratio of densitometry measured band densities between respective Sfp, PCC and DEBS strain pairs (as defined in Table 2): n = 3 and numbers in parentheses are standard deviations.
5m gl–1 erythromycin was added to re-dissolve samples. In a similar manner, 6dEB standard samples were prepared; cultures of BAP1 were acetate. After centrifugation at 2500 r.p.m. for 5 min, 0.75 ml of the ethyl acetate extract was dried overnight and 50 µl of methanol containing 5 mg l–1 erythromycin was added to re-dissolve samples. The production cultures were incubated at 22°C and 250 r.p.m. for 3 days, and final culture optical density (OD) was measured at 600 nm. Triplicate samples were used for each batch and three batches were conducted to ensure statistical analysis and reproducibility. After the culture period was completed, the different strains were clarified by centrifugation and the supernatant analysed using MS and has also been described previously (Wang et al., 2007). The second method of 6dEB analysis relied on MS analysis was used as an internal standard. Final E. coli production cultures were extracted with 1.5 ml ethyl acetate. After centrifugation at 2500 r.p.m. for 5 min, 0.75 ml of the ethyl acetate extract was dried overnight and 50 µl of methanol containing 5 mg l–1 erythromycin was added to re-dissolve samples. In a similar manner, 6dEB standard samples were prepared; cultures of BAP1 were allowed to proceed for 3 days as described above before a series of 6dEB standards were added prior to the extraction with ethyl acetate and sample resuspension in methanol containing erythromycin. The samples were analysed using a LTQ XL Linear Ion Trap Mass Spectrometer (Thermo Electron Corporation) by injection through a 250 µl syringe at a speed of 10 µl min–1. The 6dEB standard samples were used to develop a calibration curve, and the ratio of 6dEB peak intensity to the erythromycin internal standard was correlated to sample 6dEB concentrations by using a calibration curve made before every experimental analysis. Statistical analysis was completed for each comparison pair presented in Table 2. While the effect on 6dEB levels was not significant for the Sfp gene dosage variation comparison, changes associated with PCC and DEBS were statistically significant (95% confidence). Below the bar graph is a final summary collected from the densitometry and 6dEB titre data of Figs 1 and 2; a sensitivity parameter has been defined as the ratio of high and low gene copy 6dEB titres (Fig. 2) divided by the ratio of high and low copy protein levels (Fig. 1) for each heterologous component varied.

Fig. 2. Production of 6dEB as a function of the plasmid and strains varying Sfp, PCC and DEBS levels. Glycerol stocks (15%) were used to start overnight cultures (2 ml) of strain–plasmid combinations at 30°C and 250 r.p.m. in Luria–Bertani medium with antibiotic selection as appropriate [carbenicillin (100 mg l–1), kanamycin (50 mg l–1), chloramphenicol (34 mg l–1)]. The starter cultures were then used to inoculate 3 ml Luria–Bertani production cultures (at 2%, v/v) containing antibiotics (at the same concentrations referenced above), 100 µM isopropyl β-D-1-thiogalactopyranoside and 20 mM sodium propionate. The production cultures were incubated at 22°C and 250 r.p.m. for 3 days, and final culture optical density (OD) was measured at 600 nm. Triplicate samples were used for each batch and three batches were conducted to ensure statistical analysis and reproducibility. After the culture period was completed, the different strains were clarified by centrifugation and the supernatant analysed for 6dEB content using one of two methods. The first method relied on an Agilent 1100 series high-performance liquid chromatography (HPLC) coupled with an Alltech 800 series evaporative light-scattering detector (ELSD) and has been described previously (Wang et al., 2007). The second method of 6dEB analysis relied on MS and has also been described previously (Wang and Pfeifer, 2008). The MS method was reserved for those samples whose 6dEB titres were below the detection limit of the HPLC-ELSD method. For MS analysis, erythromycin (ACROS organics) was used as an internal standard. The Sfp gene dosage variation comparison, changes associated with PCC and DEBS were statistically significant (95% confidence). Below the bar graph is a final summary collected from the densitometry and 6dEB titre data of Figs 1 and 2; a sensitivity parameter has been defined as the ratio of high and low gene copy 6dEB titres (Fig. 2) divided by the ratio of high and low copy protein levels (Fig. 1) for each heterologous component varied.

References

Eppelmann, K., Doekel, S., and Marahiel, M.A. (2001) Engineered biosynthesis of the peptide antibiotic bacitracin in the surrogate host Bacillus subtilis. J Biol Chem 276: 34824–34831.

Hamilton, C.M., Aldea, M., Washburn, B.K., Babitzke, P., and Kushner, S.R. (1989) New method for generating deletions and gene replacements in Escherichia coli. J Bacteriol 171: 4617–4622.

Lau, J., Tran, C., Licari, P., and Galazzo, J. (2004) Development of a high cell-density fed-batch bioprocess for the heterologous production of 6-deoxyerythronolide B in Escherichia coli. J Biotechnol 110: 95–103.

Murli, S., Kennedy, J., Dayem, L.C., Carney, J.R., and Kealey, J.T. (2003) Metabolic engineering of Escherichia coli for improved 6-deoxyerythronolide B production. J Ind Microbiol Biotechnol 30: 500–509.

Mutka, S.C., Bondi, S.M., Carney, J.R., Da Silva, N.A., and Kealey, J.T. (2006) Metabolic pathway engineering for complex polyketide biosynthesis in Saccharomyces cerevisiae. FEMS Yeast Res 6: 40–47.

Pfeifer, B., Hu, Z., Licari, P., and Khosla, C. (2002) Process and metabolic strategies for improved production of Escherichia coli-derived 6-deoxyerythronolide B. Appl Environ Microbiol 68: 3287–3292.

Pfeifer, B.A., Admiraal, S.J., Gramajo, H., Cane, D.E., and Khosla, C. (2001) Biosynthesis of complex polyketides in a metabolically engineered strain of E. coli. Science 291: 1790–1792.

Sanbrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanchez, J.F., Chiang, Y.M., and Wang, C.C. (2008) Diversity of polyketide synthases found in the Aspergillus and Streptomyces genomes. Mol Pharm 5: 226–233.

© 2009 The Authors
Journal compilation © 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, Microbial Biotechnology, 2, 390–394
Wang, Y., and Pfeifer, B.A. (2008) 6-Deoxyerythronolide B production through chromosomal localization of the deoxyerythronolide B synthase genes in *E. coli*. *Metab Eng* 10: 33–38.

Wang, Y., Boghigian, B.A., and Pfeifer, B.A. (2007) Improving heterologous polyketide production in *Escherichia coli* by overexpression of an S-adenosylmethionine synthetase gene. *Appl Microbiol Biotechnol* 77: 367–373.

Zhang, H., Wang, Y., and Pfeifer, B.A. (2008) Bacterial hosts for natural product production. *Mol Pharm* 5: 212–225.