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Identification of parallel and divergent optimization solutions for homologous metabolic enzymes

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ABSTRACT

Metabolic pathway assembly typically involves the expression of enzymes from multiple organisms in a single heterologous host. Ensuring that each enzyme functions effectively can be challenging, since many potential factors can disrupt proper pathway flux. Here, we compared the performance of two enzyme homologs in a pathway engineered to allow *Escherichia coli* to grow on 4-hydroxybenzoate (4-HB), a byproduct of lignocellulosic biomass deconstruction. Single chromosomal copies of the 4-HB 3-monooxygenase genes *pobA* and *praI*, from *Pseudomonas putida* KT2440 and *Paenibacillus* sp. JJ-1B, respectively, were introduced into a strain able to metabolize protocatechuate (PCA), the oxidation product of 4-HB. Neither enzyme initially supported consistent growth on 4-HB. Experimental evolution was used to identify mutations that improved pathway activity. For both enzymes, silent mRNA mutations were identified that increased enzyme expression. With *pobA*, duplication of the genes for PCA metabolism allowed growth on 4-HB. However, with *praI*, growth required a mutation in the 4-HB/PCA transporter *pcaK* that increased intracellular concentrations of 4-HB, suggesting that flux through PraI was limiting. These findings demonstrate the value of directed evolution strategies to rapidly identify and overcome diverse factors limiting enzyme activity.

1. Introduction

Synthetic biologists frequently transfer metabolic pathways from native hosts into more-tractable production strains (Nielsen and Keasling, 2016). By combining enzymes from different organisms, they can construct novel pathways to produce valuable biochemicals (Yim et al., 2011; Galanie et al., 2015). However, combining diverse enzymes into complex pathways is challenging, since the interactions between enzymes within a pathway, or between a pathway and its host, can limit productivity (Michener et al., 2012; Kim and Copley, 2012). A common solution is to screen multiple enzyme homologs, with the goal of identifying a variant that lacks deleterious interactions (Bayer et al., 2009; Narcross et al., 2016). A deeper understanding of the reasons that enzyme homologs function poorly will increase the success rate of screening homolog libraries, and thereby allow faster and cheaper pathway assembly.

The catabolism of lignin-derived aromatic chemicals provides a representative example of this engineering challenge. The thermochemical depolymerization of lignin produces a complex mixture of aromatic compounds (Rodriguez et al., 2017). Though a process described as biological funneling, microbes can convert this low-value mixture of substrates into a small set of core intermediates and then into valuable products (Linger et al., 2014). However, no single microbe has yet been isolated that is capable of catabolizing and valorizing all the constituents of a typical mixture (Bugg et al., 2011). Instead, researchers seek to augment the catabolic capabilities of foundation organisms by introducing new metabolic pathways for conversion of recalcitrant substrates (Strachan et al., 2014). Changes in the feedstock composition and pretreatment strategy will alter the composition of the depolymerized mixture (Ragauskas et al., 2014), necessitating the construction and optimization of an entire suite of microbes, each specific to a particular substrate mixture. Consequently, the facile valorization of lignin-derived aromatics will require the ability to rapidly engineer metabolic networks using pathways from diverse microbes.

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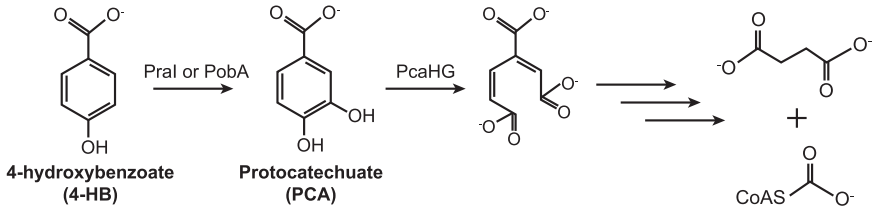

Fig. 1. Catabolism of 4-HB. The 4-HB monooxygenases PraI and PobA convert 4-HB into PCA. The first step in PCA degradation is ring cleavage by the PCA 3,4-dioxygenase PcaHG, ultimately yielding succinate and acetyl-CoA.

Heterologous metabolic pathways are frequently expressed from plasmids. These vectors are easy to construct, and the resulting high DNA copy numbers can compensate for low specific enzyme activity. However, plasmid-based pathways are unstable and difficult to scale for larger pathways, such as those that will be required for catabolism of a mixture of lignin-derived aromatic compounds (Jiang et al., 2013). Moving heterologous enzymes from plasmids to the chromosome increases stability and scaling, and due to new techniques is increasingly practical (Jiang et al., 2015). However, the reduced copy number increases the challenge of providing sufficient enzyme activity (Alonso-Gutierrez et al., 2018; Wang and Pfeifer, 2008).

In this work, we explored the challenges involved in extending a metabolic pathway using chromosomally-expressed enzyme homologs, focusing specifically on pathways for the catabolism of 4-hydroxybenzoate (4-HB) and protocatechuate (PCA) (Fig. 1). These compounds are the core metabolites for catabolism of hydroxyphenyl (H) and guaiacyl (G) lignans, respectively, and therefore are important targets for engineering biological conversion of lignin components. We previously constructed and optimized a pathway in *E. coli* that converts PCA to succinate and acetyl-CoA using the nine-gene *pca* pathway from *Pseudomonas putida* KT2440 (Supplementary Fig. 1). After optimization, the pathway enabled growth with PCA as the sole source of carbon and energy (Clarkson et al., 2017). We then extended the pathway to convert 4-HB into PCA using a 4-hydroxybenzoate monooxygenase, *praI*, from *Paenibacillus* sp. JJ-1B (Kasai et al., 2009), allowing *E. coli* to grow with 4-HB as the carbon source, with limitations that we have now discovered. In the present work, we introduced an alternative 4-hydroxybenzoate monooxygenase homolog, *pobA*, from *Pseudomonas putida* KT2440 (Harwood and Parales, 1996; Jimenez et al., 2002). Using either of the monooxygenases, we selected mutant strains that grew efficiently with 4-HB as the sole source of carbon and energy, at the same rate as with PCA, and without the previous limitations.

Characterizing the resulting evolutionary solutions identified multiple interacting factors that limited 4-HB catabolism and revealed unexpected differences between enzyme homologs. Similar RNA secondary structures, unintentionally introduced during codon optimization, initially decreased expression of both 4-HB monooxygenases. After resolving this issue, we found that the monooxygenases are not fully interchangeable, as different optimization solutions produced varied results with the two homologs. Ultimately, we identified pathway modifications for both homologs that allowed similar levels of growth with either PCA or 4-HB. These modifications included duplications of the core PCA degradation pathway and point mutations in the PCA/4-HB transporter, PcaK. Unexpectedly, these modifications had very different impacts on *praI* and *pobA* strains. Either allowed *pobA* strains to grow on 4-HB, but only the PcaK mutations allowed *praI* strains to grow. The results provide examples of the modifications required to optimize the function of chromosomally-expressed enzyme homologs, facilitating the rapid, predictable construction and debugging of novel metabolic pathways.

2. Materials and methods

2.1. Media and chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fairlawn, NJ) and were molecular grade. All oligonucleotides were ordered from IDT (Coralville, IA). *E. coli* strains were routinely cultivated at 37 °C in LB broth containing the necessary antibiotics (50 mg/L kanamycin or 50 mg/L spectinomycin). Growth assays with PCA and 4-HB were performed in M9 salts medium containing 300 mg/L thiamine and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). PCA and 4-HB were dissolved in water at 5 g/L, filter sterilized, and added at a final concentration of 1 g/L. The pH of the substrate stock solutions was not adjusted, as PCA oxidation in air occurred more rapidly at neutral pH.

2.2. Plasmid construction

Expression for sgRNA plasmids targeting chromosomal loci were constructed as described previously (Clarkson et al., 2017). Briefly, an inverse PCR was used to amplify the vector backbone. Overlapping oligonucleotides containing the new 20-nt targeting sequence were inserted by Gibson assembly and transformed into 10-β *E. coli* (NEB, Waltham, MA). Correct assembly was verified by Sanger sequencing. The plasmids used are listed in Supplementary Table 2.

2.3. Strain construction

Strain JME17 was previously optimized for growth with PCA. For growth with 4-HB, the appropriate 4-HB monooxygenase was introduced into the chromosome of JME17. Construction of JME50, expressing the 4-HB monooxygenase PraI, was described previously (Clarkson et al., 2017). Strain JME38 was constructed in a similar fashion by introducing a commercially-synthesized *pobA* expression cassette (Gen9, Cambridge, MA) into JME17. The promoter and terminator for *pobI* expression were chosen from previously characterized genetic parts (Supplementary Fig. 1B; Chen et al., 2013; Kosuri et al., 2013). The expression cassette was integrated into the *gfcAB* locus using plasmid pJM168 (Jiang et al., 2015). To reconstruct evolved mutations, the mutant locus was amplified from the appropriate genomic DNA and introduced into the selected recipient strain in the same fashion. To introduce a second copy of *pcaHGBDC*, the expression cassette was amplified from JME17, combined with homology arms for *yiaU* by overlap-extension PCR, and transformed into JME17 together with plasmid pJM193. All modifications were verified by colony PCR and Sanger sequencing, or by whole-genome resequencing. The strains used are listed in Supplementary Table 1.

2.4. Experimental evolution

Parental strains were streaked to single colonies. Three colonies were grown to saturation in LB + 1 mM IPTG, then diluted 128-fold into M9 + 1 mM IPTG + 1 g/L 4-HB + 50 mg/L PCA and grown at 37 °C. When the cultures reached saturation, they were diluted 128-