TWIST BIOSCIENCE

APPLICATION NOTE

Pathway Engineering Through 5 kb Gene Synthesis

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INTRODUCTION

A rapid design-build-test cycle is vital to metabolic engineering applications in which researchers re-program microorganisms to produce non-native molecules of interest. To produce these molecules, researchers typically add one gene or an entire pathway of genes into a host organism. These genes encode proteins or enzymes that, when expressed in a microbial host, convert a common substrate into a molecule of interest. For example, the vanillin pathway can be introduced into a microbial host to produce vanilla as an alternative to extracting vanilla from vanilla orchids. Using microbial synthesis as an alternative to chemical synthesis or extraction can yield the same compounds more quickly, at a lower cost, and with fewer environmental impacts.

Challenges exist, however, in reprogramming cells to synthesize molecules in amounts that are sufficient for purification. Traditional methods for metabolic engineering of non-native pathways require access to sequenced genomes and the isolation of genes encoding enzymes of interest through PCR amplification. With the numbers of publicly available annotated genomes constantly growing, novel enzymes can be found with the aid of bioinformatic queries.

Often, the enzymes used in synthetic metabolic pathways are derived from different organisms. Families of homologous enzymes can have a range of activities and substrate specificities, and finding the optimal set of enzymes presents a challenge. In addition, expressing genes in different vectors or under the control of different promoters can result in inconsistent yields, or even in cellular toxicity. Therefore, to ensure success, researchers often screen multiple homologous enzymes for activity, and using synthetic DNA for this purpose eliminates the need for PCR amplification of sequences of interest from genomic sources.

We applied the Twist oligonucleotide synthesis platform, which can synthesize genes as long as 5 kb, to synthesize multiple genes involved in carotenoid synthesis pathways into a single plasmid. Carotenoids are a family of colorful pigment molecules that are commonly produced by plants and algae and are easily verified by color change. We synthesized and expressed homologous genes from multiple organisms in *E. coli*, which lacks an endogenous carotenoid pathway to demonstrate how gene synthesis can dramatically improve the throughput of pathway engineering: results were available within a few days of receiving a synthesized construct.

METHODS

Five enzymes required for the carotenoid pathway were identified using either the KEGG (Kyoto Encyclopedia of Genes and Genomes) database or through the literature (Nishizaki et al. 2007, Yoon et al. 2007): crtE, crtB, crtI, crtY, and crtZ (**Figure 1**). Both sets of sequences for each of the five genes were designed and codon-optimized for expression in *E. coli* using Twist's codon-optimization algorithm. All genes contained their individual stop codons and were ordered from Twist as clonal genes, as a single operon, and in a single Twist catalog bacterial expression vector (pET-28a+). Within each construct, the genes were separated using an 18 bp stretch of DNA and were under the control of a T7 promoter and upstream ribosome binding site (**Figure 2**).

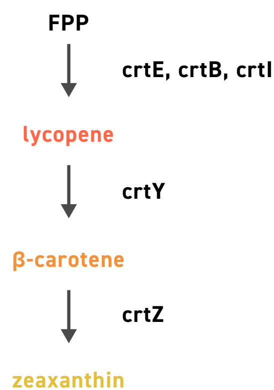
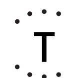


Figure 1. Carotenoid family of pathways and genes selected. The pathway converts farnesyl pyrophosphate (FPP) through several enzymatic steps to produce lycopene (red), β-carotene (orange), or zeaxanthin (yellow) (Farre et al. 2010). The genes involved in each step, and which were used in this study, are indicated.

For expression in *E. coli*, the lyophilized vector DNA was resuspended in 1x TE buffer, and 10 pg was transformed into electrocompetent *E. cloni* 10GF' (Lucigen) or T7 Express competent cells (New England Biolabs). After recovery, bacteria were plated on Lennox LB agar plates containing kanamycin and incubated overnight at 37°C. Individual colonies were picked into liquid LB and grown at 37°C for 16 hours. Cultures were diluted the next morning into 4 ml LB media with 1% glucose and allowed to grow until they reached 0.4–0.6 OD. Expression was induced with 4 mM IPTG, and after 2 hours of growth, cultures were spun down and assayed for color change.



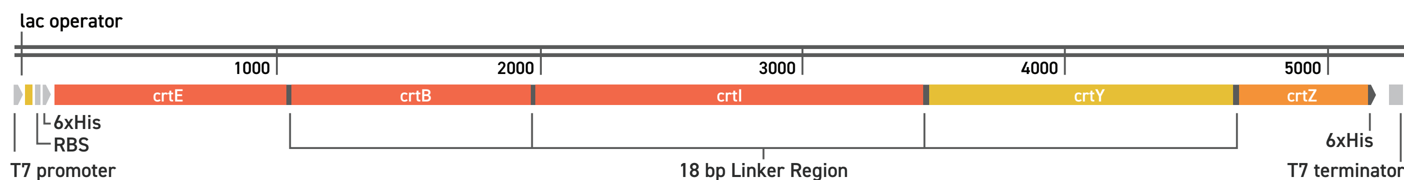


Figure 2. Example construct map of the Zeaxanthin pathway in pET Vector. Individual pathways from the Carotenoid family were constructed of multiple genes separated by an 18-bp glycine-serine linker.

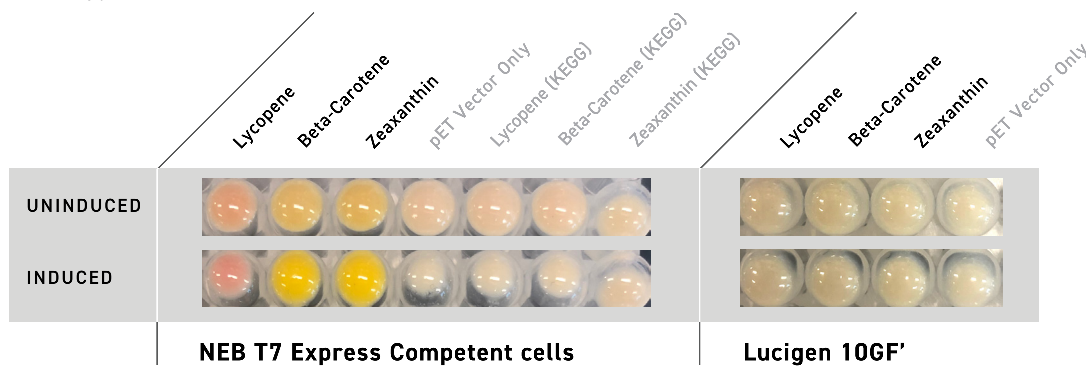


Figure 3. Results of expression studies. Competent T7 Express and 10GF' cells were transformed with vectors containing the genes required for the production of lycopene, β-carotene, and zeaxanthin. An empty pET vector control was used as a negative control. Samples coding for functional pathways are highlighted with black labels. Cells were then either uninduced or induced with IPTG. Note the prominent red, orange, and yellow colors produced by the induced T7 Express cells expressing the genes identified from the literature.

RESULTS

Five genes are involved in the carotenoid pathway, which converts farnesyl pyrophosphate (FPP, a compound created endogenously in *E. coli*) through several enzymatic steps to lycopene, β-carotene, and finally zeaxanthin (Farre et al. 2010). The three steps share common genes; for example, the difference between lycopene and beta-carotene production is the addition of the crtY gene. Each of these pigments produces a unique color, making the expression of an active pathway easy to assay at each endpoint (**Figure 1**).

We identified two sets of sequences for these genes: one set from the KEGG database, and one from the literature (Nishizaki et al. 2007, Yoon et al. 2007). When we entered these sequences into the Twist online ordering platform for clonal genes, we found four of the five pathway genes identified from the KEGG database required codon optimization for synthesis due to low GC content and stretches of homopolymers. Normalizing GC content for the pathway is important for synthesis when creating a pathway, since %GC ranges from species to species and mixing and matching enzymes is important for pathway optimization. Once the sequences were optimized, the genes were synthesized and delivered in a bacterial expression vector using the Twist solid-phase synthesis platform. Each pathway was then expressed in *E. coli* (**Figure 3**).

Figure 3 shows production of all three colored products was successful in the T7 Express cells transformed with the sequences identified in the literature, and as expected, brighter colors were produced by these cells when protein expression was induced by IPTG. The *E. coli* 10GF' cells, which lack a T7 RNA polymerase, yielded no color, indicating the pathways were tightly regulated. Interestingly, the constructs using homologous genes identified from a variety of host organisms through the KEGG database did not perform as well as pathways previously validated in the literature, a result likely indicating non-functional enzymes in *E. coli*.

DISCUSSION

Metabolic engineering enables the efficient synthesis of complex molecules in an environmentally friendly manner. Trial and error are inevitable in this process (as evidenced by the inability of the KEGG-identified constructs to yield product seen in this study), and finding the right combination of gene sequences and promoters is a critical step in optimizing the efficiency and cost-effectiveness of pathway engineering.

Twist Bioscience's silicon-based oligonucleotide synthesis platform can synthesize Clonal Genes of up to 5 kb in length with no minimum order size. This means that Twist Clonal Genes can be designed and built to contain all the genes involved in a pathway in a single plasmid. We exploited this capability and demonstrated that synthesized cloned constructs allow quick and effective evaluation of an engineered pathway through a single transformation into a host organism. This application for Twist Clonal Genes allows researchers to test multiple iterations quickly and efficiently to expedite the engineering of functional pathways, and the scale-up of such studies is now feasible through the use of Twist's high-throughput DNA synthesis technology. In addition, the fact that this technology is available at relatively low cost (15¢ per bp for a 5 kb gene compared to three times more charged by others in the market) makes this approach for engineering and validating metabolic pathways more accessible to more laboratories performing this type of research.

REFERENCES

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