One-Step Construction of a Bioplastic Production Pathway in *E. coli*

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iGEM 2009

Circular Polymerase Extension Cloning

CPEC is a much **simplified sequence-independent cloning technology based entirely on the polymerase extension mechanism**. After denaturation, both single-stranded vector and inserts overlap with each other by end sequences and extend by using the other as the primer in a typical PCR reaction (Figure 1a). **CPEC strategy is not only suitable for the cloning of an individual gene but also for gene libraries, combinatorial libraries and multi-fragment plasmid assembling (figure 1b).**

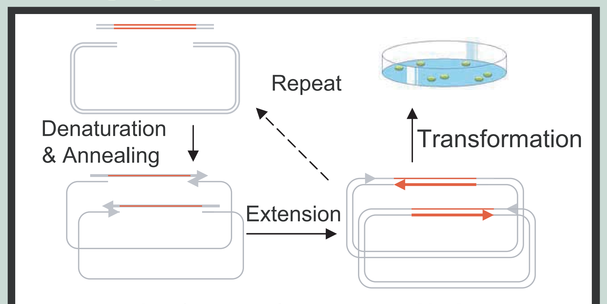

Figure 1a. Single-gene cloning using CPEC

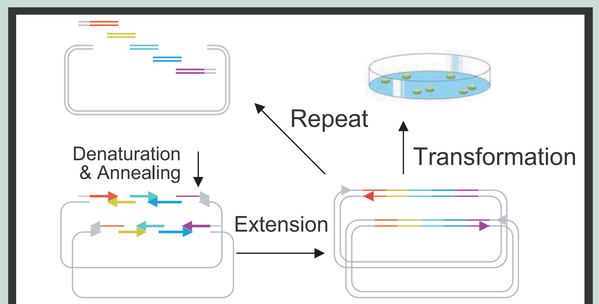

Figure 1b. Multicomponent CPEC

Biodegradable Plastic Synthesis Pathway in *E. coli*

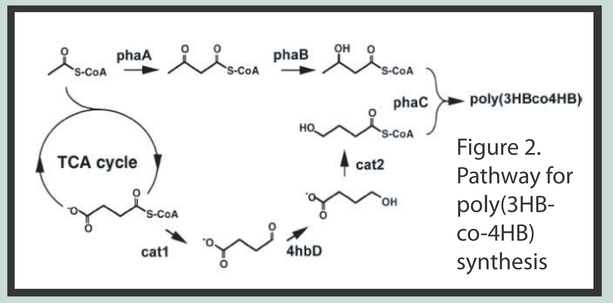

Figure 2. Pathway for poly(3HB-co-4HB) synthesis

The most common PHA is poly(3-hydroxybutyrate), or poly(3HB), which is produced by the bacteria *Cupriavidus necator*. Three genes – phaA, phaB, and phaC – make up the phaCAB operon that synthesizes the enzymes required for formation of poly(3HB), which is not elastic enough for general plastic applications. But when phaCAB is expressed with cat2, a succinate degradation gene from *Clostridium kluyveri* that produces the 4HB monomer, the copolymer poly(3-hyroxybutyrate-co-4-hydroxybutyrate), or poly(3HB-co-4HB), is formed (Figure 2). **Poly(3HB-co-4HB) is more elastic and thus has potential to be used in a variety of applications.**

Thus, this project aims to **develop a more efficient biopathway for poly(3HB-co-4HB) while increasing the 4HB monomer composition predictably.** We **designed vectors with various combinations of the phaCAB operon and the cat2 gene** so that genetic manipulations can be made to the genes for PHA synthase and precursor processing to increase bioplastic production and quality.

Methods

3HB genetic pathway

The phaCAB operon genomic DNA from *Cupriavidus necator* was cloned into PCR Blunt II-TOPO vector as the backbone and the cloning product was transformed into *E. coli* which were then plated on kanamycin (50 µg/ml) plates. **Successful insertion of phaCAB was shown by Nile Red fluorescence of the bacteria under UV light** (Figure 4, right).

4HB genetic pathway

The cat2 gene was inserted into the pSOS vector to obtain the pSOS-cat2 construct. To obtain 4HB production, though, the phaC gene must also be expressed. The 4HB precursor γ-butyrolactone (GBL) is required for 4HB production in this pathway, as the 4HB precursor does not naturally exist in *E. coli* (Figure 2).

pASK vector construct using CPEC

pASK-phaAB-TE-pLZCat2phaC was created using CPEC cloning, combining the 3HB and 4HB genetic pathways, and transformed into *E. coli* (Figure 3). All the constructs were sequenced to ensure that each component was correct.

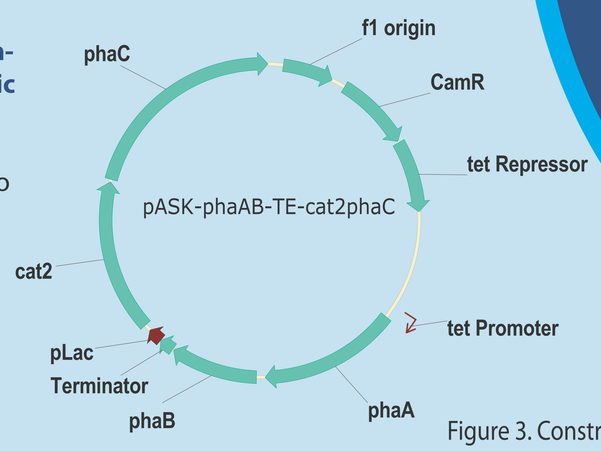

Figure 3. Construct assimulated by CPEC

Abstract

A convenient ligase-free, sequence-independent one-step cloning method is developed. This strategy, Circular Polymerase Assembly Cloning (CPEC), relies solely on polymerase extension to assemble multiple fragments into any vector. Using this method, we are able to **quickly assemble a metabolic pathway consisting of multiple enzymes and regulatory elements** for the production of a biocompatible as well as biodegradable plastic polymer in *E. coli*.

Results

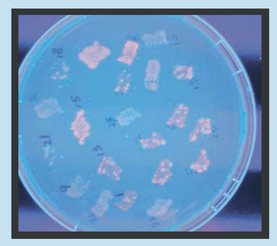
Figure 4. The reddish tint present on some colonies indicates the presence of PHA granules in the bacteria

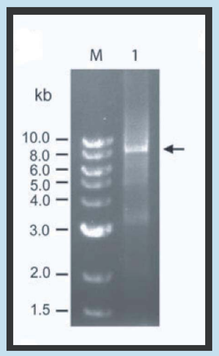

Figure 5a. Gel electrophoresis analysis of the final assembly product after a 20-cycle CPEC. 5 ml of the reaction was separated on a 0.8% agarose gel and visualized after ethidium bromide staining. The full-length plasmid was 8360 bp.

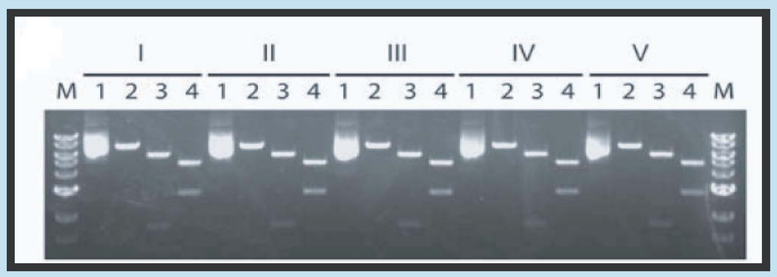

Figure 5b. Restriction mapping of the isolated plasmids derived from the CPEC reaction. Plasmid DNA from five independent colonies (I-V) were digested with BamHI (lane 2, 8.4 kb), BamHI-XhoI (lane 3, 6.6 kb and 1.8 kb), and NdeI (lane 4, 5.4 kb and 3 kb). Purified plasmids not subjected to restriction digestion are shown in lane 1. The molecular weight marker used in this figure was NEB 1 kb DNA ladder.

Acknowledgements

Maggie Jiayuan Quan
Faisal Reza

Dr. Jingdong Tian, Duke University Department of BME & IGSP
Dr. Lingchong You, Duke University Department of BME & IGSP
Dr. Fan Yuan, Duke University Department of BME

Conclusions

A bioplastic synthesis pathway in *E. coli* was assembled using a ligase-free sequence-independent plasmid assembly process, Circular Polymerase Extension Cloning. CPEC has been shown to be more cost-effective, accurate and especially versatile when cloning multi-component systems in a single step. Our future work focuses on **standardizing CPEC for use in assembling biobricks**. On a separate front, we will **continue modifying the bioplastic synthesis pathway to allow predictable regulation of 4HB and therefore produce 3HB-co-4HB with desired elastic properties**.

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