111. CRISPR-Assisted Rational Protein Engineering (CARPE) For Biofuel Production

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Project Goals: We are developing tools for rationally-based protein engineering which will allow multiple modifications from the single protein to the whole pathway levels. One of the main obstacles in high throughput genome modification via recombineering is the low efficiency of successful recombination events. This leads to low abundance of cells that have incorporated the desired DNA library relative to the whole population, and impedes library size and downstream processes such as screening and selection. Here, we use the CRISPR system to induce cell death of the unwanted, non-recombinant population and thus dramatically increase the levels of the modified library cells. To test this technology, we selected the DXS pathway that results (with added genes) in the production of lycopene and isopentenol. Lycopene is relatively easy to screen for, and isopentenol is considered to be a 'second generation' biofuel, with higher energy density and lower water miscibility than ethanol. We aim at engineering this pathway at the open reading frame level to increase metabolite flux and ultimately the level of the final product, isopentenol.

The hunt for better biofuels for industrial manufacturing via bacterial production requires the ability to perform state of the art genome design, engineering and screening for the desired product. Previously, our group demonstrated the ability to modify individually the expression levels of every gene in the *E. coli* genome[1]. This method, termed trackable multiplex recombineering (TRMR), produced a library of about 8000 genomically-modified cells (~4000 over-expressed genes and ~4000 knocked down genes). This library was later screened under different conditions, which enabled deeper understanding of gene products' activities and resulted in better performing strains under these selections. TRMR allowed modification of protein expression for two levels (overexpressed and knocked down) but did not enable the modification of the open reading frame (ORF). Here, we aim at producing large libraries of ORF modifications and engineering whole metabolic pathways for the optimal production of biofuels.

A major difficulty in producing such libraries, which are carefully designed (in contrast to random mutagenesis), is the insertion efficiency of the desired mutations into the target cells. Recombineering, the canonical method for genome modifications in *E. coli*, uses recombinant genes from the Lambda phage to facilitate the insertion of foreign DNA into the host genome. However, this process suffers from low efficiencies that are usually being overcome by either adding an antibiotic resistance gene followed by selection (as in TRMR), or by recursively inducing recombination events (i.e., by MAGE [2]). Our strategy to increase the recombineering efficiency involves the use of the CRISPR system to remove all non-recombinant cells from the population. CRISPR is a recently discovered RNA-based, adaptive defense mechanism of bacteria and archaea against invading phages and plasmids[3]. This system underwent massive engineering to enable sequence-directed double strand breaks using two plasmids; one plasmid coding for the CRISPR-associated nuclease Cas9 and the second plasmid coding for the sequence-specific guide RNA (gRNA) that guides Cas9 to its unique location[4]. Our method utilizes the CRISPR system's ability to induce DNA breaks, and consequently cell death, in a sequence-dependent manner. We produce DNA recombineering cassettes that, in addition to the desired mutation within the ORF, include a mutation in a common location outside of the gene's ORF which is targeted by the CRISPR machinery. This approach of linking desired mutations with the avoidance

from CRISPR- mediated death enables dramatic enrichment of the engineered cells within the total population.

To demonstrate our method, we selected the DXS pathway. This pathway results in the production of isopentenyl pyrophosphate (IPP) which results in the biosynthesis of terpenes and terpenoids. Interestingly, IPP can be the precursor of lycopene or isopentenol, given the addition of the required genes. While lycopene renders the bacterial colonies red, and hence is easily screenable, isopentenol is considered to be a 'second generation' biofuel with higher energy density and lower water miscibility than ethanol. In this project three genes were selected for engineering: 1) DXS, the first and the rate-limiting enzyme of the pathway, 2) IspB, which diverts the metabolic flux from the DXS pathway, and 3) NudF, which has been shown to convert IPP to isopentenol in both *E. coli* and *B. subtilis* [5 6]. Both DXS and IspB mutants will be screened for increased lycopene production with a new image analysis tool we developed for colony color quantification. NudF activity will be assayed directly by measuring isopentenol levels by GC/MS and indirectly by isopentenol auxotrophic cells that will serve as biosensors. This method will provide the ability to rationally engineer large mutational libraries into the *E. coli* genome with high accuracy and efficiency. Furthermore, this approach will result in the first proof-of-concept strain that produces high yield of isopentenol.

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