Engineering Robust Hosts for Microbial Biofuel Production

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Project Goals: **The overall goal of this project is to enhance microbial synthesis of next-generation biofuels by developing tools for improving microbial tolerance of biofuel production conditions. Research is organized around three objectives: (1) Identify novel biofuel tolerance mechanisms from microorganisms that naturally thrive in hydrocarbon-rich environments. (2) Engineer a synthetic feedback loop that responds to biofuel production. To optimize biofuel production yields, cells must balance several competing sources of stress. We are designing and constructing a novel feedback loop that senses biofuel production and turns on export pumps in response. (3) Integrate multiple tolerance strategies in a biofuel production strain. In addition to having the potential to greatly enhance biofuel yields, this work advances understanding of how multiple tolerance mechanisms interact within a cell.**

Abstract:
A major challenge when using microorganisms to produce bulk chemicals like biofuels is that the production targets are often toxic to cells. Biofuel-like compounds are known to reduce cell viability through damage to the cell membrane and interference with essential physiological processes. Thus, cells must trade off biofuel production and survival, reducing potential yields. Studies have shown that strains engineered to increase tolerance can improve biofuel production yields.

Microorganisms that survive in oil-rich environments are a valuable source of tolerance mechanisms. Using genomic DNA from the hydrocarbon-degrading microbe *Marinobacter aquaeolei*, we constructed a transgenic library that we expressed in *Escherichia coli*. We exposed cells to inhibitory levels of pinene, a monoterpene that can serve as a jet fuel precursor with chemical properties similar to existing tactical fuels. Using a sequential strategy with a fosmid library followed by a plasmid library, we were able to isolate a region of DNA from the *M. aquaeolei* genome that conferred pinene tolerance when expressed in *E. coli*. We determined that a single gene, *yceI*, was responsible for the tolerance improvements. Overexpression of this gene placed no additional burden on the host. We also tested tolerance to other monoterpenes and showed that *yceI* selectively improves tolerance. The genomes of hydrocarbon-tolerant microbes represent a rich resource for tolerance engineering. Using a transgenic library, we were able to identify a single gene that improves *E. coli*'s tolerance to the bio-jet fuel precursor pinene.

In addition to identifying novel tolerance mechanics, we are designing control systems for efflux pumps known to export biofuel. Pump overexpression inhibits cell growth, suggesting a trade-off between biofuel and pump toxicity. To counter this, we are using the protein MexR, native to *Pseudomonas aeruginosa*, as a biosensor because it detects oxidative stress such as that caused by the introduction of biofuels. In the feedback loop design, MexR represses the expression of an efflux pump derived from *M. aquaeolei* by binding to a synthetic promoter

region. We developed a library of synthetic promoters, which vary the number and location of MexR binding sites, and screened these for tolerance to pinene, a known pump substrate. The screen tests both constant and dynamic biofuel environments. The dynamic environment is important for selecting a sensor that performs well in both the presence and absence of biofuel. Our experimental findings are further supported by a mathematical model describing the dynamic sensor selection. By applying dynamic inputs to the selection, we show that it is possible to select for traits that satisfy multiple goals (such as performing well in the presence and absence of biofuel). Furthermore, we demonstrate that the underlying diversity in a library is heavily influenced by the initial circuit design. Overall, our findings argue that rational synthetic circuit design, coupled with diversity generation and dynamic selection are powerful tools for many synthetic biology applications, including biofuel production.

We have also studied whether expressing multiple pumps in combination could further increase biofuel tolerance. With multiple pumps, the combined impact of pump toxicity and benefits from increased tolerance are unclear. To address this, we measured tolerance of *E. coli* to pinene in one-pump and two-pump strains. To support our experiments, we developed a mathematical model describing toxicity due to biofuel and overexpression of pumps. We found that data from one-pump strains can accurately predict the performance of two-pump strains. This result suggests that it may be possible to dramatically reduce the number of experiments required for characterizing the effects of combined biofuel tolerance mechanisms.

Publications

1. J. M. Lindle, M. J. Dunlop. "Performing Selections Under Dynamic Conditions for Synthetic Biology Applications." *Integrative Biology*, DOI: 10.1039/c5ib00286a, 2016.
2. T. Tomko, M. J. Dunlop. "Engineering Improved Bio-jet Fuel Tolerance in Escherichia coli using a Transgenic Library from the Hydrocarbon-degrader Marinobacter aquaeolei." *Biotechnology for Biofuels*. 8:165, 2015.
3. W. J. Turner, M. J. Dunlop. "Trade-offs in Improving Biofuel Tolerance Using Combinations of Efflux Pumps." *ACS Synthetic Biology*, DOI: 10.1021/sb500307w, 2014.
4. M. Frederix, K. Hutter, J. Leu, T. S. Batth, W. J. Turner, T. L. Ruegg, H. Blanch, B. A. Simmons, P. D. Adams, J. D. Keasling, M. P. Thelen, M. J. Dunlop, C. J. Petzold, A. Mukhopadhyay. "Development of a native Escherichia coli induction system for ionic liquid tolerance." *PLoS One*, 2014.
5. M. E. Harrison, M. J. Dunlop. "Synthetic Feedback Loop Model for Increasing Microbial Biofuel Production Using a Biosensor." *Frontiers in Microbiology* 3:360, 2012.
6. M. J. Dunlop. "Engineering Microbes for Tolerance to Next-Generation Biofuels." *Biotechnology for Biofuels* 4:32, 2011.

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