219. Developing New Fatty Acid-Derived Biofuels at JBEI: Methyl Ketones and Ladderanes

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Project Goals: The Joint BioEnergy Institute (JBEI) aims to produce a chemically diverse suite of biofuels from lignocellulosic biomass. Some JBEI fuels use fatty acids as precursors, as these biomolecules are highly reduced, aliphatic compounds that, when modified (e.g., decarboxylated), can have properties comparable to those of petroleum-derived fuel components. The goals of the two projects presented here are to: (1) engineer *E. coli* to produce diesel-range methyl ketones in the gram-per-liter range with yields of at least 40% of maximum theoretical yield and (2) elucidate the biosynthetic pathway for unique and highly energetic ladderane fatty acids, which contain linearly concatenated cyclobutane rings, are synthesized by anaerobic ammonium-oxidizing (anammox) bacteria, and have promise as a new class of fatty acid-derived biofuels.

Methyl Ketones

We have engineered *Escherichia coli* to overproduce saturated and monounsaturated aliphatic methyl ketones in the C_{11} to C_{15} (diesel) range; this group of methyl ketones includes 2- undecanone and 2-tridecanone, which have favorable cetane numbers and are also of importance to the flavor and fragrance industry. We have made specific improvements that resulted in more than 10,000-fold enhancement in methyl ketone titer relative to that of a fatty acid-overproducing *coli* strain, including the following: (a) overproduction of beta-ketoacyl-coenzyme A (CoA) thioesters achieved by modification of the beta-oxidation pathway (specifically, overexpression of a heterologous acyl-CoA oxidase and native FadB, and chromosomal deletion of *fadA*) and (b) overexpression of a native thioesterase (FadM). The first generation of engineered *E. coli* (Goh et al. 2012) produced ~380 mg/L of methyl ketones in rich medium. We have subsequently made additional genetic modifications, including consolidation of all pathway genes onto a single plasmid (they were originally borne on two plasmids). We have also conducted *in vitro* assays with purified pathway enzymes, which revealed that FadM is promiscuous and has thioesterase activity not only toward beta-ketoacyl-CoAs but also toward other CoA thioester intermediates in the pathway (such as *trans*-2-enoyl-CoAs). These *in vitro* results have provided insight on how to fine-tune expression of pathway genes for further optimization of methyl ketone production. Our current methyl ketone titer is >1.5 g/L with 1% glucose, which represents 40-45% of maximum theoretical yield; these are the highest titer and yield values reported to date for methyl ketones.

Ladderanes

Ladderanes (or their derivatives) hold potential as a novel next-generation biofuel and for material science applications. These highly energetic molecules, with a carbon backbone consisting of linearly concatenated cyclobutane rings attached to an acyl chain, are incorporated into organelle (anammoxosome) membranes by anammox bacteria. To date, metagenomic data analysis (Rattray et al. 2009) identified 34 genes from an anammox bacterium, *Kuenenia stuttgartiensis*, hypothesized to be candidates involved in ladderane biosynthesis. However, the exact pathway and role of each gene product are unknown. We have developed a strategy to test these candidate ladderane biosynthesis genes in *E. coli* because *K. stuttgartiensis* is not amenable to biochemical and genetic studies, as it has a two-week doubling time and is not available as an isolate. The genes were synthesized and divided into non-native operonic groups, each gene with a unique inducible promoter, translation initiation element, and

terminator, based on putative function as well as reported expression levels from metatranscriptome data (Kartal et al. 2011).

Strategies and tools for DNA assembly, gene expression, and fatty acid (conventional and ladderane) analysis using GC/MS were developed and, to date, 17 genes have been simultaneously expressed in *E. coli* under anaerobic conditions. Based on GC/MS data, the strain expressing the potential ladderane pathway genes exhibited higher ratios of monounsaturated to saturated C_{14}, C_{16}, and C_{18} fatty acids compared to the control strain. Additionally, compared to the control, the experimental strain produced higher levels of C_{16} and C_{18} cyclopropane fatty acids relative to palmitic acid (n-C_{16}). Incorporation and expression of the remaining putative pathway genes is ongoing. This work will lay the foundation for a novel advanced biofuel pathway.

References

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