**Syntrophic co-cultures of *Clostridium* organisms to produce higher alcohols and other C6-C8 metabolites**

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**Project Goals: The goal of this project is to develop syntrophic *Clostridium* co-culture systems for producing intermediate carbon-chain length metabolites (C4-C8) and their derivatives that can be used as chemicals or serve as biofuels and their precursors. Part of the effort is to develop $O_2$-independent fluorescent reporters which will allow us to determine the population dynamics of the dual and triple co-culture system in real time. Furthermore, new fluorescent reporters will also allow us to study the unique cell-to-cell interactions between organisms, which lead to the unique co-culture phenotype and performance.**

*Clostridium* organisms are of major importance for developing new technologies to produce biofuels and chemicals. Three major types of *Clostridium* organisms have been the focus of studies for the sustainable production of fuels and chemicals. Solventogenic clostridia are capable of utilizing a large variety of biomass-derived carbohydrates such as hexoses, pentoses, disaccharides, and hemicellulose, and can produce a good number of C2-C4 chemicals.[1] Acetogenic clostridia can fix inorganic $H_2$, $CO_2$, and CO to generate C2 acids and alcohols.[1] Other specialized clostridia possess diverse biosynthetic capabilities for production of a wide variety of metabolites including C4 – C8 carboxylic acids and alcohols, which could serve as commodity chemicals, biofuels, or biofuel precursors.[1]

The majority of previous work with clostridia focused on optimizing single-organisms systems for production of biochemical. In nature, microorganisms live in complex communities where syntrophic interactions result in superior resource utilization. Here, we first examined a synthetic syntrophy consisting of the solventogen *Clostridium acetobutylicum*, which converts simple and complex carbohydrates into a variety of chemicals, and the acetogen *C. ljungdahlii*, which fixes $CO_2$.[2] This synthetic co-culture achieved carbon recoveries into C2-C4 alcohols almost to the limit of substrate-electron availability, with minimal $H_2$ and $CO_2$ release. The syntrophic co-culture produced robust metabolic outcomes over a broad range of starting population ratios of the two organisms. Finally, the co-culture exhibited unique direct cell-to-cell interactions and material exchange among the two microbes, which enabled unforeseen rearrangements in the metabolism of the individual species that resulted in the production of non-native metabolites, namely isopropanol and 2,3-butanediol.[2] Furthermore, the unique co-culture phenotype was possible only

when both organisms were allowed to physically interact, which allowed them to form unique cell-to-cell fusions. To further investigate the extent of these interactions we have developed fluorescent *C. acetobutylicum* and *C. ljungdahlii* expressing fluorescent FAST[3] and HaloTag® proteins, respectively. When co-cultured together both fluorescent strains showed the whole-cell exchange of protein material, a unique and never-observed before phenomena.

To expand our synthetic co-culture system, we will form a triple co-culture including *C. kluyveri*, which can metabolize ethanol and acetate to produce C6 and C8 carboxylic acids. Both *C. acetobutylicum* and *C. ljungdahlii* produce ethanol and acetate, which makes *C. kluyveri* and ideal partner for a triple synthetic co-culture system capable to converting biomass-derived carbohydrates to C6 and C8 biochemicals. $^{13C}$-based Metabolic Flux Analysis (MFA) will be used to gain insight into the regulation of cell growth and product formation pathways, and to identify metabolic bottlenecks. Currently, use of stable-isotope (e.g. $^{13}$C) tracers combined with measurements of isotopic labeling by mass spectrometry represents the state-of-the-art in flux determination. Metabolic fluxes will be studied using $^{13}$C MFA in *C. kluyveri*, *C. acetobutylicum*, and *C. ljungdahlii* under mono- and co-culture conditions to identify key changes in metabolism of each organism. The consortium model consisting of *C. kluyveri*, *C. acetobutylicum*, and *C. ljungdahlii* will be constructed using the SteadyCom framework. This will be done by standardizing the biomass equations and metabolite naming conventions for existing genome-scale models (GSMs), and updating each GSM using RNAseq and $^{13}$C-fluxomics procured under varying experimental conditions. $^{13}$C-fluxomics and RNAseq data will be used to infer regulatory events in each organism to simulate and compare transient monoculture and co-culture population dynamics.

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**Publications**

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2 Charubin, K. & Papoutsakis, E. T. Direct cell-to-cell exchange of matter in a synthetic Clostridium syntrophy enables CO2 fixation, superior metabolite yields, and an expanded metabolic space. *Metabolic engineering* **52**, 9-19, (2019).

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