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. Genome scale models will also elucidate the interactions of co-culture organisms.

Multiple *Clostridium* organisms are of major importance for developing new technologies to produce biofuels 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. Acetogenic clostridia can fix inorganic H_2, CO_2, and CO to generate C2 acids and alcohols. 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.

While most previous work with clostridia focused on optimizing single organisms for production of biochemicals, microorganisms naturally live in complex communities where syntrophic interactions result in superior resource utilization. Here, we first examined a synthetic syntrophy consisting of the solventogen *C. acetobutylicum* (*Cac*), which converts simple and complex carbohydrates into a variety of chemicals, and the acetogen *C. ljungdahlii* (*Clj*), which fixes CO_2.[1] 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 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.[1] 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 *Cac* and *Clj* expressing fluorescent FAST[2] and HaloTag® proteins, respectively. When co-cultured together, both fluorescent strains exhibited cell-to-cell fusion, a never-before-seen phenomena. The cell fusion facilitated the whole-cell

exchange of protein and RNA material between both organisms and led to the formation of stable 'hybrid' bacterial cells which contained the cellular material of both organisms.

To expand our synthetic co-culture system, we formed dual and triple co-cultures including *C. kluyveri* (*Ckl*), which can metabolize ethanol and acetate to produce C6 and C8 carboxylic acids. Both *Cac* and *Clj* natively produce ethanol and acetate, and they have been engineered to produce higher amounts of ethanol while producing fewer unneeded chemicals. These organisms also convert the carboxylic acids produced by *Ckl* into their respective alcohols. In order to identify genes differentially expressed in co-culture conditions, an RNAseq study was performed on the binary *Cac*-*Clj* co-culture, using *Cac* and *Clj* monocultures as controls. Preliminary analysis of genes hypothesized to play key roles in the cross-species metabolic pathways showed upregulation in co-culture, supporting prior RT-qPCR results. Ongoing global transcriptional analysis will categorize differentially expressed genes based on the Clusters of Orthologous Groups of proteins (COGs) database to determine which gene classes play important roles in the co-culture phenotype. Future RNAseq studies will focus on the *Cac*-*Ckl* and *Cac*-*Clj*-*Ckl* co-cultures.

^{13}C-based Metabolic Flux Analysis (MFA) is used to gain insight into the regulation of cell growth and product formation pathways and to identify metabolic bottlenecks. Currently, use of stable-isotope tracers combined with measurements of isotopic labeling by mass spectrometry represents the state-of-the-art in flux determination. Metabolic fluxes are being studied using ^{13}C MFA in *Ckl*, *Cac*, and *Clj* under mono- and co-culture conditions to identify key changes in metabolism of each organism. We have introduced a dynamic genome-scale metabolic modeling framework and hybrid metabolism models to explore how cell fusion alters the growth phenotype and panel of metabolites produced by the binary *Cac*/*Clj* co-culture. Computational results agree quantitatively with experimental fermentation profiles and indicate *Clj* persists in the co-culture with the aid of *Cac* metabolic enzymes acquired during fusing events. We have assembled a genome-scale metabolic reconstruction (GSM) of *Ckl* containing 1989 reactions inferred through gene homology analysis and gene-protein-reaction mapping from six existing Clostridia GSMs. We are merging our *Ckl* GSM with *Cac* and *Clj* hybrid metabolism models in our dynamic modeling framework and using this system to explore perturbation strategies for maximizing medium-chain fatty acid production.

References

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