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Program Office BER - Microbial Systems Biology Design for Bioenergy Production Project: Metabolism and evolution of a biofuel-producing microbial coculture

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Abstract. Specialized features of individual microbial species can be combined in coculture to produce useful fuels and chemicals. Cocultures can also be used to address how microbes cooperate through metabolic interactions in nature but with a level of experimental control that is not possible in natural settings. However, the challenge of maintaining stable and reproducible microbial relationships has impeded progress in characterizing and implementing synthetic communities. My lab developed a stable and reproducible H_2 biofuel-producing coculture between fermentative *Escherichia coli* and phototrophic *Rhodopseudomonas palustris*. Each species provides or 'cross-feeds' a nutrient that the other requires for growth. My lab used this coculture along with mathematical modeling to uncover basic principles governing mutualistic interactions between microbes, including (i) the potential for cross-fed nutrients to alternatively act as toxins depending on their concentration, (ii) how mutualistic cross-feeding can establish and persist in nutrient-deprived environments where the highest biofuel yields are observed, and (iii) the potential for competition between mutualistic partners for a cross-fed nutrient when that nutrient holds value for both species. In using ethanol-producing *Zymomonas mobilis* in place of *E. coli* in the coculture, my lab also discovered that *Z. mobilis* can use N_2 gas as a nitrogen source, with potential cost-savings for the industrial production of cellulosic ethanol.

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Project activities.

1. Development of a stable H_2- producing coculture. H_2 is a commodity chemical and a promising biofuel. Fermentative bacteria, like *E. coli*, produce H_2 from sugars but at low yields due to the obligate production of organic acids and alcohols. Photosynthetic purple nonsulfur bacteria, like *R. palustris*, consume fermentation products and produce H_2 via nitrogenase. For over 30 years, various groups have repeatedly demonstrated that pairing these two classes of microbes results in a higher H_2 yield from sugars. However, little progress was made on understanding the metabolic interactions that occur due to the challenge of maintaining stable relationships between fast and slow-growing species. Using defined mutations and environmental conditions my lab developed a stable coculture of *E. coli* and an *R. palustris* mutant called, Nx (Fig 1). Similar to previous cocultures of this kind, *E. coli* ferments carbohydrates and excretes essential carbon for *R. palustris.* Our coculture is stabilized by requiring that *R. palustris* fix N_2 gas and excrete essential nitrogen as ammonium (NH_4^+) for *E. coli*. A defined mutation in the *R. palustris nifA* gene or inactivation of the *R. palustris* NH_4^+ transporter, AmtB, is necessary for *R. palustris* NH_4^+ excretion.

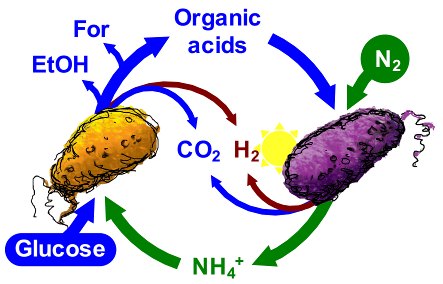

Fig 1. An obligately syntrophic coculture in which *E. coli* (orange) provides carbon (organic acids) to *R. palustris* Nx (purple) in exchange for nitrogen (NH_4^+).

The coculture supports stable coexistence as we have serially transferred cocultures over 100-times without extinction events (unpublished). The coculture is also highly reproducible, and growth and metabolic trends always converge on the same equilibrium values, even when favoring either species up to 6 or 9-orders of magnitude upon inoculation (5). My lab also took steps to make this coculture more practical as an experimental system, for example by genetically disrupting biofilm formation in *R. palustris* to accurately quantify cell densities by ensuring that colony forming units are derived from single cells (6). As described below, the reproducible nature of this coculture along with the genetic tractability of both species has made it a useful system for deciphering the factors that govern metabolic interactions between microbes, which in turn has implications for our understanding of the fate and flow of carbon in the environment and the design of synthetic communities to benefit society, for example through the production of biofuels.

2. Development of mathematical models to simulate metabolic interactions in coculture. The reproducibility of the growth and metabolic trends in the coculture inspired me to attempt to describe the interactions mathematically. I developed a Monod model (equations resembling Michaelis-Menten kinetics) using parameter values derived from experimental data wherever possible. The resulting model was relatively simple, describing only the input and output metabolites (Fig 2), yet proved to have remarkable predictive power (5, 7, 8). My lab routinely now uses the model to guide the design of wet-lab testing and experimental results are used to revise the model as necessary. This combined use of modeling and experimentation has proven to be highly effective research approach in my lab. All model versions are available at https://github.com/McKinlab/Coculture-Mutualism and can be run in R-Studio.

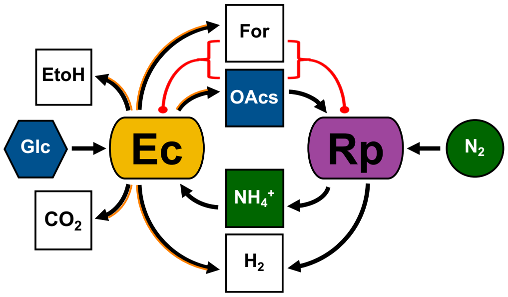

Fig 2. Schematic of the model simulating coculture growth and metabolic interactions. Boxes indicate compounds that are consumed (blue, green) or that accumulate (white). Red oval arrows indicate inhibition by organic acids if they accumulate to acidify the medium. Orange-highlighted arrows indicate the option for growth-independent fermentation.

3. Insights into dose-dependent toxicity of cross-fed metabolites. The metabolites that are transferred during mutualistic cross-feeding are often assumed to serve only as nutrients for the recipient species. In reality, the role of these metabolites can be more nuanced, as we determined to be the case for organic acids cross-feeding as organic acids can serve as a nutrient for *R. palustris* but can alternatively become toxic if they accumulate to high levels. We determined that the relative benefit *R. palustris* receives from organic acids is even influenced by its own level of cooperative NH_4^+ excretion (5). Our model predicted that more NH_4^+ excretion would result in a less efficient utilization of feedstock and a lower *R. palustris* cell density. The higher levels of NH_4^+ would stimulate rapid *E. coli* growth and organic acids would be produced faster than *R. palustris* could consume them, resulting in a growth-inhibiting acidic pH. Thus, by cooperating more, *R. palustris* would change the nature of organic acids from a carbon source to a growth inhibitor. To test these predictions, we engineered a 'hyper-cooperator' strain of *R. palustris* that excreted 3-fold more NH_4^+ than the Nx parent (5). Cocultures with the hyper-cooperator confirmed the predictions, as there were fewer *R. palustris* cells, a higher residual organic acid concentration, and a more acidic pH (5). Nonetheless, the hyper-cooperator and *E. coli* stably coexisted over serial transfers, albeit at a new equilibrium. These results inform on the potential for both positive and negative roles of organic acids in anaerobic food webs and the influence that microbial interactions can have on deciding these roles. Our results also have implications for the use of engineered NH_4^+-excreting, N_2-fixing bacteria in both industrial and agricultural settings. Varied NH_4^+ excretion rates can indirectly modify system behavior in potentially negative ways, such as altering the species ratio or misdirecting resources towards undesired products. In other words, optimizing NH_4^+ cross-feeding does necessarily mean increasing NH_4^+ cross-feeding.

4. Cross-feeding interactions in nutrient-limited environments. Most microbes experience nutrient limitation in natural environments, including those that engage in mutualistic cross-feeding. How does mutualistic cross-feeding establish and persist in the face of nutrient limitation? We hypothesized that metabolism need to provide energy to maintain a cell (maintenance metabolism) rather for cell growth could be coupled to cross-feeding. For example, to generate ATP, *E. coli* must excrete organic acids under fermentative conditions. Thus, even under complete nitrogen-starvation, *E. coli* will continue to ferment glucose to organic acids to maintain itself. Our model suggested that growth-independent fermentation is critical for sustaining coexistence at low levels of NH_4^+ transfer (7). When growth-independent fermentation was excluded from the model, the coculture was predicted to go extinct below a threshold level of NH_4^+ transfer (Fig 3). We decreased NH_4^+ transfer in our coculture by lowering the supply of N_2. Coexistence was observed at even the lowest levels of NH_4^+ transfer that we tested (Fig 3), suggesting an essential role for growth-independent fermentation in maintaining coexistence (7). The continuous excretion of organic acids stimulates *R. palustris* growth and reciprocal NH_4^+ excretion, eventually lifting both species out of starvation. Organic acids are an obligate waste product of fermentative organisms and an important carbon and electron shuttle in anaerobic communities. Thus the importance of growth-independent fermentation in establishing and maintaining cross-feeding relationships could be widespread in nutrient-limited environments. As we observed and characterized in monoculture (1), the highest coculture H_2 yields were also observed under severely nitrogen-limiting conditions, exceeding the theoretical maximum fermentative H_2 yield (7).

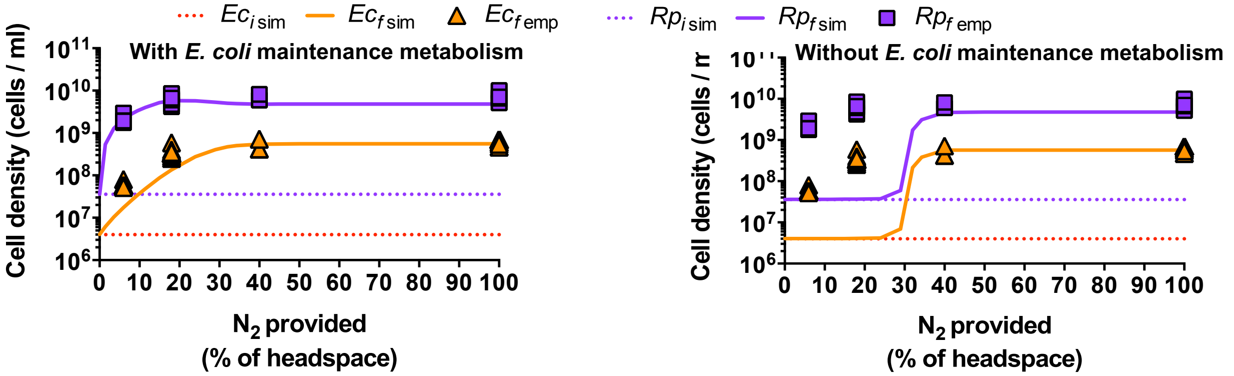

Fig 3. Simulated initial (dotted lines) and final (solid lines) cell densities in cocultures with different N_2 concentrations when the model included (left) or did not include (right) growth-independent fermentation by *E. coli*. Experimental cell density data is overlaid with symbols.

We also found that growth-independent fermentation can be detrimental to coexistence when the *E. coli* population is large (7). Large populations amplify the growth independent fermentation rate causing rapid organic acid accumulation that acidifies the medium before growth can occur (7). This result also highlights a dual role of organic acids along a continuum between being beneficial versus detrimental.

5. Competition between mutualists for cross-fed nutrients. Cross-feeding is typically viewed as being a uni-directional process; a producer population excretes a metabolite which then serves as a nutrient for a recipient population. Organic acids meet this criteria as a waste product of *E. coli* and a nutrient for *R. palustris*. However, NH_4^+ is a metabolite that both species value as a nutrient and we hypothesized that there was therefore competition for excreted NH_4^+ between *E. coli* and *R. palustris*. We decreased the affinity of each species for NH_4^+ computationally and also experimentally by genetically disrupting NH_4^+ AmtB transporters (Fig 4). We discovered that mutualism stability necessitates that the recipient, *E. coli*, have a competitive advantage against the producer in obtaining the cross-fed nutrient, provided that the nutrient is generated intracellularly (8). RNAseq and proteomics analysis, along with genetic disruption of the *E. coli* master regulator of nitrogen metabolism, NtrC, revealed that the nitrogen starvation response is crucial for *E. coli* to assume a physiological state that reliably maintains coexistence with *R. palustris* (9). We proposed that

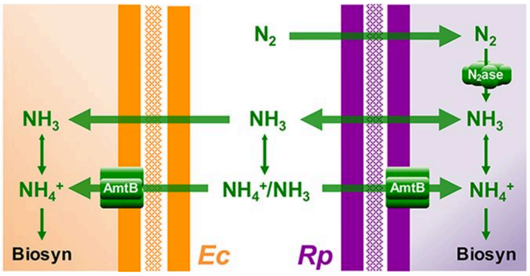

Fig 4. Mechanisms of NH_4^+ excretion and acquisition by *E. coli* (orange) and *R. palustris* (purple) set the stage for competition for NH_4^+.

the requirement for recipient-biased competition is a general rule for mutualistic coexistence based on the transfer of intracellularly generated, communally valuable resources.

6. N_2 gas as a low-cost nitrogen supplement for cellulosic ethanol production. In exploring what other fermentative bacteria could be partnered with *R. palustris* we tried *Zymomonas mobilis*, a bacterium renowned for producing ethanol from glucose at 97% of the theoretical maximum yield. In the process, our negative controls with *Z. mobilis* in monoculture with N_2 as the sole nitrogen source supported growth. This was surprising since *Z. mobilis* had been noted to be incapable of using N_2 in the literature. Using growth experiments and stable isotopes of N_2 we confirmed that *Z. mobilis* can use N_2 as a nitrogen source (3). Remarkably, the electrons needed to convert N_2 into NH_4^+ were diverted away from biosynthesis instead of ethanol production, allowing *Z. mobilis* to maintain a near-theoretical maximum ethanol yield while using N_2 (Fig 5). We estimated that if the process can be scaled up that it would save a cellulosic ethanol facility $1-2 million per year in operating costs (3).

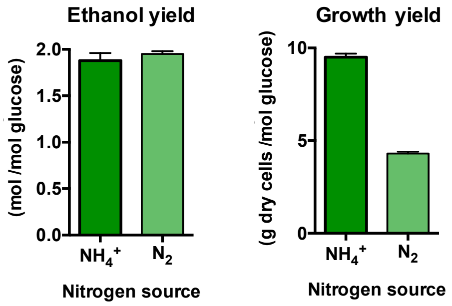

Fig 5. *Z. mobilis* ethanol and growth yields when grown in a defined medium with either NH_4^+ or N_2 as the nitrogen source.

7. Interplay between CO_2 fixation and H_2 production. In previous work, I had shown that the *R. palustris* CO_2-fixing Calvin cycle competed against H_2 production for electrons and that genetically disrupting Calvin cycle activity led to increased H_2 yields but not increased H_2 production rates. We hypothesized that the use of *R. palustris* Calvin cycle mutants might also enhance H_2-yields in coculture. While this hypothesis proved to be incorrect, the Calvin cycle mutants we generated informed on physiological role of the Calvin cycle in purple nonsulfur bacteria (2) and resulted in a better strategy for making Calvin cycle mutants to increase both H_2 yield and production rate in *R. palustris* monocultures (4).

Publications resulting from this project (9):

1. **McKinlay, JB**, Y Oda, M Rühl, AL Posto, U Sauer, CS Harwood. 2014. Non-growing *Rhodopseudomonas palustris* increases the hydrogen gas yield from acetate by shifting from the glyoxylate shunt to the tricarboxylic acid cycle. Journal of Biological Chemistry. 289: 1960-1970. http://hdl.handle.net/2022/22257

2. Gordon, GC and **JB McKinlay**. 2014. Calvin cycle mutants of photoheterotrophic purple non-sulfur bacteria fail to grow due to an electron imbalance rather than toxic metabolite accumulation. Journal of Bacteriology. 196: 1231-1237.
   - Undergraduate 1st author.
   http://hdl.handle.net/2022/22258

3. Kremer, TA, B LaSarre, AL Posto, **JB McKinlay**. 2015. N_2 gas is an effective fertilizer for bioethanol production by *Zymomonas mobilis*. Proceedings of the National Academy of Sciences USA. 112: 2222-2226.
   - Featured in the news sections of The Scientist, AAAS Science, Christian Science Monitor, and other websites and blogs. For highlights see: http://www.indiana.edu/~mckinlab/Press.html https://www.osti.gov/pages/biblio/1235509-n2-gas-effective-fertilizer-bioethanol-production-zymomonas-mobilis

4. McCully, AL and **JB McKinlay**. 2016. Disrupting Calvin cycle phosphoribulokinase activity results in proportional increases to both H_2 yield and specific H_2 production rate. International Journal of Hydrogen Energy. 41: 4143-4149.

https://www.osti.gov/pages/biblio/1345235-disrupting-calvin-cycle-phosphoribulokinase-activity-rhodopseudomonas-palustris-increases-yield-specific-production-rate-proportionately

5. LaSarre, B, AL McCully, JT Lennon, and **JB McKinlay**. 2017. Microbial mutualism dynamics governed by dose-dependent toxicity of cross-fed nutrients. ISME J. 11:337–348. http://hdl.handle.net/2022/22259

6. Fritts, RK, B LaSarre, AM Stoner, AL Posto, and **JB McKinlay**. 2017. A *Rhizobiales*-specific unipolar polysaccharide adhesin contributes to *Rhodopseudomonas palustris* biofilm formation across diverse photoheterotrophic conditions. Applied and Environmental Microbiology. 83: doi:10.1128/AEM.03035-16
   - Featured in the AEM spotlight of issue 83 (4) with accompanying cover image. Undergraduate coauthor.
   http://hdl.handle.net/2022/22260

7. McCully, AL, B LaSarre, and **JB McKinlay**. 2017. Growth-independent cross-feeding modifies boundaries for coexistence in a bacterial mutualism. Environmental Microbiology. 19: 3538-3550. http://hdl.handle.net/2022/22261

8. McCully, AL, B LaSarre, and **JB McKinlay**. 2017. Recipient-biased competition for an intracellulary generated cross-fed resources is required for coexistence in a bacterial mutualism. mBio. 8: e01620-17
   https://www.osti.gov/pages/biblio/1437774-recipient-biased-competition-intracellularly-generated-cross-fed-nutrient-required-coexistence-microbial-mutualists

9. McCully, AL, M Behringer, JG Gliessman, E Pilipenko, JL Mazny, M Lynch, DA Drummond, and **JB McKinlay**. 2018. A nitrogen starvation response is important for *Escherichia coli* to coexist in a mutualistic cross-feeding relationship with *Rhodopseudomonas palustris*. Applied and Environmental Microbiology. 84:e00404-18
   http://hdl.handle.net/2022/22262

Patent applications resulting from this project (1):

1. 2015. McKinlay, JB, TA Kremer, B LaSarre, AL Posto. Culture conditions that allow *Zymomonas mobilis* to assimilate N_2 gas as a nitrogen source during bio-ethanol production. (submitted). https://patents.google.com/patent/WO2016109286A1/en