Engineering New Tools for Non-Standard Amino Acid Biosynthesis and Reliance in Microbes

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While synthetic biology has demonstrated promise in controlling the function of live microbial systems in laboratory settings, deployment of engineered microbes outside of reactors has been slow due to poor efficacy in environmentally relevant settings and public concern about genetically modified organisms. One potential solution to overcome concerns regarding safety of deployed microbes is intrinsic biocontainment, a method to prevent the unintended proliferation of engineered microbes in the environment. Synthetic auxotrophy, the dependence of a microbe on a synthetic nutrient, like a non-standard amino acid (nsAA), has been one of the most promising methods of biological containment with undetectable escape of an *Escherichia coli* synthetic auxotroph. However, there are still limits to the existing technology that prevent deployment. First, production of relevant nsAAs for biocontainment have been restricted by what is commercially available or can be chemically synthesized, which can be cost prohibitive. We explored the natural sequence diversity of L-threonine transaldolases for the efficient metabolic production of nsAAs from readily available precursors. Next, existing *E. coli* synthetic auxotrophs are unable to persist in outdoor environments making them an unsuitable chassis for biofertilizers or bioremediation. We successfully extended synthetic auxotrophy to *Bacillus subtilis*, a rhizobacterium for persistence in soil environments. Lastly, to support the survival of a synthetic auxotroph in an applied setting for a longer period, the Kunjapur Lab combined nsAA biosynthesis and synthetic auxotrophy to develop a novel microbial interaction, orthogonal obligate commensalism. To better predict survival outcomes of this interaction, we modeled the behavior of biocontained members of the orthogonal obligate commensalism. Each component of this work builds toward the successful deployment of an engineered, biocontained microbe for addressing global engineering challenges in agriculture, human health, and waste management.

Beyond synthetic auxotrophy, nsAAs are essential for expanding the chemical repertoire of medicine. Specifically, beta-hydroxy non-standard amino acids (β-OH-nsAAs) have utility as small molecule drugs, precursors for beta-lactone antibiotics, and building blocks for polypeptides. We ascertained the specificity of the TTA enzyme class more comprehensively by characterizing 12 candidate TTA gene products across a wide range (20-80%) of sequence identities. Using an optimized

coupled enzyme assay, we identified six novel TTAs, including one that exhibits broader substrate scope, two-fold higher L-Threonine (L-Thr) affinity, and five-fold faster initial reaction rates than the previously identified TTA under conditions tested. We harnessed these TTAs for first-time bioproduction of β-OH-nsAAs with handles for bio-orthogonal conjugation from supplemented precursors during aerobic fermentation of engineered *E. coli*, where we observed that higher affinity of the TTA for L-Thr increased titer.

To further advance the field of synthetic biology for deployment of microbes into natural environments, we implemented synthetic auxotrophy in *B. subtilis,* a rhizobacterium that can form robust spores to withstand harsh environmental conditions. Excitingly, we successfully developed several *B. subtilis* synthetic auxotrophs through extensive study of nsAA incorporation across growth conditions, precise synthetic auxotrophy marker selection, and optimization of *B. subtilis* recombinant DNA transformations. While working toward generating a *B. subtilis* strain incapable of growing in the absence of an nsAA, we characterized the strain under targeted experimental conditions that mimic the environment. In these conditions, we successfully observed no escape from a synthetic auxotroph in minimal media conditions for over a week at a detection limit of 10^{-6} cells at 25°C. Notably, this strain will also readily form spores that are unable to germinate in the absence of nsAA under environmentally relevant conditions, offering a new mechanism for deployment of robust engineered microbes. To further extend the possibilities of this technology, we have demonstrated nsAA incorporation and synthetic auxotrophy in a non-domesticated *B. subtilis* strain, UD1022, which is actively used as a plant-growth promoting bacterium with field hardiness and the ability to actively colonize plant roots.

Lastly, a quantitative understanding of the representation of a synthetic auxotroph in a non-sterile environment where nsAA biosynthesis is occurring can be useful for applications in which long-term co-existence is desired. To capture the dual dependence of the synthetic auxotroph on both a carbon source and on an nsAA, we developed a computational model to predict the growth of a synthetic auxotroph in co-culture using a non-interactive, double-substrate limited form of the Monod growth kinetic model. We applied this to the obligate and orthogonal commensalism in which a second microbe biosynthesizes the nsAA for the biocontained strain. This model can provide qualitative predictions of the impact of inoculation ratio and substrate concentration on the growth of the commensal. Excitingly, we were able to generate a phase diagram of exclusion and co-existence regimes based on two engineerable properties, the rate of biosynthesis of the synthetic nutrient and the affinity for that nutrient in the biocontained strain.

In summary, this thesis develops experimental and computational advances that will help enable successful deployment of an engineered, biocontained microbe.