Techniques for *in situ* DNA delivery and targeted editing within microbial communities

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Project Goals: Understanding the interactions, localization, and dynamics of grass rhizosphere communities at the molecular level (genes, proteins, metabolites) to predict responses to perturbations and understand the persistence and fate of engineered genes and microbes for secure biosystems design. To do this, advanced fabricated ecosystems are used in combination with gene editing technologies such as CRISPR-Cas and bacterial virus (phage)-based approaches for interrogating gene and microbial functions *in situ*—addressing key challenges highlighted in recent DOE reports. This work is integrated with the development of predictive computational models that are iteratively refined through simulations and experimentation to gain critical insights into the functions of engineered genes and interactions of microbes within soil microbiomes as well as the biology and ecology of uncultivated microbes. Together, these efforts lay a critical foundation for developing secure biosystems design strategies, harnessing beneficial microbiomes to support sustainable bioenergy, and improving our understanding of nutrient cycling in the rhizosphere.

Abstract

Knowledge of microbial gene function classically is derived from modifying the DNA of individual microbial strains in isolation from their natural environments and communities. Currently the ability to directly characterize individual microbial strains in the context of a natural microbial community, as well as assay their individual impacts on these communities using genetic experiments is limited. This is a highly limiting hurdle for the forward advancement of microbiology as most microorganisms naturally exist in complex communities, the vast majority of microbial species are difficult to or have not been cultivated, and the true behaviors of individual microbial cells as well as communities as a whole can likely only be assayed by interrogating cells in these natural contexts. Here we present a suite of integrated methods that allow for the *in situ* assay of genetic tractability of microbes to specific DNA delivery methods, targeted and locus specific delivery of DNA, and genetic modification, and application of targeted phages to specific microbial species existing in a community context. Additionally, we demonstrate a number of practical applications of these techniques by directly assaying and genetically perturbing specific microbial species directly within a synthetically assembled microbial consortia.

To approach the direct perturbation of individual microbes within communities we are developing three complementary isolation and selection independent approaches that address the following hurdles: (i) Isolation independent determination of genetic tractability; (ii) Targeted and isolation independent delivery of genetic cargo; (iii) High penetrance editing and species specific removal using engineered phages. To address the first hurdle we have developed a novel technique, Environmental Transformation Sequencing (ET-Seq), in which barcoded non-targeted transposons are delivered to a mixture of microbial cells, and discrete integration events are quantified without selection via sequencing, using three different DNA delivery approaches (conjugation, electroporation, and natural transformation without induced competence). Next, we present our development of a DNA-editing All-in-one RNA-guided CRISPR-Cas Transposase (DART) system that allows for the species and locus specific delivery of DNA into organisms identified as tractable by ET-Seq. Finally, given the low penetrance of DNA edits using classical transformation techniques we have developed engineered phage systems as repurposed nucleic acid delivery vectors for strain specific ablation in a dynamic community context.

To demonstrate the practical applications as well as synergistic combinations of these technologies we used ET-Seq to detect and quantify targeted site-specific integrations of DNA cargo delivered to a specific member of a microbial consortia with our DART vector without selection or isolation. Subsequently we demonstrate that this targeted quantification can be used to assay gene fitness within a microbial consortia by using DART to target and disrupt the *pyrF* locus in *Klebsiella michiganensis*, which is known to confer a fitness advantage in the presence of 5-fluoroorotic acid (5-FOA). Quantification of the *pyrF* inserts using ET-Seq show that after the community is treated with 5-FOA a positive selection can be observed *in situ*. We additionally demonstrate the utility of the DART vector for both targeted isolation of a single member of a microbial consortia through targeted delivery of an antibiotic resistance cassette, as well as for conferring novel metabolism to a targeted community member by delivery of a *lacZY* cassette allowing growth on lactose. Furthermore, we demonstrate optimal locus selection for high-efficiency phage engineering and report successful integration of varying-sized exogenous payloads for delivery to a target strain.

Overall, these techniques provide a novel avenue for the targeted and isolation independent analysis of specific microorganisms within complex microbial consortia thereby providing a route for genetic testing of microbiome-based hypotheses.

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