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Recoding the metagenome: microbiome engineering *in situ*

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Synthetic biology has enabled a new generation of tools for engineering the microbiome, including targeted antibiotics, protein delivery, living biosensors and diagnostics, and metabolic factories. Here, we discuss opportunities and limitations in microbiome engineering, focusing on a new generation of tools for *in situ* genetic modification of a microbiome that hold particular promise in circumventing these limitations.

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Introduction

Commensal microorganisms play a critical role in maintaining human health [1–7]; interactions between microbiota and the host play a key role in metabolizing nutrients [8], maintaining homeostatic immunity [9,10], regulating local tissues [11], and protecting against pathogens [12–15]. Conversely, perturbations in microbial communities have been associated with a vast number of diseases as diverse as skin disease [16,17] to mental disorders [18,19], cancer [20,21], metabolic diseases [22], and inflammation [23]. In recent years, the microbiome field has rapidly progressed from inferences and microbial correlates toward a mechanistic understanding of the microbiome's role in disease risk. As these studies identify key host–microbiome interactions underlying disease (i.e. potential therapeutic targets), manipulating our native microbial flora has emerged as a unique opportunity to upsell the benefits of the commensal flora and reduce potential pro-disease elements.

Correspondingly, microbe-centric therapeutic strategies have emerged to either provide or restore a desirable function to the microbial community of interest or suppress or eliminate a specific undesirable or pathogenic element. Such strategies may involve the delivery of a microbe or cocktail of microbiota of interest. For example, bacteriotherapy has been used successfully in *Clostridium difficile* infections, where fecal microbiome transplants (FMT) engraft healthy human donor microbiota into patients [24]. In other cases, systems biology approaches have identified individual microbes and simple defined consortia that can largely recapitulate the effects of the FMT. A phylogenetically diverse consortium of four species or one top candidate, *Clostridium scindens*, was nearly as effective in reducing mortality as a full FMT in mice [25], or an 11-strain consortium acted in concert to elicit an inflammation-free interferon-γ-CD8 T cell response in the gut to increase immunotherapy efficacy and resistance to *Listeria monocytogenes* infection in mice [26]. However, progress in identifying species, strains, and consortia that provide a universal or lasting protection against infection remission has been limited by our understanding of the underlying biology of microbe–microbe and host–microbe interactions, which is perpetuated and compounded by the extensive interindividual variation of the microbiome.

Complementary synthetic biology-based approaches are seeing rapid ideation and iteration to generate microbes with an increasingly sophisticated suite of sensors and metabolic factors that too can be supplemented in the community of interest. While the genetic manipulation of *Escherichia coli* has been a staple of molecular biology for decades, the toolbox for microbial engineering has vastly diversified over the last decade, including clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 for precision genome engineering [27], vastly more affordable and accessible DNA sequencing and synthesis tools [28], tools to increase the genetic tractability of natural isolates [29–31], high-throughput genomic integration [32], heterologous protein expression in nonclassical bacteria [33], phages [32], and optimized promoters [34,35], among others. In addition, engineering principles have been leveraged for modular design for ready swapping of genetic parts to build sensors, switches, circuits, and other response regulators to diversify the utility of the engineered microbe of choice, and such tools have been effectively used to probe different biological functions of the underlying ecosystem [36]. In turn, these tools have greatly broadened the landscape for potential strategies for microbiome remodeling.

However, using live microbes as therapies – or 'live biotherapeutic products' – faces challenges in successful integration into and sustained competition against the native flora. Foreign probiotic organisms have a low (and highly variable [37,38]) efficacy of integration into a native community, often requiring systemic antibiotic treatment for engraftment [39]. Given this central limitation, emergent approaches for genetic manipulation of a microbial community *in situ* are of strong interest for a more durable engineering of the microbiome. Here, we will discuss two recent approaches for *in situ* genetic manipulation of a microbiome. Both are predicated on mechanisms of natural genetic exchange between microbes, and both are amenable to a variety of different genetic payloads, ranging from heterologous expression of metabolic pathways, delivery of sensors, or CRISPR/Cas9 systems for targeted modification or removal of strains of interest from a microbiome.

Phage-mediated engineering

Phages, as natural bacterial predators and mediators of horizontal gene transfer, are versatile for both targeted bacterial removal as well as delivery of genetic payloads to the microbiome. Natural, 'unmodified' phages or phage cocktails [40] have been used to eliminate undesired bacteria, ranging from infectious disease agents to agricultural applications [41,42], albeit with limited clinical success. In these cases, such therapeutic phages are typically identified by screening libraries of phages that effectively target the etiological agent of interest [43]. This is necessary because phages have exquisite host specificity, even to the strain level, a major factor which has significantly limited their utility as therapeutics as a phage for each outbreak strain typically must be identified then optimized [44].

Different approaches have sought to increase the versatility and potency of phage approaches. For example, shotgun sequencing of human-associated microbiomes has identified prevalent and diverse phage populations [1,45,46], and sequencing isolates cultivated from microbiota have also increased the search space for phage diversity. Microbiome analyses can thusly generate new candidates for phage therapy, even personalized to an individual or ecosystem of interest. Phage engineering and recoding can further increase host range, increase longevity, decrease potential side effects, or modify the target bacterial genome to remove or add potential functions of interest [47]. Host range can be increased by changing tail fiber diversity, for example by swapping tail fibers from different phages, or creating libraries of tail fiber variants to select for a desired host range [48–50]. Because of their relatively small size, whole genome synthesis [51] may also have promise for creating phage libraries with genetic diversity that can increase host range and circumvent innate resistance mechanisms of bacteria to phage infection.

More recently, phages have been demonstrated to have promising utility for delivering genetic payloads for *in situ* modification of a metagenome. For example, phages have been used to deliver CRISPR/Cas9 payloads with guides that target specific strains [52,53]. The CRISPR system is encoded on a plasmid packaged into a target phage as a 'phagemid', which is then delivered to all susceptible bacteria in a microbial community. Because guides can target any gene of interest with very high specificity, genetic manipulation (in this case, cleavage of the genome resulting in cell death) only acts on strains of interest. This approach has been used to target carbapenem-resistant and enterohemorrhagic *E. coli* [53] and methicillin-resistant *Staphylococcus aureus* [52] in mixed communities. The use of Cas3, which has endonuclease activity for greater genome degradation can have similar effects [54]. Such approaches are promising for targeted removal of pathogenic strains in a microbiome, rather than wholesale removal of the species, potentially resulting in an undesirable microbial dysbiosis. Other CRISPR-based approaches have been used to re-sensitize antibiotic resistant microbes by targeting resistance genes and plasmids [55].

Functionalization of the microbiome by delivery of genes or pathways of interest is another exciting application of phage-based *in situ* microbiome engineering. Such genes of interest can be delivered by phagemid, or by modifications integrated into the genome of the phage itself, if temperate. This approach has been used to restore antibiotic susceptibility and deliver biofilm dispersal enzymes to increase efficacy of adjuvant treatments *in vitro* or in mice [56–58]. However, restrictions on the size of packaged DNA can limit transfer of larger and more complex metabolic pathways, in addition to structural considerations in disrupting highly ordered phage genomes with the addition of genetic elements.

Conjugation-mediated engineering

Conjugation via plasmids and transposable elements through direct cell–cell contact, similar to phages, can deliver genetic elements directly to cells in a microbial community. A major advantage of conjugation over phage-based approaches is a broader host range and potential transfer of more complex genetic elements. Two recent studies have developed conjugative systems with demonstrated broad host range of natural 'undomesticated' isolates.

Brophy *et al.* [59] developed miniaturized integrative and conjugative elements (ICEs) from *Bacillus subtilis* to engineer a diversity of natural isolates and propagate the conjugation machinery. They characterized the ability of their *B. subtilis* donor strain, termed the XPORT donor, to deliver ICEs to 57 Gram-positive strains isolated from human skin, gut, and soil. Transfer was observed for 35 of these microbes with varying efficiency (ranging over

several orders of magnitude), albeit with impressive phylogenetic diversity. They furthermore characterized the ability of an IPTG-inducible system to be active in recipient strains, demonstrating effective expression in all but three strains with low leakiness and adequate (though variable) dynamic range. They then demonstrated the ability of the XPORT system to deliver multi-gene pathways to recipient bacteria, transferring a 10 kb nitrogen fixation gene cluster between species. Finally, they demonstrated the ability of this system to modify a synthetic 'gnotobiotic' soil community (presterilized, then colonized with defined soil consortia) *in situ* under ambient conditions, showing effective transfer to 4/6 strains.

Ronda *et al.* [60] created a platform for *in situ* conjugative delivery, termed Metagenomic Alteration of Gut Microbiome by *in situ* Conjugation (MAGIC). This platform, based on the IncPα-family RP4 conjugation system [61], is compatible with both Gram-positive and Gram-negative cells and was introduced into a modular suite of plasmids with different replicative origins and integrative potential via the Himar transposon system. They tested the host range and versatility in genetic payloads starting with fluorescent markers and antibiotic resistance genes, using libraries of these different delivery vectors expressed in *E. coli*. With remarkable rapidity (six hours post gavage), they observed conjugation to 297 different species from four phyla and estimated transfer to 5% of all bacterial cells in the mouse gut. However, donor cells and transconjugants were rapidly lost from the community (48–72 hours, suggesting a potential toxicity or instability of the vector design). Nevertheless, the use of an autologous system, that is, previous transconjugants isolated from the gut and repurposed to be a donor strain, resulted in long-term and stable colonization (15 days), albeit with reduced host range. This striking demonstration of *in situ* genetic modification of an *in vivo* microbial community opens exciting new applications for microbiome engineering, particularly as MAGIC has demonstrated versatility for both Gram-negative and Gram-positive organisms. Future iterations will likely demonstrate both recipient-specific as well as broad remodeling applications, flexible to be inclusive of many of the engineering approaches previously mentioned.

Challenges for *in situ* microbiome engineering

While several examples of *in situ* microbiome engineering have shown some early translational promise, many challenges still remain, as current limitations of *in situ* approaches are shared with those inherent to any horizontally transferred DNA element. The use of phage therapy in a clinical setting was first described in 1931 [62]; however, this approach has had difficulty in showing success, and several recent trials using phage therapy have failed to show efficacy, including clinical trials in *E. coli* diarrheal diseases [63] and *Pseudomonas aeruginosa* burn wound infection [64].

One limitation is penetrance of the donor, as transduction, conjugation and other transformation events require physical proximity for DNA delivery. While a surprising penetrance and taxonomic breadth in transfer of donor DNA were observed in Ronda *et al.*, penetrance may be increasingly limited in likely more structured communities like the skin, oral pockets or biofilm-like structures. In addition, donor range will likely require significant optimization, both for cellular attachment as well as expression of the genetic payload. The high range of transduction and conjugative efficiency observed in these studies underscore challenges to broad efficacy *in vivo* or in complex consortia, including physical proximity, donor-host compatibility, and innate host defenses like CRISPRs and restriction endonucleases. Following delivery, expression of the desired product in recipient cells will be further complicated by host factors such as plasmid copy number, efficiency of genomic integration, promoter incompatibility, codon usage, protein folding, and secretion systems that may need species-specific optimization.

Moreover, subspecies, or strain considerations in microbiome engineering is an important emerging concept. Recent algorithms have attempted to characterize strain diversity in metagenomic data [65,66]. Both population-level and within-individual strain diversity [67] will alter efficacy as strains can have different resistance mechanisms and compatibility to donors, which themselves must be screened for strain-specific effects. Finally, colonization resistance of a community may change favorably or unfavorably in conditions of high versus low strain diversity, in which a larger and potentially more saturated functional complement provided by heterogeneous strains may be more resilient to foreign microbes or genetic pathways.

We envision that microbial community engineering is best complemented with a deep understanding of the metagenome gained through genomic surveys, systems analyses, imaging, and modeling at the taxonomic and functional level. At the most basic level, the metagenome provides a blueprint to identify potential therapeutic targets (e.g. antibiotic resistance or virulence genes) and guide selection of a delivery vehicle that could have maximum host range. Metagenomes could also facilitate design and iteration on delivery vehicles; plasmids have been identified with increasing resolution and quality with *de novo* assembly and mapping-based approaches [68,69], chromatin linkage analysis [70,71], and long-read sequencing [72]. Phages (most commonly lysogens) are commonly reconstructed together with their host genome. Advances in synthesis technologies obviate the requirement for cultivation of microbes to obtain or modify these potential delivery vehicles [51]. Metagenomic analyses can be also used to create predictive models for understanding the impacts of *in situ*

engineering. While few ecological models have accounted for species complexity of a microbiome [73], ecological models that define stable states of a community and resilience to perturbation will be valuable for inferring the stability of an exogenous microbe or genetic pathway that alters the metabolic complement of a microbiome on a population-scale. Such models must also account for the different rubrics of an application. For example, phage-based approaches might have versatility for both acute, point-of-care (e.g. infection control, antibiotic resistance) as well as chronic or preventative treatments, while conjugative approaches might be preferred for the latter's long-term implications.

Advances in imaging complex microbial communities will also be critical in optimizing delivery and microbiome modulation. Combinatorial Labeling and Spectral Imaging–Fluorescence *in situ* Hybridization (CLASI–FISH) uses fluorescent probes that in combination, uniquely label dozens of different bacteria [74,75]. This method showed that human oral structures are polymicrobial and complex, which will likely reduce access to certain microbes or limit penetrance of the genetic payload. Imaging of *in vivo* dynamics and microbial flow in the mouse gut using fluors and click chemistry [76] showed different colonization dynamics for different Bacteroides species depending on location in the gut. In addition, live imaging of fluorescently labeled Proteobacteria in the zebrafish intestine defined a spectrum of different growth modes and microbial superstructures [29]. These data underscore that relative abundance data reconstructed by metagenomic sequencing can be misleading in terms of biological inferences and potential regions where *in situ* delivery may be more or less effective.

Conclusions

Adding to the toolkit of broad-spectrum remodelers like antibiotics, pre-biotic, pro-biotic, and post-biotic approaches for modulating the microbiome, *in situ* approaches have extraordinary potential for introducing a broad range of different functions into existing communities, with two major advantages, first circumventing requirements for stable integration of an exogenous microbe, and second, increasing the genetic repertoire that can be introduced. While these approaches are in their infancy, synthetic biology allows rapidly iteration on these initial platforms for improved stability, dosing, spatial and temporal control as well as containment and control. Would *in situ* engineering approaches yield a microbiome for which containment be more challenging? We note that while the approaches discussed here are amenable to many containment approaches developed by the synthetic biology community, including kill switches [77,78], auxotrophies [79], metabolic dependencies [80], or use of unnatural amino acids [81–84], they may be challenging to implement as the underlying chassis characterized to date are inherently size-restricted—for example, in the DNA fragment packageable as a phagemid, or deliverable through conjugative machinery. Nonetheless, as the underlying biology of host–microbiome and microbe–microbiome interactions is better understood, new opportunities for re-coding, optimizing, and re-programming the microbiome for a wide range of different applications will continue to emerge.

Declaration of Competing Interest

Travis Whitfill is an employee of Azitra, Inc. Julia Oh is on the scientific advisory board of Azitra, Inc.

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