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Toward Microbiome Engineering: Expanding the Repertoire of Genetically Tractable Members of the Human Gut Microbiome

James W. Marsh, Christian Kirk, and Ruth E. Ley

Department of Microbiome Science, Max Planck Institute for Biology, Tübingen, Germany; email: ruth.ley@tuebingen.mpg.de

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Abstract

Genetic manipulation is necessary to interrogate the functions of microbes in their environments, such as the human gut microbiome. Yet, the vast majority of human gut microbiome species are not genetically tractable. Here, we review the hurdles to seizing genetic control of more species. We address the barriers preventing the application of genetic techniques to gut microbes and report on genetic systems currently under development. While methods aimed at genetically transforming many species simultaneously in situ show promise, they are unable to overcome many of the same challenges that exist for individual microbes. Unless a major conceptual breakthrough emerges, the genetic tractability of the microbiome will remain an arduous task. Increasing the list of genetically tractable organisms from the human gut remains one of the highest priorities for microbiome research and will provide the foundation for microbiome engineering.

Contents

1. INTRODUCTION

Microbiome: the bacteria, fungi, archaea, viruses, and their genes that synergistically interact with each other and their host in a well-defined habitat

Genetic system: a set of tools and techniques that are optimized for the reliable genetic engineering of a particular organism

Genetic manipulation: the modification or engineering of an organism's genes using genetics

Model organism: a well-studied organism with an established genetic system and experimentally validated gene annotations

The fields of genetics and microbiology developed in tandem, significantly advancing our understanding of gene function and the complex interplay between microbes. Classical bacterial genetics, which centers on introducing mutations to specific microbes in isolated environments and analyzing the resulting phenotypic changes, has laid the foundation for expanding genetics to the community level. Interest has now shifted to transposing these techniques into physiologically relevant contexts, such as the human gut microbiome, to unravel both the functional genetics of individual microbes and their contribution to the system's emergent chemical, biological, and physical properties. This integrated approach is the foundation for microbiome engineering—a burgeoning field that seeks to enable a more comprehensive and mechanistic understanding of these intricate systems.

Integrating genetic tools into microbiome research has proven to be particularly challenging, even though well-developed genetic systems already exist for abundant gut bacteria, such as *Bacteroides* spp. (see the sidebar titled A History of *Bacteroides* Genetics). Wexler and Goodman (146) described *Bacteroides* as "a window into the microbiome" due to its amenability to genetic manipulation, which has enabled valuable metabolic and ecological insights into the functional dynamics of the gut microbiome. For example, the genetic determinants for *Bacteroides* adaptation, colonization advantage, and nutrient acquisition in the gut have been established, as well as an expanding understanding of the genus's metabolic contributions to community behavior (48, 94). This extensive body of research has positioned *Bacteroides* spp. as model organisms for studying the genetics of the human gut microbiome, prompting a desire to develop comparable tools to expand this "window" and explore a more diverse and representative microbiota. However, to date, the number of microbial species that can be genetically manipulated is only a small fraction of the species found in the human gut (146).

Why does the majority of the microbiota diversity within the human gut remain genetically intractable? Transferring a set of genetic tools from one species to another is rarely successful, and

A HISTORY OF *BACTEROIDES* GENETICS

The first indication that *Bacteroides* species were genetically tractable was their ability to be transformed with plasmids (124). Initially, strain-specific barriers prevented plasmids from being reliably transferred between related *Bacteroides fragilis* strains. However, the design of an *E. coli–Bacteroides* spp. shuttle vector enabled reliable constructs to be developed for the transformation of *B. fragilis* (133), *Bacteroides distasonis* (117), *Bacteroides uniformis* (131), *B. thetaiotaomicron* (131), and *Bacteroides ruminicola* (44). Additionally, the identification of transposons that were active in *Bacteroides* spp. enabled transposon mutagenesis to be applied to *B. uniformis* (130), with other vectors later designed for *B. thetaiotaomicron* (54) and *B. fragilis* (134). The combination of a counter-selectable *Bacteroides thetaiotaomicron* strain and the development of conjugative suicide plasmids led to multiple knockout studies for this organism (70), as well as *B. fragilis* (8) and *Bacteroides vulgatus*, which provided a powerful basis for subsequent genetic manipulations. These included tyrosine integrase–mediated chromosome integrations (94), CRISPR-based approaches, and the development of synthetic gene circuits (75). At the same time, endogenous and synthetic promoters have been engineered for inducible control of in vitro or in vivo expression (94). These genetic capabilities afforded *Bacteroides* spp. a unique place among gut-associated bacteria, and their application has provided valuable insight into their functional role in the gut microbiome.

certain strains are resistant to the genetic manipulation techniques developed for other strains within the same species (124). The human gut is composed of hundreds to thousands of mostly bacterial species and some archaeal species [more than 7,000 unique bacterial strains (110) and up to 18 unique archaeal strains (19)]. We are far from being able to manipulate even a representative set of bacterial species, and no genetic systems have been established for human gut–associated archaea. For example, out of the latest collection of microbial species proposed to model the human gut microbiome, only 15–20% have an established genetic system (18). Several efforts are underway to optimize and adapt traditional microbial genetics tools to others; however, progress is slow. Breakthroughs at several levels will be necessary to make substantial progress. This review examines the barriers and challenges that exist for broadening the scope of microbial genetic tools, as well as the recent developments and novel approaches to this problem.

2. MICROBIAL GENETICS MEETS THE MICROBIOME: A SHORT HISTORY

Microbial genetics dates back to the first description of *Escherichia coli* by German pediatrician Theodor Escherich in 1885. This discovery of a hardy, versatile, nonpathogenic microorganism with simple growth requirements led to *E. coli* becoming a molecular biology workhorse and one of the best-characterized model systems in microbiology. Many major microbial genetics advances and discoveries were made using an *E. coli* host, including conjugation, plasmids, bacterial competence, recombinant DNA, molecular cloning, transposons, bacteriophage λ, in vivo mutagenesis, site-directed mutagenesis, and allelic replacement. *E. coli* remains the first choice for commercial genetic engineering, pharmaceutical production, experimental evolution, and biotechnology. As a result, it can be regarded as a microorganism with the ultimate and archetypal genetic system, one that is stable, reliable, and enjoys a well-characterized arsenal of compatible tools and parts.

Building on the principles established for *E. coli*, genetic systems for many other microorganisms followed over the years, including *Salmonella enterica* Typhimurium (1951), *Pseudomonas aeruginosa* (1955), *Bacillus subtilis* (1958), *Streptococcus pneumoniae* (1962), *Staphylococcus aureus* (1970), and *Vibrio cholerae* (1993). However, although the development of new systems often prioritized microorganisms with straightforward isolation, culturing, and handling requirements, they

Microbiota: collective microbial content, usually in a defined environment like a microbiome

Genetically intractable: the multidimensional incompatibilities that negatively impact the fitness of the genetic-engineering recipient, restricting the microbe's ability to be reliably genetically manipulated

Conjugation: the transfer of genetic material from one cell to another via cell-cell contact

Plasmid: a small, usually circular mobile genetic element that is physically separated from chromosomal DNA and can replicate independently

nevertheless required years of tedious trial-and-error and labor-intensive optimization. Persistent obstacles for establishing new systems soon compelled the field to adopt the term genetically intractable to distinguish those microorganisms that could be coerced to reliably take up foreign DNA and those that refused. In response, researchers began to search for alternative tools and genetic parts, beyond those established for *E. coli* and other model organisms, to determine the missing ingredient(s) required for a microorganism's transition to tractability. As research into the human microbiome gained traction, driven by next-generation sequencing technologies, a trove of unprecedented microbial diversity was uncovered at a rate that could not possibly be matched by development of genetic systems. This was compounded by the fact that the human gut microbiome harbors predominantly anaerobic microorganisms that have specific, complex nutrient requirements and are often not currently culturable in vitro. As a result, the microbiome and microbial genetics fields advanced along largely independent trajectories.

Despite this division, enthusiasm for transferring the power of genetics to the microbiome slowly intensified. While *E. coli*, which was originally isolated from the human gut, was useful for some microbiome genetics applications (56, 61, 123), it typically constitutes only 0.1–5% of a community that is instead dominated by members of the *Bacteroidota* and *Bacillota* phyla (38). However, thanks to the pioneering work of Abigail Salyers (124) to establish a genetic system for the prominent human gut symbiont *Bacteroides thetaiotaomicron* (see the sidebar titled A History of *Bacteroides* Genetics), microbial genetics was decisively brought to the gut microbiome. Since then, several groups have contributed to a growing repository of genetic tools and techniques for *B. thetaiotaomicron* and its close relatives, including genetically enhanced type-strains (68), counterselectable conjugative plasmids (70), a suite of promoters and ribosome-binding sites (RBSs) with a broad range of activity (94), plasmid vector suites (46), genome-wide mutant libraries (48, 80, 152), and inducible promoters (94). This made *B. thetaiotaomicron* the canonical model organism of gut microbiome genetics and the envy of those currently relegated to being "intractable." Informed by the successes, tools, and techniques established for *Bacteroides* spp., reliable genetic systems for additional gut-associated microorganisms have started to appear (**Table 1**), albeit at a slow rate. Now, a major priority is to accelerate the process of overcoming barriers to tractability to make these tools accessible to the majority of gut-associated microorganisms.

Competence: the ability of cells to take up exogenous DNA. Competence can be induced in vitro or can occur naturally for some organisms

Promoter: a sequence of regulatory DNA that initiates transcription of a downstream gene

Ribosome-binding site (RBS): a sequence of nucleotides upstream of the start codon for a gene that are involved in the initiation of translation for bacteria

Vector: a plasmid that has been harnessed or exploited for genetic engineering

Gain-of-function: genetic alteration of an organism to enhance the biological functions of gene products

Knockout: describes the use of genetic tools to inactivate, remove, or silence one or more specific genes from an organism

3. APPLICATION OF BACTERIAL GENETIC SYSTEMS TO THE MICROBIOME

3.1. Reverse-Engineering the Gut Microbiome

Understanding the mechanics of any complex system benefits from reverse-engineering to determine individual component function and the contribution of that function to overall system behavior. For the gut microbiome, manipulating its collective gene content can enable mechanistic insight into both microorganism physiology and community behavior (**Figure 1**). The human gut microbiota harbors 150 times more genes than its host (60), representing the major genetic determinants for microbiome dynamics and the primary targets for microbiome genetics. To characterize gene-function relationships, specific genes are targeted for deletion, inactivation, silencing, or activation. For genetically tractable gut-associated microbes, such as *Bacteroides fragilis*, *Bacteroides vulgatus*, *B. thetaiotaomicron*, *Shigella flexneri*, *Helicobacter pylori*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Clostridium sporogenes*, and *Bifidobacterium breve* (**Table 1**), these genetic loss- and gain-of-function strategies have validated several genes predicted from metagenomic data and identified novel functions for many more (53, 155).

Single-gene knockouts have proved very useful for identifying genetic determinants for microbial colonization, resilience, and niche partitioning. For example, in *E. coli*, single-gene knockouts

Table 1 Notable genetic systems for gut-associated bacteria

Phylum	Species	Subspecies	Reference
Pseudomonadota	*Escherichia coli*	All	86
		Nissle	72
		K12	114
		NGF-1	93
	Salmonella enterica		5
	Shigella flexneri		132
Bacteroidota	*Bacteroides thetaiotaomicron*		94
	Bacteroides fragilis		139
	Bacteroides ovatus		158
	Bacteroides vulgatus		158
	Bacteroides uniformis		158
Bacillota	*Lactococcus lactis*		97
	Lactobacillus casei		97
	Lactobacillus gasseri		36
	Lactobacillus paracasei		119
	Lactobacillus plantarum		116
	Lactobacillus reuteri		85
	Clostridium difficile		16
	Clostridium sporogenes		53
	Enterococcus faecalis		26
	Streptococcus pneumoniae		37
	Streptococcus thermophilus		11
	Eubacterium rectale		129
	Roseburia inulinivorans		129
Actinomycetota	*Bifidobacterium longum*		81
	Bifidobacterium breve		95
Campylobacterota	*Helicobacter pylori*		136

have led to the identification of kinase-response regulators and bile salt hydrolases that are required for colonization in the mouse gut (123), while the modulation of quorum sensing led to downstream effects on the abundance of *Firmicutes* (141). Various genetic factors responsible for in vivo phenotypes for *Bacteroides* spp. have been discovered: ribose utilization system (RUS) genes have been linked to diet-dependent, competitive colonization (47); a porphyran utilization locus was linked to abundance control (128); *O*-glycan genes were responsible for the metabolism of host-derived glycans as a determinant of colonization (88); a genetic colonization factor was linked to the availability of sucrose and glucose (142); and an indole-producing tryptophanase was shown to be responsible for producing a circulating metabolite in the mouse gut (32).

Alternatively to single-gene perturbations, genome-wide functional screens have become powerful tools to assess the functional consequence of many genes at once, often under multiple experimental conditions. This is evidenced by the frequent application of functional genomics, transposon insertion sequencing, and CRISPR interference screens in microbiome studies, especially in the context of *Bacteroides* spp. For example, gain-of-function metagenomic screens have identified the genetic factors required for the stable colonization of *B. fragilis* in the mouse gut (77), while a *B. thetaiotaomicron* metagenomic library in an *E. coli* host revealed a colonization-dependent glycoside hydrolase for this organism (154). In various *Bacteroides* species, genome-wide knockout libraries were used to determine the genetic factors required for stable colonization and nutrient

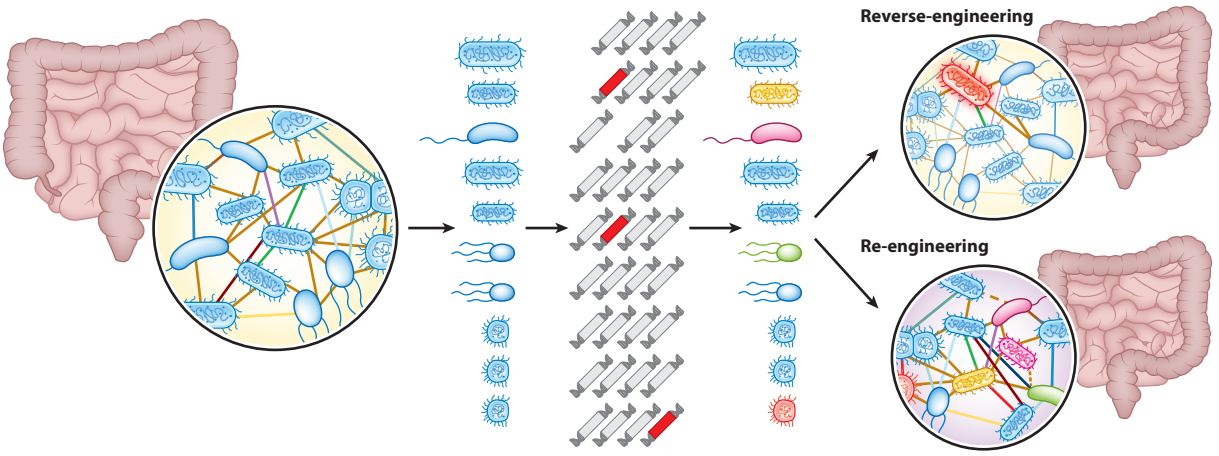

Figure 1

Reverse-engineering and re-engineering the human gut microbiome. Reverse-engineering enables the microbiome to be broken down into its constituent parts (microorganisms and their genes) to genetically perturb their function and observe the phenotypic effects. These phenotypic effects enable the functional elucidation of the targeted genes on an organism and/or microbiome level. Re-engineering enables the microorganisms to be genetically manipulated to introduce novel functions or capabilities to the microbiome for its exploitation, change, or control. Figure adapted from images created in BioRender.com.

acquisition (48); shared, species-, strain-, and diet-dependent metabolite and nutrient processing (151); and diet-dependent ammonium fluctuations in the mouse gut (80).

Genetic monitoring strategies are gaining traction for their ability to report microbial gene expression histories in the gut. For example, using transcriptional recording by CRISPR spacer acquisition from RNA (Record-seq) in *E. coli* sentinel cells, the history of gene expression in the mouse gut could be monitored and recorded under different dietary and disease-related conditions (126).

3.2. Re-Engineering the Gut Microbiome

Beyond understanding the genetic determinants of microbiome function and behavior, genetic engineering principles can be applied to re-engineer the gut microbiome by introducing novel capabilities or altering native functions. Several genetically tractable "probiotic" microbes such as lactic acid bacteria, *Bifidobacterium* spp., and *E. coli* Nissle 1917 have been engineered as noninvasive diagnostics or therapeutics. By overexpressing a genetic payload in these hosts, community dynamics could be modulated or controlled (23). These approaches represent the earliest applications of genetics to the microbiome and are designed to correct or reverse undesirable changes in host-microbiome interactions by regulating virulence, producing antimicrobial molecules, targeting toxins or adhesins, rewiring metabolism, or modulating the immune system (89).

Genetic payload: the genetic material being transferred to a new recipient

Synthetic biology: use of genetic tools to redesign an organism to have new or altered capabilities

As genetic tools become more sophisticated, focus is shifting toward the development of synthetic biology–based tools, including sensors, switches, circuits, and other response regulators, which offer increased utility for controlled microbiome remodeling and therapeutic intervention (147). For example, one-component system sensors that rely on allosteric transcription and a target promoter have been co-opted for in vivo sensing in the gut (76), with *Streptococcus thermophilus*, *Bacteroides ovatus*, and *B. thetaiotaomicron* engineered to sense and respond to the presence of different sugars (35, 55, 94). Alternatively, two-component systems combine a sensor histidine kinase with a response regulator to enable multistep signaling capabilities and have been exploited for

more controlled engineering applications. For example, a thiosulphate sensor from *Shewanella halifaxensis* was coupled with a fluorescent reporter and used in *E. coli* Nissle 1917 to report thiosulphate levels in the presence of inflammation in the mouse gut (27), while a light-activated two-component system was engineered in *E. coli* to secrete colanic acid when activated (57).

Finally, some groups have combined sensor and reporter genes with a genetic circuit designed to execute a genetic response when an appropriate signal is sensed. For example, *E. coli* was engineered with a bistable genetic memory system to sense, remember, and report its exposure to anhydrotetracycline in the gut (71). A similar system induces a permanent DNA recombination event upon detection of the gut inflammatory signal nitric oxide (3). Two-component system strategies have also been adapted for sense-and-kill functionalities to sense the presence of pathogens and coordinate a targeted release of antimicrobial peptides for *Pseudomonas aeruginosa* (61), *Vibrio cholerae* (63), and enterococci (13).

3.3. Emerging Strategies for In Situ Targeting

Traditional genetic techniques are applicable only to microorganisms that are already cultured and genetically tractable. However, the natural ability for microorganisms to share DNA in a community setting has suggested the possibility for in situ engineering. Several studies have monitored the transfer potential of conjugative plasmids in complex communities (69, 99), and this capability has been recently exploited for genetic engineering strategies based on synthetic conjugative mobile genetic elements (14, 118). However, while promising, only untargeted insertions have been possible, and their capacity to transfer to taxonomically diverse hosts has been limited.

Alternatively, focusing on targeted manipulations, Rubin et al. (121) developed DNA-editing all-in-one RNA-guided CRISPR-Cas transposase (DART) for site-specific genome editing in a community context. DART consists of a conjugative plasmid with a Tn7-like transposon encoding a nuclease-deficient CRISPR-Cas system derived from *V. cholerae* that is able to integrate DNA payloads at genomic sites specified by the CRISPR RNA. As proof-of-principle, the authors targeted an *E. coli* propanediol utilization gene cluster within a mixed community of 1,005 microbial species and subspecies and showed that two of the five *E. coli* strains present were successfully engineered. While it is unlikely that this efficiency would extend to broader, nonmodel targets given that *E. coli* is a well-established recipient for the conjugative system utilized, this genetic technology demonstrates promise for targeted, mixed-community editing.

Farzadfard et al. (42) used an alternative targeting strategy: High-Efficiency Synthetic Cellular Recorders Integrating Biological Events (HiSCRIBE), an in situ DNA editing system. With this system, single-stranded DNA is intracellularly expressed via reverse transcription from an engineered retroelement cassette and recombined into homologous sites in the recipient genome via recombination (42). To validate this system within a bacterial community, the authors encoded HiSCRIBE on an M13 phagemid that was engineered to revert the *E. coli* gene responsible for the metabolism of galactose from an off to an on state. As a result, more than 99% of the reporter cells within the community were successfully engineered. Novel tools such as these hold significant promise for engineering the microbiome, but they are contingent on overcoming the barriers to DNA uptake.

4. DEVELOPING MICROBIAL GENETIC SYSTEMS IN THE AGE OF THE MICROBIOME

These applications highlight the significant progress that is being made to reverse- and re-engineer the microbiome. However, these collective studies also highlight a largely narrow focus on a small number of tractable microorganisms (**Table 1**). These microbes remain important

pieces of the microbiome's functional puzzle, but they permit only a limited view of the complete system when the contribution of the remaining pieces cannot be considered. Similarly, the reliance on *E. coli* strains (i.e., Nissle 1917) and lactic acid bacteria as chassis for therapeutic strategies is limiting, as these often can only transiently colonize the mammalian gut and require antibiotic treatment, frequent administration, or high titers for long-term engraftment. While some studies have engineered gut-adapted *E. coli* strains to promote more stable colonization (123), the freedom to genetically engineer any gut-associated microorganism would provide greater flexibility for understanding and exploiting the diverse metabolic and spatial capabilities of a diverse microbiota. Thus, a complementary priority to the functional characterization of genetically tractable gut microbes is to simultaneously broaden our access to the genetically intractable majority. As a result, there has been great interest in determining precisely which gut-associated microbes are amenable to genetic manipulation, and in developing strategies to overcome the barriers for those that are not.

Transduction: the process by which a virus transfers genetic material from one bacterium to another

Transformation: transferring exogenous DNA into a recipient organism

4.1. Minimum Requirements When Applying Genetics to New Microbes

The requirements for genetically engineering a microorganism have been reviewed previously (78). Briefly, amenability to genetic manipulation is defined by an organism's ability to take up and maintain foreign DNA, and this is a key classifier for defining a model and genetically tractable organism (65). Decades of microbial genetics research using *E. coli* models has produced a strong framework for defining these requirements, which comprise the following: (*a*) an appropriate DNA transfer mechanism; (*b*) the capability for the recipient microorganism to maintain the DNA construct; and (*c*) predictable gene expression from (or as a result of) the DNA construct (**Figure 2**). The genetic construct usually includes an antibiotic selection cassette, the genetic payload required to execute the engineering strategy (i.e., CRISPR *cas* genes, guide RNAs, transposases, transposons, fluorescent markers), and the regulatory elements required for predictable expression of the coding sequences in the recipient (i.e., promoters, RBSs, terminators). The form of the DNA construct (i.e., replicative plasmid, integrative plasmid, linear DNA, transposable genetic element) and the specifics of the payload and regulatory elements will depend on the engineering strategy and the method of DNA transfer. DNA is usually transferred via conjugation, transduction, in vitro transformation, or natural transformation. Integrative plasmids (nonreplicating "suicide" plasmids) or linear DNA fragments can facilitate integration of the payload into the recipient chromosome using endogenous or foreign integrases, recombinases, transposases, or homologous recombination-based methods. For confirming the genetic tractability of new gut-associated species, however, the conjugative transfer of replicative plasmids is usually preferred, because of its broad application for genetic engineering strategies, the ability to transfer large constructs, and the potential for in vivo or in situ transfer within complex communities (78). In this case, an additional consideration is the identification of an appropriate origin of replication for the plasmid to ensure it is recognized by the recipient's cell replication and partitioning machinery. Similarly, optimizing these variables for specific species has driven early efforts to create new genetic systems for gut-associated microbes. However, this single-species approach has proven inefficient for making significant progress. Consequently, innovative and alternative strategies are essential to modernize this process to accommodate a larger number and greater diversity of microbes.

4.2. Multifactorial Screening Approaches

As the known diversity of species in the human gut microbiome continues to expand, a need for high-throughput strategies for simultaneously elucidating the genetic requirements for many

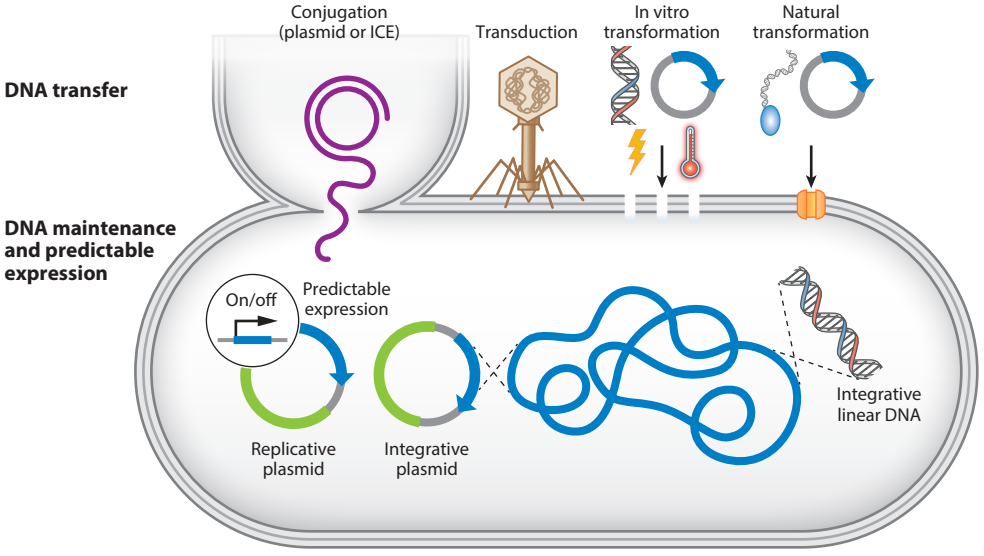

Figure 2

Minimum requirements for a new genetic system. DNA transfer methods for inducing the uptake of foreign genetic material include plasmid- or ICE-based conjugation, transduction via bacteriophages, artificially induced competence followed by in vitro transformation of plasmid or linear DNA using electroporation or heat shock, and transformation via the natural competence of the microorganism. Once inside the cell, the DNA construct is maintained as either a replicative plasmid containing a compatible origin of replication or an integrative plasmid or linear DNA fragment that is inserted into the chromosome. Peptide nucleic acid constructs (or other constructs expressed by a plasmid, e.g., gRNA for CRISPR applications) are maintained by binding to homologous regions of the chromosome. The final requirement is the predictable (constitutive or inducible) expression of the genetic payloads. Abbreviations: gRNA, guide RNA; ICE, integrative and conjugative element. Figure adapted from images created in BioRender.com.

microorganisms has become a priority. For example, several plasmid-based screening tools have recently been developed that are designed to incorporate a selection of promoters and RBSs, different antibiotic resistance markers, and plasmid origins of replication, including environmental transformation sequencing (ET-Seq), magic pools, metagenomic alteration of gut microbiome by in situ conjugation (MAGIC), and an unnamed gene transfer pipeline (64, 79, 118, 121). As a proof-of-principle, these were used to simultaneously screen multiple microbes to determine their specific regulatory requirements and DNA transfer methods. All methods relied on the conjugation of their constructs to recipients using the IncPα family RP4 conjugation mechanism and an *E. coli* donor, while the ET-Seq and gene transfer pipeline methods included additional DNA transfer approaches. Only MAGIC included replicative plasmids in its approach, although the corresponding origins of replication were predominantly of *E. coli* origin. Overall, these methods included a broad cross section of components and parameters and did successfully identify the conditions required for some new microorganisms, but their efficiency rates were mixed. Among these strategies, only MAGIC was tested against an undefined, complex community that was derived from a mouse gut, where it successfully identified the plasmid and transfer conditions for 5% of this population. While the other methods achieved higher success rates, the regulatory components of their constructs were largely tailored to the taxonomic scope of their target populations. These values are confounded by the varied contents of their constructs and the number and range

of recipients targeted, yet they highlight the diversity of tractability barriers even for closely related microorganisms. Accordingly, one additional screening strategy developed by Brophy et al. (14) relies on a *Bacillus subtilis* donor to transfer a payload to recipient organisms via an engineered integrative and conjugative element (ICE). Called XPORT, this method was able to successfully transfer a payload to 35 of 55 bacteria tested. While 20 of these recipients were *Bacillus* species, the shift toward non–*E. coli*–based conjugation donors, and the effort to rationally engineer the DNA transfer mechanism to broaden its effectiveness and scope, represents a promising advance toward more promiscuous DNA transfer in gut-associated microbes.

4.3. Portfolio Diversification: Expanding the Scope of the Genetics "Parts List"

Although successful in some cases, these high-throughput approaches have so far proven to be insufficient. This is most likely due to the reliance on a conventional "parts list" (see Section 4.1), leading to standardization bottlenecks where the pool of variables is not sufficiently diverse to accommodate the microbial diversity of the targets (87). However, a number of promising developments have recently been described that represent either more nuanced optimizations to satisfy species-specific requirements for DNA uptake, or novel DNA transfer techniques for reaching broader microbial targets.

4.3.1. Regulatory elements. To identify regulatory elements such as promoters, RBSs, and terminators beyond those established for model organisms, an initial approach is often sequence-based identification of these putative elements flanking known microbial housekeeping genes (70). If available, RNA-Seq data can help establish the genes that are highly expressed to guide the selection and prediction of regulatory elements (149), or more targeted methods like cappable-seq can precisely identify the genome-wide transcription start sites for an organism (41). Metagenomic mining and high-throughput techniques for identifying strain-specific components have also been useful (1). Structured systems have been established to allow reliable expression of heterologous genes in multiple bacteria, including the TREX and UBER systems that rely on an orthogonal T7 RNA polymerase for host-agnostic replication (73, 82), although effectiveness of these systems across broad phyla has yet to be determined. Finally, in silico predictions are also increasing in their predictive capabilities and reliability (29).

4.3.2. DNA transfer methods. Transformation methods such as heat-shock and electroporation are widely applicable for microorganisms that can be cultured in vitro across diverse phylogenetic groups, although some require further optimizations (45). Transformation efficiency is significantly lower in bacterial species containing a cell wall (4), which can be weakened by the addition of glycine or Tween-80 prior to transformation (157) or removed altogether to create spheroplasts or protoplasts (140). DNA transfer via the natural competence of the recipient is a common approach for microorganisms such as *B. subtilis* (143), *S. pneumoniae* (52), *Haemophilus influenzae* (90), *H. pylori* (34), and *V. cholerae* (28). When all traditional forms of DNA transfer were inadequate, innovative and alternative strategies were developed including sonoporation (135), biolistic bombardment (127), nanofiber piercing (148), and microfluidic electroporation (59). These optimizations highlight the frequent need to adapt or substitute traditional methods to reach diverse targets. Three methods in particular have shown promise for microbiome genetics that warrant further discussion: conjugation, transduction, and programmable RNA.

4.3.2.1. Conjugation. Conjugative transfer of plasmids or transposable elements via direct cell-cell contact frequently occurs between microbes to increase microbial fitness and facilitate niche adaptation (104). Thus, the potential for exploiting this capability in a microbiome context is

enormous. However, while thousands of conjugative plasmids have been described for the gut, their scope of transfer is strongly defined by phylogenetic boundaries (112), implying that for microbiome genetics, diverse representatives will be required to reach diverse recipients.

In contrast, current microbiome genetics applications mostly utilize the canonical RP4 conjugative machinery, and this was the sole conjugation mechanism employed by each of the multifactorial screening approaches described above. While RP4 was originally isolated from *P. aeruginosa*, it is the canonical conjugation mechanism used for microbial genetics and is primarily used in conjunction with an *E. coli* host (conjugation donor). Due to its ability to transfer plasmids to both gram-negative and gram-positive microorganisms, it is commonly regarded as a broad–host range mechanism. However, this designation was established prior to the advent of high-throughput sequencing of microbiome samples and is not an accurate classification at this level of diversity (64, 118, 121). Indeed, this conjugative strategy relies on a specific interaction between donor and recipient, which likely determines its taxonomic scope (49), and its limited value for in vivo microbiome studies is commonly reported (83). Additionally, anaerobic conditions have been shown to significantly reduce the efficiency of RP4 (156). Thus, novel conjugation systems with a diversity of host ranges and mechanisms are required to broaden the taxonomic potential of this method.

Many additional conjugative plasmids have been identified that could be transferred to gut-associated recipients including *Salmonella enterica* and *E. faecalis* (101), but these have not yet been characterized as minimal-plasmid or chromosomally integrated engineering tools. Furthermore, a naturally occurring conjugative plasmid was recently isolated and shown to have significantly higher transfer rates in the mouse intestinal tract compared to all others tested, including RP4 (100), but its origin of replicative transfer has not yet been identified. Nevertheless, this finding highlights the potential for characterizing more diverse, microbiome-specific conjugative tools beyond RP4. For this to be achieved, the growing collection of data on alternative conjugative plasmids, including the recent description of the taxonomic scope of bacterial plasmidome transfer (112), and the ability to computationally predict novel systems in metagenomic data (102) will become valuable resources.

In addition to plasmid-based conjugative transfer, attention is also shifting to ICE-based conjugative transfer as exemplified by the XPORT system discussed above (14). Similar to the case of conjugative plasmids, specific interactions between an ICE and its host influence the efficiency of acquisition by new hosts (9), but ICEs are also more frequently observed in sequenced genomes compared to conjugative plasmids (51). Furthermore, it has been demonstrated that functional components from different ICEs can be combined to form hybrid elements that alter their transfer efficiencies and host range (9), suggesting the potential for altering their specificity for different targets.

4.3.2.2. Phage-based transfer. Upon infection of a host microbe, bacteriophages hijack the replication machinery to facilitate their reproduction via chromosomal integration or dissemination following host lysis. These capabilities, coupled with the high specificity of phages for their microbial hosts, have made them promising vehicles for the transfer of genetic payloads. The high specificity of phages has routinely been exploited to target pathogens within mixed communities, including *Klebsiella pneumoniae* (50) and *Clostridium perfringens* (92). Furthermore, phage host range and function can be engineered by modifying receptor-binding proteins that interact with bacterial surface receptors. This can be achieved via random mutagenesis or rational engineering or by swapping different tail fibers (78). For example, CRISPR-Cas9 payloads with guides to target specific microbes have been encoded on a plasmid and packaged into a target phage, which was delivered to a microbial community (147). Using this approach, carbapenem-resistant

and enterohemorrhagic *E. coli* (22) and methicillin-resistant *Staphylococcus aureus* (10) have been successfully targeted in mixed communities. Furthermore, genes of interest have been delivered by phages to restore antibiotic susceptibility and deliver biofilm dispersal enzymes (84).

4.3.2.3. Programmable RNA. Other groups have also turned to programmable RNA for the targeted transfer of synthetic DNA in microbial communities. In these cases, the genetic payload is in the form of antisense peptide nucleic acids (PNAs) that are coupled to short carrier peptides to facilitate their transfer into recipient cells. Once inside the cell, the PNAs silence the mRNA of target genes by binding to their corresponding RBSs. The carrier peptides can be selected according to specificity for the organism of interest (109), and an increasing list of phylogenetically diverse bacteria have been successfully targeted (106, 109). While their species- and strain-level specificity and off-target effects require further characterization, PNAs represent a novel and encouraging strategy.

5. THE GENETIC INTRACTABILITY BARRIER

It is evident that the strategies for designing new genetic systems must be modernized to enable compatibility with a broader range of gut-associated microbes. By characterizing more versatile and orthogonal genetic elements, and developing more advanced tools and species-specific techniques beyond those of traditional model hosts, significant progress can be achieved. This will require a deep understanding of the underlying causes of genetic intractability to enable us to predict or surmount these challenges, both on a species-specific or community level.

5.1. Factors Contributing to Intractability

Genetic intractability is the inability to manipulate the genetic content of an organism. This is usually due to evolutionary incompatibilities that manifest as physiological or technical conflicts that restrict an organism's ability to be reliably genetically modified (**Figure 3**). For microorganisms, physiological barriers include the compatibility or fitness costs incurred by taking up foreign genetic material, the metabolic condition of the recipient, the activity of defense systems, and the outer membrane structure of the recipient, while technical barriers refer to the growth and culture conditions of the recipient, as well as the protocol specifics for DNA transfer. These incompatibilities are often species- or even subspecies-specific, making it difficult to anticipate and overcome their effects.

Physiological fitness costs can occur for several reasons. For example, foreign DNA transferred via conjugation, transformation, or transduction often activates the SOS stress response (7, 6, 15), leading to retarded cell division (91). Construct-encoded genes and transcription factors can result in toxicity, the dysregulation of host pathways (43), or cause a codon-usage imbalance between the foreign genes and the available tRNA pool in the recipient (108). Payloads with disparate nucleotide biases compared to the recipient (i.e., AT and GC content) can be metabolically costly (103), while host amino acid pools can be prematurely depleted (12). Additionally, these metabolic burdens will scale with plasmid or payload size and copy number. Conjugation efficiency will be dependent on the proximity and interaction between donor and recipient (24). For plasmids designed to replicate in the recipient, the replicon must be compatible with host cell partitioning and replication machinery (105). Even when this is the case, the replicating plasmid will additionally sequester host DNA polymerases and helicases that can stall chromosomal replication, an effect that will be heightened for plasmids recognized as having high copy number (91). The presence of existing plasmids in the recipient can also accelerate the drainage of limited cellular resources (24).

Technical issues can include culture and growth conditions (31), which are not always optimal for fastidious gut microbiota (74), while the size and complexity of microbial cell envelopes,

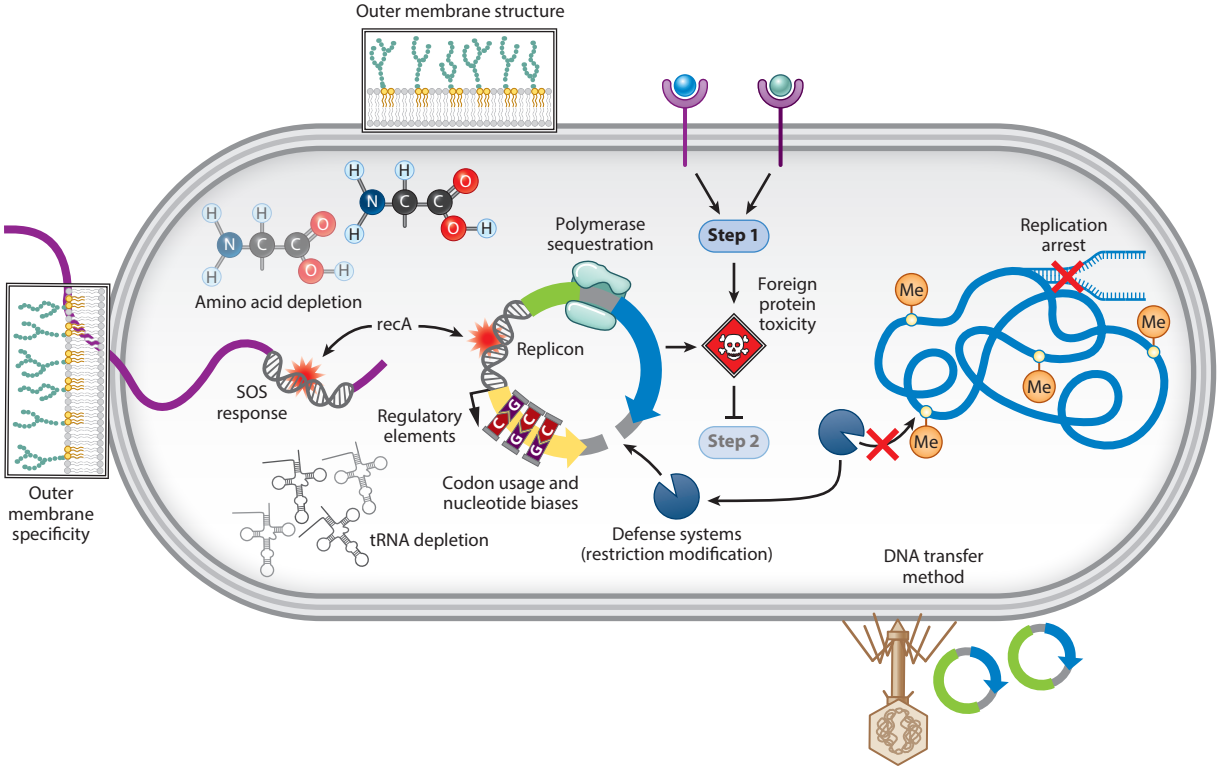

Figure 3

Physiological sources of genetic intractability in microorganisms include the activation of the host SOS stress response following DNA transfer, premature depletion of recipient amino acid or tRNA pools due to the increased genetic requirements, dysregulation of host pathways or toxicity induced by exogenous gene expression, codon usage and nucleotide bias between DNA construct and host, incompatible plasmid replicons, host replication stalling due to the sequestration of host polymerases, diverse outer membrane structures that affect transformation efficiency, outer membrane specificity for conjugation donors, host defense systems, and ineffective DNA transfer methods. Figure adapted from images created in BioRender.com.

including the peptidoglycan layer, can lead to incompatibilities with the DNA transfer method used (58). In these cases, the taxonomic range of conjugative donors will be limited (20) as will be the ability of DNA constructs to penetrate unique membrane structures. Other technical barriers include the experimental and environmental conditions used for DNA transfer, including temperature, pH, chemical and physical composition, redox status, organic or inorganic pollutants, cell density, growth phase, carbon and metal concentrations, oxygen levels, reaction times, donor-to-recipient ratios, and mating times (for conjugation strategies), all of which require optimization for the recipient (91). Furthermore, the optimal conditions for most gut-associated microorganisms are likely to be vastly different to those for *E. coli*, complicating the coculturing required when carrying out conjugation experiments with *E. coli* conjugation donors.

5.2. Defense Systems

One additional tractability bottleneck that warrants further discussion is the influence of microbial defense systems for eliminating foreign DNA from new hosts. An increasing number and variety of microbial defense systems have recently been discovered. Several are implicated in inhibiting DNA (linear and plasmid) transfer: restriction modification (RM), CRISPR

(107), bacteriophage exclusion (BREX) (120), Wadjet (30) prokaryotic Argonautes (pAgo) (138), MksBEFG (145), defense island system associated with restriction–modification (DISARM) (2), and DdmDE (62). For many of these emerging systems the mechanism of action is not fully characterized, making it difficult to predict, alleviate, or overcome their defenses. Nevertheless, the expanding repertoire of known antidefense proteins, including anti-CRISPR, antirestriction, anti-BREX, and anti-SOS, presents a promising avenue for devising strategies to effectively counteract these defense mechanisms (125).

Conversely, microbial RM systems have been studied in detail and are the most common cause of genetic intractability in bacteria (65). Identified in ∼90% of known bacterial genomes, RM systems are the most abundant microbial defense systems known. These systems utilize restriction endonucleases that cleave foreign DNA according to the recognition of specific nucleotide motifs. In addition, cognate methyltransferases methylate the same sequence motifs on the host chromosome. This ability to differentiate self from nonself DNA has been shown to prevent DNA transfer to diverse microorganisms, including *Prevotella* spp. (66), *Clostridium* spp. (115), *Yersinia* spp. (67), *H. pylori* (33), *Bifidobacterium* spp. (96), and *Staphylococcus* spp. (25).

Strategies to overcome this barrier include (*a*) the use of genetically tractable hosts that express RM systems with compatible recognition motifs (98, 113); (*b*) expression of heterologous methyltransferases to mimic the methylation profile expected by the recipient (153); (*c*) in vitro methylation with commercially available enzymes or cell-free extracts (17); (*d*) engineering plasmids and other DNA constructs to avoid known RM recognition sites (65); (*e*) heat treatment of recipient cells to inactivate endonucleases (39); and (*f*) genetic inactivation of recipient endonucleases (21). A common misconception is that all plasmids that are transferred to a recipient via conjugation are immune to RM systems (122); however, this oversimplified perspective does not accurately reflect the diverse majority of microbes. Several studies have demonstrated that successful plasmid conjugation to a recipient was dependent on either the inactivation of RM endonucleases or the mimicking of RM methyltransferases (40, 137, 150). Additionally, others have shown that observed reductions in conjugation efficiencies were proportional to the number of RM recognition sites present on the plasmid (111). More recently, the discovery of orphan methyltransferases and anti-restriction proteins encoded by the leading regions of conjugative elements (125) has highlighted an evolutionary adaptation that was required to safeguard conjugative plasmids from recipient RM systems. Nevertheless, our otherwise in-depth understanding of RM systems has enabled us to establish extremely effective countermeasures, providing a benchmark for combatting other emerging defense systems when establishing new genetic systems.

6. FUTURE DIRECTIONS: MICROBIOME ENGINEERING

Bringing genetics to the microbiome creates challenges that require a combination of optimization, adaptation, and innovation to overcome the initial struggles. By definition, microbiome genetics demands that strategies designed for individual microbes be scaled to the community level. This ambition, while essential for our ability to understand and control the microbiome, faces the existing tractability challenges that are frequently reported for single microorganisms, multiplied by the diversity of the gut microbiome. The *Bacteroides* genetic systems have demonstrated the power of genetics for the microbiome and are models for new systems. For that to happen at the scale and speed that are demanded, we must prioritize the following:

- An expansion in the number of characterized conjugative plasmids that are derived from the gut microbiome for subsequent use as engineering tools is needed. This includes the laboratory domestication, characterization, and engineering of their native host microorganisms to serve as robust conjugation donors. This will enable the establishment of

phylum-level (or lower) classes of DNA transfer tools for more controlled targeting of specific microbial groups.

- Likewise, a considerable increase in the number of characterized plasmid replicons to support plasmid replication in both novel conjugation donors and new, gut-associated microbes.
- In addition to conjugation, the continued development of novel DNA transfer methods for both individual microbe and targeted community editing.
- An improvement in our capability to characterize and predict microbial regulatory components on a much larger scale so that they may be compiled into taxonomically defined collections for targeting specific microbial groups.
- Characterizing the mechanisms of action for emerging microbial defense systems to enable us to overcome, inactivate, or bypass them.
- Improving our ability to draw together experimental, genomic, metagenomic, and metabolic data for predicting genetic intractability factors down to the strain level. This will enable these factors to be ranked according to their putative significance so that we can focus and accelerate the screening process and avoid the random testing of factors.
- Advances in automation and high-throughput strategies to facilitate the screening of large numbers of taxonomically defined groups of microorganisms.

Furthermore, there is much interest in establishing a model microbiome that represents the common, minimal compositional requirements for it to function. A natural extension to this parts list is to establish an equivalent genetic or functional parts list, enabling a more granular global map of the functional dependencies for a model system. As members of the microbiota are the custodians of microbiome function via the specifics of their genetic content, this would provide a more informed basis for both determining the drivers of microbial composition and establishing a framework for more sophisticated reengineering strategies to control microbiome behaviors.

Once these barriers can be overcome, we will significantly expand the number of tractable microbes and establish unprecedented capabilities for microbiome engineering. The ability to genetically perturb a microorganism and determine the functional consequence remains one of the key advantages when applying microbiome genetics. However, to date, genetics has been applied to identify single-microbe determinants for colonization. This strategy remains valuable for functionally characterizing the gut microbiota one-by-one, but this segregated, multispecies view of the microbiome is insufficient to truly tease out the intertwined molecular interactions that drive the emergent properties of the community. The human gut microbiome is more than the sum of its microbial parts and represents a complex, interacting biological entity rather than a random assortment of individual microbes (144). A true microbiome engineering strategy would be to genetically perturb an organism and observe the phenotypic effects beyond the engineered cell's physiology as they reverberate throughout the system. Therefore, we must advance our capabilities for detecting and measuring the community-level consequences of microbiome genetics, beginning with defined, compartmentalized microbiome models, to drive a more synergistic understanding of the system as a whole.

SUMMARY POINTS

1. Genetics strategies can be used to reverse-engineer the microbiome to determine its functional mechanics or to re-engineer the microbiome to instill novel functions.

2. Emerging microbiome genetics techniques enable the in situ targeting and transfer of DNA between microorganisms.
3. Developing genetic systems for microbiome genetics relies on a "parts list" of the minimum items that need to be adapted for each microorganism.
4. Multifactorial screening approaches enable high-throughput strategies that can determine the DNA transfer conditions for many organisms at once, but these are often not enough to overcome significant tractability barriers.
5. Physiological and technical restrictions to the successful genetic manipulation of an organism are intractability barriers.
6. Restriction modification defense systems are one of the largest barriers to tractability.
7. New strategies are needed to enable genetic manipulation of the gut microbiome.

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Errata

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