Translating dynamics of human-microbe interactions
By
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Abstract

High-throughput genetic sequencing revolutionized our ability to systematically quantify and analyze biological systems. These methods have been particularly fruitful in understanding the composition and dynamics of the microbial communities that inhabit the human body and how our cells interact with these microbes to maintain health or generate disease. In this thesis, I describe the results of four projects that used high-throughput sequencing to interrogate the dynamics of four systems at the boundary of man and microbe. In the first project, I and my coauthor discovered a mechanism by which marine bacteria dynamically become hypermutators—allowing for rapid adaptation—and we discovered similar mechanisms in clinically-relevant pathogens. In the second project, I developed a new method for targetedly profiling living bacteria in a sample, allowing me to assess the effects of fecal processing on the viability of bacteria in fecal microbiota transplantations. In the third project, I characterized the longitudinal dynamics of the T cell receptor repertoire in healthy people, providing a critical baseline for interpreting changes in the adaptive immune system—our first line of contact with commensals and pathogens. In the fourth project, I tracked the dynamics of engrafting bacteria in a clinical trial of patients with inflammatory bowel disease who received fecal microbiota transplant, demonstrating that patients differ not only in their capacity to accept donor bacteria, but also in their ability to maintain those bacteria. Aside from contributing scientific conclusions about each system, these studies exemplify how genetic sequencing can allow us to directly study the complexity of human subjects, providing a shorter path to translatable clinical insights.

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Chapter 1

Introduction

1.1 Measuring complexity

From the flora of the tropical rainforest canopy to the proteins on a meiotic chromatin scaffold, biological systems are uniformly complex and dynamic. No matter the scale, these systems host a dizzying array of individual players, each with its own host of interactions and each with its own ability to change over time. These two forces — interaction (ecology) and change (evolution) — underpin the ability for organisms to achieve a diversity of functions, whether invading host tissue or sharing limited nutrients.

Traditionally, to extract meaning from such complex systems, researchers needed to focus on specific entities in these complex systems, since attempting to quantify and organize all of them was unmanageable. Such a reductionist approach revealed a broad array of mechanistic insights, like the role of pollinators in plant reproduction and the central dogma of DNA to protein. But despite these fundamental contributions, these reductionist studies largely ignored the greater biological context in which these phenomena unfolded. Without the methods to more comprehensively assess communities of molecules, proteins, or species, researchers continued to reduce problems into tractable experimental designs.

In the past two decades, so called “-omic” technologies have revolutionized our ability to assess biological systems in high-throughput. Developments in DNA sequencing [1], protein sequencing [2], and chemical mass spectrometry [3] suddenly opened the opportunity to move beyond measuring single biological parameters to simultaneously quantifying hundreds to millions of chemicals, proteins, or organisms at once. For the first time, researchers could now directly tackle the messy, convoluted interactions within their environment of interest, from cytoplasms to oceans.

These methods were particularly critical in a rapid expansion of research on the human microbiome—the bacteria that live on and within the human body [4]. Although previous host-microbe research primarily focused on pathogens—as they fit into reductionist experimental designs—high-throughput sequencing suddenly allowed for characterization of entire microbial communities [5]. Much of this attention has focused on the hundreds of species of bacteria that inhabit the human gastrointestinal tract, and changes in this “gut microbiota” have been implicated in diseases ranging from inflammatory bowel disease to autism [6–8]. In parallel, genetic sequencing also open new ways to characterize the host immune system [9,10]. Like the microbiome, the immune system is made up of thousands of fluctuating cell types, each with different functions and roles [11–13]. And also like the microbiome, high-throughput genetic sequencing opened the ability to characterize these populations as a whole and not disparate parts.
We now have the ability to systematically interrogate both sides of the host-microbe interface, with the potential to address a broad spectrum of infectious and autoimmune diseases.

In this thesis, I present the results from four projects that used high-throughput experimental and computational tools to ask questions that fall along this axis of host and microbe, with the aim of informing our understanding of human health and disease. In Chapter 2, I discuss the use of whole-genome sequencing and experimental evolution to discover a novel mechanism of hypermutation driven by a mobile element [14]. In Chapter 3, I discuss the development of a new experimental sequencing method to profile living bacteria in a microbial sample and its application to understanding the effects of processing treatments on fecal microbiota transplants [15]. In Chapter 4, I discuss a longitudinal analysis of the healthy human immune system, specifically the T cell receptor repertoire [16]. And finally in Chapter 5, I discuss a longitudinal analysis of the gut microbiome in patients with inflammatory bowel disease who received fecal microbiota transplant therapy. In the rest of this chapter, I introduce and contextualize these contributions. In the final chapter, I discuss the limitations and extensions of these studies.

### 1.2 Genomic sequencing reveals a novel mechanism of mutation

Mutation is the raw material on which evolution operates, allowing both for the generation and fixation of new traits. Generally, mutations are rare, in part because the vast majority of mutations are deleterious [17]. But some individuals break this mold. So called “hypermutators” or “mutators” exhibit an elevated rate of mutations, allowing them to quickly adapt to changing conditions [18].

Before the development of high-throughput sequencing, discovering hypermutators meant quantifying the accumulation of mutations in individual genes, as measuring genome-wide mutation rates was impractical [17,19]. This limitation meant that only extreme hypermutators could be identified, since they would have to accumulate enough mutations to manifest in a tiny slice of the overall genome.

In Chapter 2, I describe the results of an experimental evolution project in the marine bacterium *Vibrio splendidus*, where because of our ability to rapidly and cheaply sequence whole genomes, we were able to identify (a) the presence of hypermutators rapidly evolving in the face of salt stress and (b) a novel mobile-element driven mechanism generating this mutation phenotype. Only by integrating full genome information were these simultaneous discoveries possible. The mechanism in particular required finding a particular region of the genome missing in the hypermutator strains, which would have been likely impossible to identify if the study had been limited to single-gene analysis.

### 1.3 Microbial sequencing shapes fecal microbiota transplant practices

One of the most powerful aspects of high-throughput sequencing is the number of ways it can be paired with various biochemical reactions to target questions of interest. For example, 16S rDNA
sequencing, whereby targeted amplification of the bacterial 16S gene is followed by sequencing, provides both the abundance and the identity of various microbes in a sample, since the 16S gene acts as a “barcode” for each bacterium [20]. Before the development of this method, quantifying microbial communities generally relied on culturing microbes, which—among other artifacts—limits the species of bacteria that can be measured to those that can be easily cultured [21]. As a culture-independent method, 16S sequencing exposed the “dark matter” of the microbial world, prompting the discovery of many new bacteria [22].

A potential drawback of 16S is that it does not distinguish between living or dead microbes. Any genetic material—whether housed in a living cell or floating in solution—will be amplified and sequenced all the same. For many applications, this artifact is not a problem. But in others, there is a need to know specifically what bacteria are alive in a given sample. For example, in the context of fecal microbiota transplants—the transplant of fecal microbes from a healthy donor to a sick recipient—one can reasonably assume that it is the transplant of living microbes that is most likely to generate the desired clinical outcome, not dead ones.

To address this limitation, I developed PMA-seq, which pairs 16S rDNA sequencing with pretreatment with the molecule propidium monoazide (PMA) to targetedly profile living bacteria in a microbial sample. In Chapter 4, I describe the development of this method as well as its application to assess how processing procedures (oxygen exposure, freeze-thaw cycles, and lag time between defecation and sample processing) affect the abundances of living bacteria in fecal microbiota transplants.

1.4 Immune sequencing uncovers the ecology of adaptive immunity

Although ecologists are generally most comfortable thinking about the interactions of different species, this perspective is somewhat arbitrary. Almost every biological systems is made up of individual entities that interact with each other (ecology) and can be differentiated into discrete populations (species). Thus, by using frameworks from microbial ecology, we might be able to provide new insights into biological systems that are not traditionally viewed from such a perspective.

The adaptive immune system is one such system, where different populations of immune cells interact with each other and their environment to maintain host health. Like microbiology, our understanding of the immune system rests on a foundation of knowledge built by targeted experiments. But again, like microbiology, these targeted experiments overlooked and ignored the immense diversity of immune cells, each with very different behavior. For example, a key attribute of the adaptive immune system is its ability to specifically respond to a given antigen—such as a pathogen—and to later remember that same antigen upon reexposure [13]. This specificity and recognition depends on the activity of two receptors on two types of immune cells: B cell receptors (BCR) [23] and T cell receptors (TCR) [24]. But the body does not know beforehand what antigens it will encounter in its lifetime. The adaptive immune system can recognize new antigens because it first creates an extraordinary diversity of these receptors (a potential diversity of $10^{15}$ different receptors) by pseudo-randomly shuffling the genes that encode them [25]. Thus, when the body faces a new antigen, it is likely that one of the millions of different receptors available will specifically recognize the new threat.
With high-throughput sequencing, we can now systematically quantify the abundance of different receptors, using “immunosequencing” or TCR-seq and BCR-seq [10]. Drawing comparisons to microbial ecology, instead of species, we now observe individual receptors. Much of the work to understand the dynamics of the immune repertoire has perhaps rightly focused on disease [25]. In the pursuit of immune cells that improperly recognize certain antigens, researchers have asked how the immune repertoire differs in healthy controls versus diseased patients or before and after immune treatment. But the research community lacked information about the baseline dynamics of immune repertoires in healthy people. Such foundational knowledge is critical context for understanding changes in the adaptive immune system.

In Chapter 4, I describe the analysis of a time-series of T cell receptor sequencing data from three healthy subjects over the course of one year. In addition to setting baseline expectations for the rates of change and turnover in the adaptive immune system, I discovered a subset of immune receptors that were both widely shared across people and maintained within people across time. Such “public” and “persistent” receptors may play an important role in immune maintenance.

1.5 Metagenomics track the dynamics of fecal transplants

Beyond 16S, high-throughput sequencing has provided a number of other ways to analyze microbial communities. In particular, shotgun-metagenomics sequencing—where all DNA in a sample is indiscriminately sequenced—provides additional layers of information beyond who is there and how many [26]. Most frequently, researchers turn to metagenomics for three reasons: (1) to know what the community is doing (i.e., gene functions); (2) to assembly unknown microbial genomes directly from the sample; and (3) to obtain strain-level resolution on the identities of community members. Thus, metagenomics sequencing allows us to deeply characterize the dynamics of microbial communities well beyond the capabilities of 16S. Such knowledge will likely be crucial to translate much of the current research on the gut microbiome into actionable clinical therapies and diagnostics.

In Chapter 5, I describe an analysis of longitudinal stool samples taken from a small cohort of patients with inflammatory bowel disease who received an intensive course of fecal microbiota transplant. Despite the widespread therapeutic use of fecal microbiota transplants in the treatment of gastrointestinal infections, we still understand little about their effects on the recipient’s gut microbiome. In particular, we lack a clear picture of how the introduction of these new bacteria and genetic functions unfolds across time in each of these patients. When strains colonize their new host, how long do they persist? How dynamic are their abundances in the recipient? To answer these questions, I analyse 16S and metagenomic sequencing data, uncovering the diversity of engraftment trajectories possible during the course of these treatments.
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Chapter 2

A mobile element in mutS modulates hypermutation in a marine Vibrio

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Supplementary Information is in Appendix A.
ABSTRACT

Bacteria face a trade-off between genetic fidelity, which reduces deleterious mistakes in the genome, and genetic innovation, which allows organisms to adapt. Evidence suggests that many bacteria balance this trade-off by modulating their mutation rates, but few mechanisms have been described for such variable rates. During a whole-genome sequencing experiment on the marine bacterium *Vibrio splendidus* 12B01, we discovered one such mechanism, which allows this bacterium to switch to an elevated mutation rate. This switch is driven by the excision of a mobile element residing in *mutS*, which encodes a DNA mismatch repair protein. When integrated within the bacterial genome, the mobile element provides independent promoter and translation start sequences for *mutS*—different from the bacterium's original *mutS* promoter region—which allow the bacterium to make a functional *mutS* gene product. Excision of this mobile element rejoins the *mutS* gene with host promoter and translation start sequences but leaves a two-base-pair deletion in the *mutS* sequence, resulting in a frameshift and a hypermutator phenotype. We further identified hundreds of clinical and environmental bacteria across Beta- and Gammaproteobacteria that possess putative mobile elements within the same amino acid motif in *mutS*. In a subset of these bacteria, we detected excision of the element but not a frameshift mutation; the mobile elements leave an intact *mutS* coding sequence after excision. Our findings reveal a novel mechanism by which one bacterium alters its mutation rate and hint at a possible evolutionary role for mobile elements within *mutS* in other bacteria.
DNA mutations are a double-edged sword. Most mutations are harmful; they can scramble precise genetic sequences honed over thousands of generations. But in rare cases, mutations also produce beneficial new traits that allow populations to adapt in changing environments. Recent evidence suggests that some bacteria balance this trade-off by altering their mutation rates to suit their environment. To date, however, we know of few mechanisms that allow bacteria to change their mutation rates. We describe one such mechanism in the marine bacterium *Vibrio splendidus* 12B01, modulated by the action of a mobile element. We also found similar mobile genetic sequences in the *mutS* genes of many different bacteria, including clinical and agricultural pathogens. These mobile elements might play an as yet unknown role in the evolution of these important bacteria.
INTRODUCTION

To adapt to changing environments—whether changes in pH or exposure to antibiotics—bacteria rely on mutations to produce new genetic variants that can survive under new conditions. The majority of possible mutations, however, are deleterious, so all organisms face a trade-off between genetic fidelity, which enables accurate gene replication, and genetic innovation, which provides new genetic diversity (1). Observed mutation rates in bacteria studied in the laboratory are thought to balance this trade-off, because a wide range of microorganisms have similar, low mutation rates: about one mutation per genome per 300 cell divisions (2).

Nevertheless, bacterial strains with higher-than-expected mutation rates—known as mutators—have been found in diverse habitats. These habitats include the respiratory tracts of patients with cystic fibrosis (3,4), the mouse gut (5), and the human gut and urinary tract (6–8). In clinical settings, mutator bacteria are believed to contribute to the rise of antibiotic resistance, which is increasingly recognized as a critical burden on global health (9,10). In seeking to reconcile the prevalence of bacterial mutators with the deleterious nature of mutations, many past theoretical studies found that constitutive mutators—bacteria with a fixed, elevated mutation rate—can be maintained at low frequencies in asexual populations (11–13). But even though mutator cells as a group can persist in a community, each individual mutator cell can pass on its genes only by reverting to a nonmutator state or by recombining its genes with nonmutator members of the community via horizontal gene transfer (14). Otherwise, unchecked, rapid accumulation of mutations would make each mutator cell an evolutionary dead end, a paradigm akin to Mueller's ratchet (15).

It has been proposed that many bacteria might resolve the fidelity-versus-innovation dilemma by altering their mutation rates. One strategy involves active regulation of mutation rate, or stress-induced mutagenesis (16). In this strategy, bacteria respond to stress by increasing their mutation rates (17). Such bacteria can thus harness genetic innovation during periods of environmental change—like the application of antibiotics—while maintaining genetic fidelity during periods of stability; their mutation rate is thus actively linked with a need to adapt. This active strategy is distinct from stress-driven mutagenesis, where a given stress triggers a change in mutation rate irrespective of active bacterial response (e.g., ultraviolet radiation) (17).

Despite this theoretical framework, few mechanisms are known that allow bacteria to alter their mutation rates, and the mechanisms uncovered to date have largely been constrained to well-studied model organisms. One mechanism that increases mutation rates is upregulation of error-prone DNA polymerases, which introduce more errors than typical polymerases (18,19). Another mechanism is downregulation of genes in the mismatch repair pathway, a set of genes encoding proteins that proofread DNA during replication, recombination, and damage (20). Most described mutator bacteria with deficits in mismatch repair genes have partial or full loss-of-function mutations in one of the mismatch repair genes (e.g., mutS, mutL, or mutH) (7,21–24). Researchers have previously observed elevated rates of polymorphism and recombination in this chromosomal region, which in many bacteria also includes the stress response gene rpoS (25,26). Such variation and instability have led some to propose that mutS is a "contingency gene," a highly mutable locus that allows an organism to rapidly alter its genotype (27).

To our knowledge, only three cases of mismatch repair mutations have been described that do not involve constitutive mutations and thus a constitutive mutator phenotype. Some strains of Escherichia coli downregulate mutS expression during the transition to stationary-
phase growth, increasing their mutation rate by an order of magnitude (28). Strains of *E. coli*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* also downregulate *mutS* via a *rpoS*-dependent response to antibiotics (29). *Streptococcus pyogenes*, a common cause of skin infection, offers another example. Strains of *S. pyogenes* contain a prophage that, when integrated into the bacterial chromosome between *mutS* and *mutL*, halts transcription of *mutL*, increasing mutation rate 100-fold (30). The prophage excises itself during exponential growth and reintegrates itself when cells reach stationary phase (30). Thus, integration and excision of this prophage cause a temporary and reversible increase in mutation rate in response to the environmental stress of entering into stationary phase.

Here, we report a mechanism that allows *Vibrio splendidus* 12B01, a common marine bacterium, to increase its mutation rate. We also identified diverse mobile elements within the *mutS* sequences of hundreds of environmental and clinical strains, suggesting that these elements might play a role in regulating mutation rates in many bacteria.

**RESULTS**

**Whole-genome sequencing revealed elevated, variable, and transition-biased mutation rates in *V. splendidus* 12B01**

During serial selection for salt-tolerant mutants in *V. splendidus* 12B01 (see Materials and Methods, Fig S1), we discovered a mobile element that modulates the bacteria's mutation rate. We performed whole-genome sequencing on 40 isolated colonies from 8 independent bacterial lineages (lineages 1–8), which originated from the same ancestral strain over five rounds of selection on high-salinity plates (data access: NCBI BioSample SAMN05560410). We identified *de novo* mutations by calling high-confidence single-nucleotide polymorphisms (SNP) between each genome assembly and a reference genome for the ancestral strain (see Materials and Methods). We checked for contamination by confirming that each strain maintained all mutations that arose in the previous selection round. We also quantified deletions, insertions, and inversions but focused on SNPs in further analyses because they were more common, easier to quantify, and simple to compare across lineages.

We found that two lineages (subsequently referred to as hypermutators) had accumulated a surprising number of mutations—more than 1,500 single-nucleotide mutations over an estimated 250 generations (e.g., lineage 2 and 6, Fig 1a, Table S1). This mutation rate (6–7 mutations per generation) is three orders of magnitude higher than reported averages for bacteria (~0.003 mutations per generation) (2). All other lineages (subsequently referred to as mutators) still had mutation loads two to three orders of magnitude higher than expected (e.g., lineage 8, Fig 1a, Table S1). Lineages did not accumulate mutations evenly across selection rounds but instead did so sporadically (Fig 1b). Previous studies have shown that mutations often follow a Poisson distribution, which assumes a stable average mutation rate (31,32). But we found that in all lineages (including the hypermutators), the variance in the number of new mutations across selection rounds was far greater than that expected under a Poisson model (Fig 1c), suggesting that the mutation rate in this strain varied substantially more than rates in other commonly studied bacteria, such as *E. coli* (31,33,34).

The mutations in all lineages displayed a dramatic bias toward transition mutations, which accounted for 96% of observed mutations, or a transition-to-transversion mutation ratio of 22.6 (Table S1, $\chi^2 = 3461.6677$, df = 7, $P < 10^{-308}$). Moreover, when rounds of selection
generated more mutations, we observed even greater bias (Fig 1d; ordinary least squares: \( F \)-statistic = 29.68, df = 35, \( P < 10^{-5} \), \( R^2 = 0.459 \)). Using a binomial model of transition frequency and a maximum-likelihood estimator, we found that mutations among our two hypermutator lineages were best described by a transition-to-transversion mutation ratio of 24.6, mutator lineages by 16.5, and all lineages by 22.8—values substantially higher than typically reported values for wild-type strains (2.1–3; Fig S2a) (35). These values are also much higher than the transition-to-transversion ratio of SNPs when comparing 12B01 and the closely related strain 12F01—which is 2.1 (71/105 SNPs, Fig 1d, S2a)—or the more distantly related 13B01—which is 2.8 (30,975/42,012 SNPs). On the basis of these results, we speculated that all strains had developed a defect in DNA mismatch repair, which is known to produce similar, characteristic increases in mutation load and transition frequency (36,37). In addition, we posited that this defect could be transient, given the extreme variability in mutation load across selection rounds.

Excision of a mobile element results in mutS scarring and a hypermutator phenotype

From our whole-genome sequencing reads, we found that both hypermutator lineages shared a deletion of a 27 kb region adjacent to mutS (Fig S3a). On closer inspection of the wild-type sequence, this deletion appeared to be a mobile element (Fig 2a). In fact, it appeared that the mobile element resided within a conserved amino acid motif at the beginning of the mutS sequence. The mobile element effectively provided a new translation start sequence and promoter region for the mutS gene, which appeared to result in a functional mutS coding sequence. Upstream of the mobile element, we identified a putative N-terminal coding region for mutS. Unlike the starting mutS sequence provided by the mobile element, this orphaned mutS starting sequence matched that of closely related Vibrio strains, which suggests it is the ancestral, host-derived starting sequence of mutS (Fig S3b). In hypermutator lineages that had lost the mobile element altogether, we found that this host-derived start sequence was rejoined with the rest of the mutS sequence, but excision of the mobile element had also removed two additional base pairs, leaving a frameshift mutation, which resulted in an early stop codon and a disrupted mutS sequence (Fig 2b).

We observed sequence similarity between the terminal ends of the mobile element and its insertion site in 12B01's mutS sequence, which resembled the direct repeats of mobile elements that excise themselves by site-specific recombination (Fig 2b, S3c). We therefore hypothesized that 12B01's mobile element may also excise itself by this means into a circular DNA element. To test whether (a) the mobile element excised itself into a circular DNA product and (b) whether it did so under conditions other than salt selection, we designed a PCR assay that amplified different sequences, depending on whether the mobile element was integrated within mutS or was excised as a circular DNA element (Fig 2c).

We found that V. splendidus 12B01 exhibited low levels of mobile element excision during growth in standard rich media (Fig 2d, S3d). Lineages that had lost the mobile element (e.g., lineage 2), produced PCR products exclusively from the scarred mutS host sequence (Fig 2d). DNA sequencing of each PCR product indicated that mobile-element excision in the ancestral strain under standard laboratory conditions resulted in the same two-base-pair frameshift mutation we observed in the hypermutators and that these two base pairs were located in a circular DNA element containing the mobile element.

On the basis of qPCR results (see Materials and Methods), we estimated that the
The frequency of excision in the original ancestral strain was $7.95 \times 10^{-5} \pm 1.25 \times 10^{-5}$ (± SE; approximately 1/12,500 genomes) during the exponential growth phase and $1.27 \times 10^{-4} \pm 1.07 \times 10^{-5}$ (± SE; approximately 1/8,000 genomes) during the stationary phase (Fig 2e, S3d). Thus, this mobile element appeared to have excised itself in a small fraction of the population during all stages of growth and perhaps did so slightly more often during the stationary phase (Student's dependent t-test, $t = -8.36$, $P = 2.39 \times 10^{-6}$).

**Mobile elements within mutS exist across Vibrio and Beta- and Gammaproteobacteria**

To establish whether this mobile element was unique to *V. splendidus* 12B01, we looked for similar elements in other bacterial strains. Using the mobile element's putative integrase as a search query, we performed a protein BLAST search of bacterial genomes to identify related integrases. We then screened for those related sequences also adjacent to *mutS*. From all bacterial genera that contained a genome identified by our search, we manually checked representative genomes to confirm that the integrase-like sequences were part of a putative mobile element present within the bacterial host *mutS* sequence. To determine that these elements had inserted themselves into host *mutS* sequences, we checked for upstream host-derived *mutS* start sequences homologous to *mutS* genes from other closely related strains (Table S2).

We initially confined our search to the genus *Vibrio*, identifying a number of strains with putative mobile elements in *mutS*; many of these were closely related to 12B01 (Fig 3a, S4a). Within close relatives (>98% similarity in 16S rRNA), the phylogeny of hosts did not match the phylogeny of the elements. We found evidence for horizontal mobile element transfer between strains 12F01 and 13B01, which had nearly identical mobile element sequences (>99.99% nucleotide similarity for >19 kb; Fig 3a, Table S3). It appeared to be a transfer of only the mobile element, as adjacent genes followed the overall divergence of the host strains (Fig S4c, Table S3). Other, less closely related *Vibrio* strains had elements of varying length and structure in *mutS*. For example, *Enterovibrio norvegicus* FF-162 appeared to have a 7 kb element with few genes (Fig S5a). Using a PCR strategy similar to the one we used with 12B01, we detected no excisions in liquid FF-162 cultures (Fig 3c), which could indicate (a) that the 7 kb element may have degraded over time, so that it could no longer excise itself from the host genome or (b) that its excision requires specific conditions we did not test.

We then broadened our search to all bacteria whose genome sequences are available. The most similar sequences from our BLAST search (before screening for sequences near *mutS*) came from a diverse set of strains in Beta- and Gammaproteobacteria; these sequences fell predominantly adjacent to *mutS*. For example, all 100 of the 100 most-similar sequences from the full NCBI nucleotide database appeared in putative mobile elements within host *mutS* sequences. The bacteria containing these sequences included opportunistic pathogens of human lungs, skin, and urinary tract, as well as pathogens of major crops (Fig 3b, Table S2).

The putative mobile elements varied in length (4 kb to >150 kb) and structure. For example, some of the elements appeared to be prophages and contained multiple open reading frames annotated to phage proteins (e.g., *Burkholderia multivorans* CF2, Fig S5a). A phylogeny of putative mobile elements based on the elements' integrase genes indicated that elements within bacteria from the same phyla largely clustered together (Fig S4b). But as among close relatives of 12B01, the phylogeny of the elements often did not reflect the phylogeny of the host bacteria, implicating possible horizontal transfer of mobile elements between diverse bacteria.
The unifying feature of these elements was the location of their insertion sites near a well-conserved amino acid motif (HTPMMQQ) that helps MutS bind mismatched DNA base pairs [(38)Fig 3d, S5b]. We found other putative mobile elements that had integrase sequences very similar to that of 12B01’s mobile element but that occurred in genomic locations other than mutS. We also found elements within the same amino acid motif in mutS with dissimilar integrase sequences. For instance, the genome of Pseudomonas putida F1 contains a very similar integrase to that of 12B01’s mobile element (amino-acid-sequence similarity of > 88%) but in a completely different genomic location. Meanwhile, P. putida F1's mutS sequence does contain a putative mobile element in the same amino acid motif in which we found 12B01's mobile element, but its integrase is quite different (amino-acid-sequence similarity of < 24%).

We experimentally tested a subset of strains outside of Vibrio (E. coli 536, B. multivorans CF2, and P. putida F1) for element excision and frameshift mutations. We chose these strains because the hosts are scientifically important and phylogenetically diverse and the elements are structurally disparate (Fig S5a). Using a PCR assay design similar to what we used for V. splendidus 12B01, we detected excision in all three strains (Fig 3c). An additional PCR product for the postexcision sequence (attB) of P. putida F1 came from a nonspecific binding site, as confirmed by DNA sequencing. Sequencing of the postexcision sequences indicated that, unlike in 12B01, excision did not leave a two-base-pair deletion in the mutS sequence. We therefore inferred that mutS was intact and functional, regardless of whether these mobile elements were integrated or excised.

**DISCUSSION**

**A mobile element modulates mutation rate in V. splendidus 12B01**

Although theoretical and empirical evidence suggests that many bacteria have variable mutation rates, we know of few mechanisms for generating this variability. Using whole-genome sequencing, we discovered a novel mechanism of hypermutation in V. splendidus 12B01—a mechanism brokered by the excision of a mobile element found within a conserved amino acid motif near the start of mutS. By residing in the start of this gene, the mobile element might regulate mutS in two ways. One way is to provide, when integrated, an entirely new amino acid start sequence and upstream regulatory region for mutS. This mobile element–provided regulatory region might respond to different environmental and cellular cues than the original host sequence does, potentially altering mutS expression patterns. Another way, exhibited in our hypermutator lineages, is to inactivate the mutS coding sequence by excision and a two-base-pair deletion. In 12B01, the introduction of a frameshift mutation by mobile element excision adds a new mechanism for mutS to act as a contingency gene (27). In this case, mutability in mutS is brokered by the activity of a mobile element, not constitutive mutations, thereby allowing 12B01 to rapidly switch to a hypermutator genotype. Such co-option of prophages or mobile elements for regulating host genes has been called "active lysogeny" and might be widespread across bacteria (39).

One plausible model consistent with our mutation results is one in which the mobile element within mutS reversibly excises itself from the genome, producing a variable and characteristic mutation rate. In our mutator lineages that retained the mobile element, mutation
rates were higher and more variable than would be expected if their mutation rate were stable. This variance stemmed primarily from transition mutations, which are more affected by changes in mutS than are transversion mutations (Fig 1c) (20). Further, although our mutator lineages retained the mobile element, they showed ratios of transition to transversion mutations very similar to those of our hypermutators, particularly in rounds of selection with more mutations. These ratios were also much higher than those between 12B01 and other closely related isolates of V. splendidus (Fig 1, S2a). Thus, it is possible that 12B01’s mobile element may have temporarily excised itself during the growth of our mutator lineages, giving rise to characteristic mutation profiles, but then reintegrated itself shortly afterward, limiting the total number of mutations.

Our results do not directly demonstrate reintegration of this mobile element after excision. Because we were unable to genetically modify the bacterium or isolate the mobile element in its circular form, we could not introduce genetic reporters or transform naive strains. We can, however, see evidence from isolates closely related to 12B01 that this element is capable of transferring horizontally between carrier and naive cells and insert itself into a host genome—also consistent with the hypothesis that these mobile elements can reinte...
results do not preclude the possibility that excision of this mobile element is regulated by an unknown cellular cue, perhaps linked to stress. For example, studies in other bacteria have identified mechanisms that link mutation rate (41) and mobile element recombination (42) to the SOS response.

From the mobile element's perspective, the phenomena we observed might have several benefits. The most compelling benefit might be that the bacterial host depends on the mobile element to prevent rapid accumulation of deleterious mutations. Any bacterium that permanently loses the mobile element, like our hypermutators, would bear a heavy cost in fitness. Although our experimental selection favored two hypermutator lineages, we expect that the burden of deleterious mutations would eventually decrease fitness, particularly in natural environments. Furthermore, disrupting mutS and the mismatch repair pathway increases rates of recombination (43-45). Thus, when this mobile element excises and invalidates mutS, it may prime its host cell for reintegration of the same mobile element by recombination at the now vacated homeologous (having a similar nucleotide sequence) attachment site in the host genome. These two factors—dependence and priming for recombination—might stabilize the interaction between host and mobile element.

**Widespread mobile elements within mutS in Beta- and Gammaproteobacteria**

Although mutS is well-conserved (46) and well studied, the presence of putative mobile elements inserted into this gene—as opposed to adjacent to mutS (30)—has not been reported. We identified putative mobile elements within mutS in a wide variety of Beta- and Gammaproteobacteria from human and environmental sources, including important human and agricultural pathogens (e.g., bacteria that cause pneumonia, urinary tract infections, and fire blight crop disease). We also found evidence that these elements are horizontally transferred between diverse bacteria and that multiple, possibly independent, families of putative mobile elements have taken advantage of the well-conserved mutS gene.

Although we observed mobile element excision in *E. coli* 536, *B. multivorans* CF2, and *P. putida* F1, we did not find that excision left a scar in these bacteria, indicating that scarring might be specific to *V. splendidus* 12B01. Element excision in *E. coli* 536, *B. multivorans* CF2, and *P. putida* F1 might nevertheless alter mutS expression in these bacteria. As in 12B01, the integrated mobile element in these strains provides an independent start sequence and promoter region for each bacterium's mutS gene. But instead of inactivating it, excision returns mutS to control of the bacterium’s own mutS promoter. Thus, excision switches control of mutS between two regulatory regions, which might encode different levels of expression or respond to different environmental and cellular cues. Further study into these strains could reveal whether these elements alter mutation rates and drive adaptation in bacteria other than *V. splendidus* 12B01.

**MATERIALS AND METHODS**

**Serial salt selection**

As part of a larger effort to characterize the microbial ecology of the coastal ocean, we isolated *V. splendidus* 12B01 from seawater collected at the Plum Island Estuary Long-term Ecological Research site (Table S5, (47)). Cultures were grown at room temperature with shaking (200 rpm) in lysogeny broth (LB) supplemented with 0.5 M NaCl unless otherwise specified.

We prepared solid media plates with a gradient of salinity so that we could select for
mutants of 12B01 with higher-than-normal salt tolerance. We made these plates by first elevating one edge of a 241 mm x 241 mm x 20 mm square culture plate by 5 mm and pouring a wedge-shaped, high-salinity (2.2 M NaCl) layer of LB. Once this high-salinity layer solidified, we then placed the plate on a flat surface, and poured a wedge-shaped layer of LB without additional NaCl. We allowed the plates to equilibrate for 48 h to allow diffusion from the high-salinity layer to the low-salinity layer, thereby establishing a salinity gradient.

To grow and select salt-tolerant mutants, we spread overnight cultures of V. splendidus 12B01 on one half of a salinity-gradient plate and incubated the plates for 48 h. We picked colonies that grew at higher salinities than the majority of cells and restreaked them at the same gradient position in the other half of the culture plate to confirm salt tolerance and prevent contamination with cells that were not salt tolerant. To further eliminate contamination and maintain selection pressure, we restreaked these colonies again on LB agar plates supplemented with salt of increasing concentration for each selection round (round 1, 0.7 M total NaCl; round 2, 0.9 M; round 3, 1.0 M; round 4, 1.1 M; round 5, 1.2 M). We then picked colonies from these final plates and used them to inoculate liquid LB of the same salinity as the plate media from which we picked the colonies. After incubating these liquid cultures overnight, we used them to extract DNA for genomic analysis, store a subsample at -80 °C with 15% glycerol, and start the next round of selection on new salinity-gradient plates. In total, we collected and analyzed eight independent lineages of 12B01 over five rounds of salt selection.

Whole-genome sequencing and analysis
To analyze genomewide mutations among these isolates, we used a Qiagen Genomic-tip 500/G kit to extract DNA from overnight liquid cultures. We fragmented genomic DNA by sonication with a Bioruptor (Diagenode) with 30 s cycles for 18 min. Illumina whole-genome sequencing libraries were prepared at MIT's BioMicroCenter and sequenced on a single Illumina HiSeq lane using 40 bp, paired-end reads. Average coverage across all lineages was 35x ± 16x (± SD).

We searched for SNP mutations as indicators of salt adaptation, using a custom Galaxy pipeline (48). We cleaned sequencing reads using FASTQ Groomer (49) and aligned reads to the wild-type reference genome of V. splendidus 12B01 (NCBI genome assembly GCA_000152765.1, Gordon and Betty Moore Foundation Marine Microbiology Initiative) using Bowtie 1.0.0 (50). We identified fixed SNPs (more than 80% of reads) using SNP Finder (49) and filtered these SNPs for ambiguous or erroneous mapping, in particular, reads mapping to the ends of contigs and deleted regions. By checking for shared SNPs, we confirmed that each strain from each selection round was the progeny of the previous selection round. To check that sequencing coverage across our isolate library did not bias our SNP calling procedure, we confirmed that read coverage did not trend with the number of SNPs called (Fig S2). We classified each SNP as a transition or transversion, coding or noncoding, and synonymous or nonsynonymous mutation. To identify deletion mutations, we searched alignments for regions of the genome that lacked read coverage for more than 100 bp.

To model the transition frequency best describing our SNP mutation data, we used a binomial model to calculate the likelihood (P) of each data point (e.g., x transition mutations out of y total mutations during a given selection round for a given lineage of 12B01) given a probability (p_t) that a mutation will be a transition.

\[ P(\text{binomial}(k \text{ transitions}, n \text{ total SNP mutations}, p_t)) = \binom{n}{k} p_t^k (1 - p_t)^{n-k} \]
Thus, the probability of the model given \( p_{ij} \) is the product of the probability of each data point:

\[
P(\text{model } p_{ij} | \text{data}) = \prod_i \binom{n_i}{k_i} p_{ij}^k (1 - p_i)^{n_i - k}
\]

**PCR and qPCR assays**

To establish whether mobile elements within \( \text{mutS} \) excised from the host chromosome, we used KAPA 2G Fast Ready Mix for all PCR reactions, applying the manufacturer's suggested reaction settings. We used KAPA SYBR FAST qPCR Master Mix for all qPCR experiments on a Roche LightCycler. To measure the frequency of mobile element excision, we employed a previously reported qPCR strategy (51). We created a dilution series of standards using DNA from lineage 2, which had 100% excision. We designed qPCR primers for attachment site attB (attB_12B01_qpcr145_fwd and attB_12B01_qpcr145_rev) and a nearby (~10 kb distant) genomic control locus (ctrlL_12B01_qpcr145_fwd and ctrlL_12B01_qpcr145_rev; Table S5). We then compared abundance measurements of attB and ctrlL from each of our samples against our 100% excision standards to obtain a relative frequency of mobile element excision.

**Phylogenetic analysis**

To search for other bacteria with similar mobile elements, we used the amino acid sequence of the integrase from 12B01's mobile element as a query for TBLASTN and BLASTP searches of the National Center for Biotechnology Information's (NCBI) nucleotide and protein databases for similar integrases. For each hit with an e-value less than or equal to \( 1 \times 10^{-6} \), we checked whether the hit lay within 300 bp of \( \text{mutS} \). For a phylogenetically diverse subset of these strains, we manually checked whether the integrase was part of a putative mobile element that orphaned an original \( \text{mutS} \) starting sequence (Table S2). To test for mobile element excision in a subset of these strains, we designed PCR assays as for 12B01 (Table S5).

To visualize the phylogenetic distribution of these elements, we first built a tree for 530 Vibrio genomes based on 52 ribosomal proteins that are well conserved across this genus (52). To improve the resolution of this tree for the clade of 12B01's close relatives, we trimmed the multiple sequence alignment using trimAl (53). We used iTOL to plot the tree and annotate the presence of mobile elements within \( \text{mutS} \) (54). We aligned integrase genes from the \( \text{mutS} \) mobile elements of four Vibrio strains closely related to 12B01 and inferred a neighbor-joined phylogeny of these genes using Clustal Omega (55). For strains across Beta and Gammaproteobacteria with mobile elements within \( \text{mutS} \), we downloaded each genome from NCBI and extracted each strain's 16S rRNA sequences using RNAmmer (56). We aligned 16S rRNA sequences from each strain using Clustal Omega (55) and inferred a maximum-likelihood tree using RAxML with 1,000 bootstraps (57).

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**REFERENCES**


FIGURES
Fig 1. Serial selection for salt tolerance identifies a hypermutator phenotype, with a distinct mutation profile, in *Vibrio splendidus* 12B01.

(a) We grew eight independent lineages on hypersaline media and sequenced genomes from each selection round. Strict SNP calling indicated that two hypermutator lineages had rapidly accumulated a large number of mutations, but all lineages had accumulated many more mutations (37–1,802) than the single mutation expected, given literature averages of spontaneous mutation (2).

(b) The number of new mutations varied greatly across selection rounds in both mutator and hypermutator lineages. (c) This variability was much higher than the expected Poisson distribution variance, although the disparity was much smaller among transversion mutations alone. (d) Selection rounds with more mutations tended to have larger ratios of transition versus transversion mutations; these ratios far exceeded averages from the literature (28), indicated by a dashed blue line and the transition-to-transversion ratio of 12B01 compared with a closely related strain, *V. splendidus* 12F01.
**Fig 2. Excision of a mobile element within mutS disrupts the mutS genetic sequence.**

(a) We identified a mobile element adjacent to mutS that was missing in both hypermutator lineages. Further inspection revealed that, when present, the mobile element appeared integrated within the mutS sequence, separating the original host-encoded mutS starting sequence. (b) When integrated, the mobile element provided a new start and upstream regulatory region to the mutS coding sequence. After excision, the mobile element left a two-base-pair frameshift deletion in the host's mutS sequence, resulting in a premature stop codon. (c) We designed a PCR assay to detect the excision of this mobile element. When the mobile element is integrated into the host mutS sequence, the left (attL) and right (attR) attachment site junctions of the mobile element and host genome are amplified. When the mobile element is excised, the rejoined host mutS gene (attB) and the circular excised mobile element (attP) are amplified. Expected amplicon lengths: attL-819 bp, attR-836 bp, attB-613 bp, attP-1042 bp. (d) We found that in rich media (LB), the mobile element excised itself at low frequency. Sanger sequencing of PCR products attB and attP confirmed the two-base-pair frameshift deletion in the host mutS sequence and the transfer
of these base pairs to a circularized mobile element. In hypermutator lineages (e.g., lineage 2), we could no longer detect the mobile element, only the scarred host mutS sequence. (e) qPCR assays indicated that the frequency of excision was approximately 1/10,000 genomes, with moderately higher excision frequency during stationary phase.

Fig 3. Mobile elements within mutS occur across Vibrio, Betaproteobacteria, and Gammaproteobacteria.

(a) Phylogeny of close relatives of 12B01 (>98% similarity in 16S rRNA). Strains with mobile elements within mutS are not a monophyletic clade, and comparison between host phylogenies
and mobile element phylogenies indicate that these elements have been horizontally transferred (dotted line). (b) Broader BLAST searches identified other bacterial genera that contained strains with mobile elements within \textit{mutS}. (c) Using a PCR assay similar to what we used for 12B01, we found mobile element excision in some, but not all, of a subset of these bacteria when grown to stationary phase. Sanger sequencing of the attB and attP PCR products from \textit{E. coli} 536, \textit{P. putida} F1, and \textit{B. multivorans} CF2 indicated that mobile element excision in these bacteria did not result in any deletions. Expected amplicon lengths: \textit{E. norvegicus} FF-162 attL-894 bp, attR-891 bp, attB (hypothetical)-380 bp, attP-1405 bp; \textit{E. coli} 536 attL-404 bp, attR-416 bp, attB-393 bp, attP-427 bp; \textit{P. putida} F1 attL-601 bp, attR-583 bp, attB-596 bp, attP-588 bp; \textit{B. multivorans} CF2 attL-811 bp, attR-723 bp, attB-451 bp, attP-1083 bp. (d) These mobile elements were all integrated into the HTPMMQQ amino acid motif in MutS, although the precise location varied.
Chapter 3

Using propidium monoazide sequencing (PMA-seq) to develop data-driven best practices in fecal microbiota transplantations

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Supplementary Information is in Appendix B.
ABSTRACT

Fecal microbiota transplantation is a compelling treatment for recurrent *Clostridium difficile* infections, with potential applications against other diseases associated with changes in gut microbiota. But variability in fecal bacterial communities—believed to be the therapeutic agent—can complicate or undermine treatment efficacy. To understand the effects of transplant preparation methods on living fecal microbial communities, we applied a DNA-sequencing method (PMA-seq) that uses propidium monoazide (PMA) to differentiate between living and dead fecal microbes, and we created an analysis pipeline to identify individual bacteria that change in abundance between samples. We found that oxygen exposure degrades fecal bacterial communities, whereas freeze-thaw cycles and lag time between donor defecation and transplant preparation had much smaller effects. Notably, the abundance of *Faecalibacterium prausnitzii*—an anti-inflammatory commensal bacterium whose absence is linked to inflammatory bowel disease—decreased with oxygen exposure. Our results suggest that (1) some current practices for preparing microbiota transplant material adversely affect fecal microbial content, and (2) PMA-seq could serve as a valuable tool to inform best practices and evaluate the suitability of clinical fecal material.
INTRODUCTION

Fecal microbiota transplantation—the transfer of fecal microbes from a donor to a patient—has emerged as an extremely effective therapy for recurrent infections of *Clostridium difficile* (~90% cure rate), a common hospital infection that kills nearly 30,000 patients each year (1,2). Fecal transplants also hold promise for treating other gastrointestinal diseases, like inflammatory bowel disease and irritable bowel syndrome, and even systemic diseases linked to the gut microbiota, like obesity (3). To date, however, fecal transplants have been proven effective only for recurrent *C. difficile* infections, and clinical trials using fecal microbiota transplants to treat inflammatory bowel disease (4–9), irritable bowel syndrome (10,11), and insulin resistance (12) have produced mixed results.

One of the greatest challenges in fecal microbiota transplantation is variability of therapeutic material, which stems from both biological variation and variation introduced by sample handling. Unlike pharmaceuticals, human stool—its microbial and chemical content—varies widely between people and between samples from the same person (13,14). Many diseases, however, are associated with specific microbes and chemicals (13), suggesting that the composition of a fecal transplant could influence clinical efficacy and side effects. Living microbes are believed to be the therapeutic agent in fecal microbiota transplants (15), since these microbes colonize the recipient patient, potentially leading to lasting changes in the patient's gut bacterial community (16). As a result, transplant preparation, transportation, and administration—which may kill certain bacteria—could affect clinical efficacy, and best practices are actively debated (17). For example, although current standard practices involve aerobic preparation, exposure to oxygen is known to alter the viability of fecal bacteria, given that most species are obligate anaerobes (17).

In the case of recurrent *C. difficile* infection, such presumed aerobic degradation apparently has little impact on clinical efficacy (2). For other indications, however, where the therapeutic component is poorly understood, variance in living bacteria could significantly affect clinical efficacy. For example, fecal microbiota transplant trials in ulcerative colitis showed fourfold differences in efficacy among different donors, suggesting that specific bacterial communities play a crucial role (9).

We sought to characterize the impacts of typical transplant preparation methods on fecal microbial communities. We found that freeze-thaw cycles and lag time did not greatly alter the community composition of living bacteria, but oxygen exposure during sample mixing did have a significant effect on the viability of different bacteria. In addition, our results validate PMA-seq as a useful tool for comparing fecal microbiota samples.

MAIN

To understand how transplant handling might alter fecal microbial communities—which may affect therapeutic efficacy—we investigated three potentially sources of degradation: oxygen exposure during homogenization, freeze-thaw cycles during transplant storage and transport, and lag time between defecation and transplant preparation. For each experiment, we prepared two separate stool samples from the same donor and divided each sample into subsamples for analysis under different transplant preparation methods, thus controlling for variance across fecal samples. After transplant preparation, we then further divided each subsample into three technical
replicates. We used qPCR to estimate total 16S rRNA abundance. We then and evaluated the replicates' resulting microbial composition using standard 16S rRNA sequencing (18,19) and PMA-seq, which selectively sequences DNA from bacteria with intact cell membranes—a proxy for living cells (20–22).

From our sequencing data, we generated two tables of operational taxonomic units (OTU), one with 1,362 OTUs clustered at 97% similarity (Data S1) and another with 77 high-confidence OTUs—ones present in all sequencing samples—clustered at 100% similarity (Data S2).

**Oxygen exposure during fecal homogenization alters the composition of living fecal bacteria**

To test the effects on fecal bacteria of oxygen exposure during stool sample homogenization, we prepared subsamples from two stool samples from a single donor using five different procedures, each with a different level of oxygen exposure (Materials and Methods; Fig S1). To ensure that any patterns we observed from PMA-seq were not procedural artifacts, we also sequenced PMA-seq controls that replaced PMA with water for some transplant preparations (see Materials and Methods; Fig S2).

We found that total 16S rRNA abundance decreased with increasing exposure to oxygen, indicating that oxygen exposure decreases the number of viable cells (Fig S3). This degradation was reflected in both untreated replicates—which captured DNA from living cells, dead cells, and free-floating DNA not associated with a cell—and replicates treated with PMA—which captured only DNA within living cells.

To understand which bacteria were affected, we analyzed 16S rRNA sequencing results. Standard 16S rRNA sequencing indicated a slight increase in beta diversity (Bray-Curtis dissimilarity) with increasing oxygen exposure, but these differences were much clearer in the PMA-seq data across all comparisons (Fig 1a, S4). Comparison with controls confirmed that the changes we observed were largely due to PMA's exclusion of unprotected DNA, not other steps in the PMA-seq process (Fig S2). These results confirmed that PMA-seq more clearly reflects changes in bacterial composition due to differential oxygen exposure than does standard 16S sequencing.
Fig 1. **PMA-seq reveals changes in bacterial community composition with oxygen exposure.**
(a) Beta diversity (Bray-Curtis dissimilarity) between subsamples prepared with varying levels of oxygen exposure indicates that PMA-seq detects higher dissimilarity than standard 16S sequencing. (b) Beta diversity between standard 16S rRNA sequencing and PMA-seq results also reflects the degree of oxygen exposure. Data were generated from the same stool sample and same oxygen preparation, sequenced using either standard 16S rRNA sequencing or PMA-seq. (c–d) PMA-seq also detects changes in the abundance of individual OTUs to a greater extent than standard 16S sequencing. (c) OTUs from the genera *Faecalibacterium* and *Megamonas* largely decreased in relative abundance when exposed to oxygen, while those from *Bacteroides* increased. This signal was stronger in results from PMA-seq. Each point represents the mean
change in relative abundance of a single OTU across three technical replicates. (d) Our analytical method identified individual OTUs that changed significantly in relative abundance between different oxygen preparations, many of which would have not been detected using standard 16S sequencing. Abbreviations of transplant preparation methods: ANC, anaerobic + cysteine; ANA, anaerobic; AEC, aerobic + cysteine; AER, aerobic; ARS, aerobic + sparging.

Given that PMA-seq reflects only living bacteria, and standard 16S rRNA sequencing ought to more closely reflect the entire bacterial community, we hypothesized that comparing sequencing results from each of these methods might provide a proxy for how much a bacterial community has been degraded. We found that beta diversity values between 16S rRNA sequencing and PMA-seq results from the same subsample increased with greater oxygen exposure (Fig 1b). This result suggests that comparing standard 16S rRNA sequencing and PMA-seq results could provide a proxy for the degradation of living bacteria within fecal material.

Our PMA-seq results also shed light on how specific bacterial taxa respond to short-term oxygen exposure, which could ultimately affect therapeutic efficacy. Oxygen appeared to have the greatest negative effect on the abundances of bacteria from the phylum Firmicutes (Fig 2). In particular, two of the four most abundant genera from one donor—Megamonas and Faecalibacterium (sp. prausnitzii)—uniformly decreased in abundance in both stool samples tested (Fig 1c, S4; comparison of anaerobic + cysteine and aerobic preparation, two-tailed Student’s t-test, \( t = 6.293, P = 0.0033 \) and \( t = 7.494, P = 0.0017 \), respectively). Little is known about the role that Megamonas plays in the gut microbiota (23,24). Faecalibacterium prausnitzii is believed to have anti-inflammatory properties in the gut and to help moderate or prevent illnesses like inflammatory bowel disease (25,26). F. prausnitzii produces short-chain fatty acids, which help regulate host immune cells (27) and are the preferred energy source of colonic epithelial cells (28). Thus, by decreasing the viability of F. prausnitzii cells during oxygen exposure, we may be compromising the therapeutic value of fecal transplant material.
Fig 2. Responses to oxygen exposure cluster taxonomically.
Phylogeny of high-confidence OTUs with their changes in abundance from ANC to AER transplant preparations. PMA-seq revealed clustered responses to oxygen exposure. Firmicutes, particularly those from *Megamonas* and *Faecalibacterium*, decreased in abundance with oxygen exposure, while those from *Bacteroides* increased. Branches with greater than 90% bootstrap support are annotated.

We also identified oxygen-resistant bacteria—such as *Bacteroides, Parabacteroides, Barnesiellaceae*, and *Rikenellaceae*—that increased in relative abundance (Fig 1c, 2, S4). We hypothesized that these increases were compositional effects—which arise because we are measuring proportions rather than counting directly—since we expected little to no growth during our brief sample handling and freezer storage. Indeed, many of these apparent increases were flattened by normalizing the data to total community size (Fig S3). OTUs from the genus *Bacteroides* were most often identified as oxygen resistant in our data, which aligns with previous evidence that some *Bacteroides* species can survive or even grow during short periods of oxygen exposure (29).
To identify individual OTUs that significantly changed in abundance between different transplant preparations, we created an analysis pipeline based on texmex (30), which models microbial community analyses using a Poisson log-normal distribution (see Materials and Methods, Fig 1d, S5). Using this pipeline and our table of high-confidence OTUs, we found that OTUs that decreased significantly in abundance during oxygen exposure most often belonged to the genera *Faecalibacterium*, *Megamonas*, and *Bifidobacterium*, mirroring the overall taxonomic shifts (Table S1). OTUs that increased often belonged to *Bacteroides*, including *B. ovatus*, *B. uniformis*, and *B. caccae* (Table S1).

Although responses to oxygen exposure by individual OTUs largely reflected the patterns of larger taxonomic groups, PMA-seq detected some heterogeneity in these responses (Fig S6). For example, the most abundant OTUs within the genus *Oscillospira* did not all exhibit the same dynamics in response to oxygen exposure (Fig S6).

**Freeze-thaw cycles and lag time have smaller effects on fecal bacterial composition**

In addition to oxygen exposure during homogenization, we also used PMA-seq to evaluate the effects of freeze-thaw cycles and lag time—common concerns when working with gut microbiota samples. For freeze-thaw experiments, we prepared two separate stool samples from the same donor according to our anaerobic + cysteine protocol (see Materials and Methods) and allowed them to freeze and thaw for the indicated number of cycles. For lag time experiments, we left subsamples of two stool samples in a biosafety cabinet for an allotted time before preparing them with our anaerobic + cysteine protocol. As we had done for oxygen exposure, we repeated each of these experiments with two separate stool samples from a single donor.

We found that bacterial community composition remained largely stable in response to freeze-thaw cycles (Fig 3, S7) and was not drastically altered by lag time (Fig 4, S8), even after as many as 20 freeze-thaw cycles or 7 hours of lag time. Beta diversity results from both experiments indicate that the communities as a whole did change with more freeze-thaw cycles (Fig 3a) and longer lag times (Fig 4a), but the changes with freeze-thaw cycles and lag time were smaller than those observed in our oxygen exposure experiments (Fig 1). Beta diversity between 16S sequencing and PMA-seq results reflected the number of freeze-thaw cycles (Fig 3b) but not the duration of lag time (Fig 4b). These results further suggest that comparing these two sequencing methods might provide a proxy for overall community disturbance but may not capture all types of stress, particularly stresses affecting all bacteria equally, which would not be captured by measurements of relative abundance.
Fig 3. PMA-seq registers little alteration of the living bacterial community with more freeze-thaw cycles.
(a) Beta diversity between subsamples from different freeze-thaw preparations reflected community perturbation, but the dissimilarity values were lower than for oxygen exposure. (b) Beta diversity between standard 16S rRNA sequencing and PMA-seq results reflected the number of freeze-thaw cycles. (c) OTUs of three dominant genera did not show uniform reactions to freeze-thaw cycles.
Fig 4. PMA-seq registers little alteration of the living bacterial community with longer lag time.

(a) Beta diversity between subsamples from different lag time preparations also reflected community perturbation, but the dissimilarity values were lower than for oxygen exposure. (b) Beta diversity between standard 16S rRNA sequencing and PMA-seq results did not reflect longer lag times, suggesting that lag time did not greatly alter overall community composition. (c) OTUs of three dominant genera did not show uniform reactions to different lag times.
From our normalized data, it appears that freeze-thaw cycles had a small negative effect on the total abundance of living bacteria, with much of that effect occurring after one freeze-thaw cycle (Fig S9). Our fecal microbiota preparations contained glycerol as a cryoprotectant, which ought to have dampened any effect freeze-thaw cycles would have had on bacterial composition and overall abundance. The lack of strong changes in community composition suggests that stress due to freeze-thaw cycles with this preparation method is less specific to certain taxa than is oxygen stress.

Longer lag times appeared to reduce total bacterial abundance (Fig S10). We suspect that the increase in overall community size in stool sample 6 (Fig S10) is an artifact of handling: since we left subsamples completely exposed in a biosafety cabinet, they dried considerably during the experiment, resulting in thicker, more rigid materials. We imagine that lag time had little effect on the composition of bacteria because this formation of a dried, stiff outer layer may have shielded the inner microbial community from the effects of oxygen, while degrading all microbes caught within the outer layer.

Reflecting our diversity analysis, few individual OTUs or taxa were reliably identified as significantly decreased or increased in abundance with different numbers of freeze-thaw cycles or length of lag time (Fig S5, Table S1). One OTU of *Bifidobacterium* did appear to decrease with freeze-thaw cycles, while some OTUs from *Faecalibacterium* and *Megamonas* were sensitive to lag time, potentially because of oxygen exposure (Table S1). Our statistical methods revealed that the distributions of changes in abundance of individual OTUs between freeze-thaw and lag-time preparations resembled our null model, further indicating that the community was largely intact (Fig S5). In contrast, the same distributions for different oxygen preparations diverged dramatically (Fig S5), emphasizing a shift in community composition.

Our results suggest that moderate numbers of freeze-thaw cycles and moderate lag times—particularly if stool samples are covered—do not alter fecal microbial communities as much as oxygen exposure during homogenization and may in fact not greatly affect therapeutic efficacy.

**DISCUSSION**

Our experiments on preparation methods for fecal microbiota transplants suggest that PMA-seq could provide a valuable tool to assess how we identify, prepare, and administer fecal microbiota transplants for recurrent *C. difficile* infections. As we learn more about specific microbe-host interactions that trigger or ameliorate diseases other than *C. difficile* infection, PMA-seq could help practitioners develop and refine new applications of fecal microbiota transplants and methods for manipulating the human gut microbiota.

We observed degradation of the microbial community with increasing oxygen exposure, indicating that therapeutic efficacy for fecal microbiota transplants—particularly in diseases other than *C. difficile* infection—may be best preserved by maintaining anaerobic conditions during sample processing (for example, by using anaerobic chambers and oxygen-free buffers) and storage (for example, by using oxygen-impermeable containers and monitoring for oxygen exposure). Freeze-thaw cycles and lag time did not greatly alter the microbial community, which could indicate that flexibility in these aspects of treatment processing and delivery might not greatly affect downstream efficacy.
MATERIALS AND METHODS

Sample preparation
To prepare fecal microbiota transplants for PMA-seq analysis, we used modified protocols based on standard practices at OpenBiome (http://www.openbiome.org/), the largest stool bank in the United States. All human stool collections and subject consent procedures were reviewed and approved by the Institutional Review Board of the Massachusetts Institute of Technology, approval number 1510271631. All participants provided written consent.

To examine how oxygen exposure alters the fecal microbial community, we used PMA-seq to evaluate stool samples processed in different oxygen conditions. We prepared two stool samples from a single donor with five different fecal microbiota-preparation protocols with varying levels of oxygen exposure (Fig S1). We transferred each stool sample into an anaerobic chamber (Coy) within 30 min of passage. We split the stool into four 30 g subsamples. For each of these four subsamples, we prepared the fecal microbiota transplant by varying two factors: the buffer used to homogenize the stool and the environment in which the stool was homogenized. We used two similar buffers: one of 50% glycerol, 50% saline solution (0.9% NaCl), and 0.1% L-cysteine buffer and another of 50% glycerol plus 50% saline solution. L-cysteine is a reducing agent, which reacts with oxygen to remove it from solution. For the homogenization environment, we homogenized the stool either within the anaerobic chamber or in ambient aerobic conditions. For subsamples prepared in anaerobic conditions, we prerduced each homogenization buffer by leaving it in the anaerobic chamber for at least 48 hr. This procedure led to four different preparations: anaerobic + cysteine, anaerobic, aerobic + cysteine, and aerobic. For the final preparation (aerobic + sparging), we then placed half the aerobic fecal homogenate into a sealed glass media bottle and sparged the homogenate with air for 30 min.

We homogenized all 30 g subsamples in separate Whirl-Pak filter bags (Nasco) with 150 ml buffer. We transferred each filter bag into an easyMix automated homogenizer (AES Chemunex) and homogenized the contents for 60 s. We then transferred aliquots of 498.75 μl of stool homogenate into screw-cap tubes, which we sealed in secondary screw-top glass containers and froze at −80°C to await further processing.

To test the effects of freeze-thaw cycles on fecal bacteria, we subjected fecal microbiota transplants to 0, 1, 5, and 20 freeze-thaw cycles. For this experiment, we prepared two stool samples from the same donor, following our anaerobic + cysteine protocol. We prepared six tubes of fecal homogenate from each of these stool samples as our zero freeze-thaw-cycles subsample preparation, and the rest we transferred to a freezer at −80°C for at least 3 h. We then allowed these subsamples to thaw at room temperature for 30 min before returning them to the freezer for 3 h for the indicated number of cycles.

We also tested the effects of lag time on the fecal microbiota. For these experiments, we transferred each of two stool samples to an anaerobic chamber and split each sample into five subsamples of 50 g each. One subsample was processed immediately as a zero time point. We removed the remaining subsamples from the anaerobic chamber and transferred them to a biosafety cabinet. We then prepared each subsample after the indicated exposure time had lapsed (0.5, 1, 3, and 7 h; 1 h is a standard limit at OpenBiome). At that time, we then prepared each subsample according to our anaerobic + cysteine protocol.
PMA to exclude unprotected DNA
To identify the living bacteria in each fecal microbiota preparation from all three experiments, we used PMA to exclude DNA from bacteria with compromised membrane structure, using the manufacturer's suggested protocol. We further divided subsamples from all experiments into six technical replicates, three of which we analyzed using PMA-seq and three of which we analyzed using standard 16S sequencing. For the PMA-seq replicates, we added 1.25 µl of 20 mM PMA dye (Biotium) to each aliquot of stool homogenate, to a final volume of 500 µl and final concentration of 50 µM PMA. We covered aliquots in aluminum foil and incubated them at room temperature for 5 min, vortexing every minute. We then removed the aluminum foil and photolysed the aliquots on ice under an LED light (Taotronics TT-AL09) for 30 min, rotating them every 10 min. After photolysis, we extracted DNA using a PowerSoil DNA extraction kit (MoBio). In parallel, we also extracted DNA from unaltered aliquots of fecal homogenate for standard 16S rRNA sequencing. To ensure that any signal we observed in the PMA-seq results did not come from incubation and photolysis, we also ran controls that replaced 1.25 µl of 20 mM PMA dye with water and underwent the same procedure as for PMA-seq.

Illumina library preparation and analysis
From these PMA-treated and untreated DNA samples, we then prepared 16S rRNA libraries using a two-step PCR protocol (19) to identify bacterial community composition. First, we quantified extracted DNA concentrations using a standard SYBR Green qPCR protocol with Phusion polymerase (New England Biolabs; used for all PCR reactions) and primers PE16S_V4_U515_F and PE16S_V4_E786_R (Table S2). We diluted all DNA samples to the concentration of the most dilute DNA sample and used 2 µl of each DNA sample for a PCR reaction with primers PE16S_V4_U515_F and PE16S_V4_E786_R (Table S2) and a program of 98°C for 30 s [98°C for 30 s, 52°C for 30 s, 72°C for 30 s] for 20 cycles, 4°C hold. For each DNA sample, we ran four 25 µl PCR reactions, which we then pooled, cleaned using SPRI AmpureXP beads, and eluted in 40 µl of elution buffer. For the second PCR reaction, we used 4 µl of the previous PCR product with primers PE-PCR-III-F and PE-PCR-IV-barcode in four 25 µl reactions with a PCR cycle of 98°C for 30 s [98°C for 30 s, 83°C for 30 s, 72°C for 30 s] for 7 cycles, 4°C hold. We pooled each set of four PCR reactions and cleaned the reactions using SPRI beads. We quantified library concentrations using another SYBR Green qPCR with primers BMC Final F and R (Table S2). We multiplexed DNA libraries so that they had equal DNA input. Libraries were sequenced on a single Illumina MiSeq lane set for paired-end, 250-base-pair reads.

We cleaned, merged, and filtered raw paired-end sequence reads using default parameters in UPARSE (34) and clustered the data into OTUs using a 97% identity threshold in QIIME (35). We used default QIIME settings to remove chimera sequences (36), pick de novo OTUs (37), and assign taxonomy (38,39). We assigned taxonomy to each OTU on the basis of the August 2013 release of the Greengenes rDNA database (38). We excluded OTUs that did not appear in at least two samples. We used qPCR results from the initial qPCR of each DNA sample to normalize our relative-abundance results.

Identifying OTUs that changed significantly in abundance
To identify OTUs that differed significantly in abundance between transplant preparations, we built an analysis pipeline that involved two primary steps: first, fitting OTU abundance data from each DNA sample to the Poisson log-normal distribution, which allows for cross comparisons, and second, fitting these comparisons to a generalized normal distribution, which provides a null model against which we could compare, and thereby identify, OTUs with significant changes in abundance.

For the first analysis step, we started by creating an OTU table with high-confidence OTUs. To create this table, we first clustered sequencing data using DADA2 and default parameters and procedures (40). We then filtered for OTUs that were present in all DNA samples, resulting in a table of 77 high-confidence OTUs, where the rarest OTU represented 0.15% of all reads. We fit OTU abundances from individual DNA samples to a Poisson log-normal distribution using pytextmex, a Python implementation of texmex (30). Previous studies have found that the Poisson log-normal distribution is an appropriate statistical model for microbial communities in many different habitats, including the human microbiome (30).

Using this model, we then calculated two metrics for each OTU, \( z \) and \( F \), which could then be compared across DNA samples to evaluate changes in an OTU's abundance in one sample compared with another. The \( z \) metric reflects each OTU's normalized abundance within a Poisson log-normal framework. The \( F \) metric reflects how each OTU's abundance compares with the abundances of all other OTUs in that DNA sample (i.e., the OTU's position in the Poisson log-normal distribution for that DNA sample). For a given OTU, comparisons of these metrics \( (\Delta z \text{ and } \Delta F) \) between two DNA samples thus reflected the change in an OTU's normalized abundance and the change in an OTU's position within the distribution of abundances from each sample (30).

In the second analysis step, we sought to build a statistical framework to identify OTUs with putatively significant \( \Delta z \) and \( \Delta F \) metrics between different transplant preparations. To build our null model, we used variation between technical replicates of the same transplant preparation and the same stool sample. Thus, for a given pair of transplant preparations (from the same stool sample), we first calculated all possible \( \Delta z \) and \( \Delta F \) values—for every OTU—among technical replicates of each preparation. We then fit these values to a generalized normal distribution (Fig S5) to make our "null model" of values expected from technical variation but not biological variation.

To identify OTUs whose \( \Delta z \) and \( \Delta F \) values were significantly greater or less than values expected from technical variation, we then calculated \( \Delta z \) and \( \Delta F \) values for each OTU across transplant preparations. We considered those OTUs whose \( \Delta z \) and \( \Delta F \) values fell outside of 95% of the null model distribution—values that had only a 5% chance of occurring in comparisons of technical replicates—as putatively significant (Fig S5). We further trimmed these OTUs to include only OTUs that had significantly greater (or smaller) \( \Delta z \) and \( \Delta F \) values in all pairwise replicate comparisons. We identified these final OTUs as changed significantly in abundance. Values of \( \Delta z \) were generally not affected by the overall abundance of each OTU (Fig S11), but \( \Delta F \) values were (Fig S12), meaning that \( \Delta F \) values may be more prone to bias.

Data can be accessed on the US National Center for Biotechnology Information SRA database under BioSample SAMN04962333.
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REFERENCES


Chapter 4

Longitudinal immunosequencing in healthy people reveals persistent T cell receptors rich in highly public receptors

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Supplementary Information is in Appendix C.
Abstract:

Background

The adaptive immune system maintains a diversity of T cells capable of recognizing a broad array of antigens. Each T cell’s specificity for antigens is determined by its T cell receptors (TCRs), which together across all T cells form a repertoire of millions of unique receptors in each individual. Although many studies have examined how TCR repertoires change in response to disease or drugs, few have explored the temporal dynamics of the TCR repertoire in healthy individuals.

Results

Here we report immunosequencing of TCR β chains (TCRβ) from the blood of three healthy individuals at eight time points over one year. TCRβ repertoires of all peripheral-blood T cells and sorted memory T cells clustered clearly by individual, systematically demonstrating that TCRβ repertoires are specific to individuals across time. This individuality was absent from TCRβs from naive T cells, suggesting that the differences resulted from an individual’s antigen exposure history, not genetic background. Many characteristics of the TCRβ repertoire (e.g., diversity, clonality) were stable across time, although we found evidence of T cell expansion dynamics even within healthy individuals. We further identified a subset of “persistent” TCRβs present across all time points. These receptors were rich in clonal and highly public receptors and may play a key role in immune system maintenance.

Conclusions

Our results highlight the importance of longitudinal sampling of the immune system, providing a much-needed baseline for TCRβ dynamics in healthy individuals. Such a baseline will improve interpretation of changes in the TCRβ repertoire during disease or treatment.

Background

T cells play a vital role in cell-mediated immunity, one branch of the adaptive immune response against foreign and self-antigens. Upon recognizing an antigen from an antigen-presenting cell, naive T cells activate and proliferate rapidly. This process stimulates an effector response to the immediate challenge, followed by generation of memory T cells, which form a lasting cohort capable of mounting more-efficient responses against subsequent challenges by the same antigen.

The key to the flexibility and specificity of T cell responses lies in the cells’ remarkable capacity to diversify their T cell receptor (TCR) sequences, which determine the antigens those cells will recognize. Most T cells display TCRs made up of two chains: an α and a β chain. Sequence diversity in these chains arises during T cell development, through recombination of three sets of gene segments: the variable (V), diversity (D), and joining (J) segments [1]. Random insertions and deletions at each genetic junction introduce still more diversity, resulting in a theoretical repertoire of $10^{15}$ unique receptors in humans [2]. Selective pressures during and after T cell
development, as well as constraints on the number of T cells maintained by the body, limit this diversity to an observed $10^7$ (approximately) unique receptors per individual [2–5].

This TCR repertoire forms the foundation of the adaptive immune response, which dynamically responds to disease. Each immune challenge prompts expansions and contractions of different T cell populations, and new T cells are continually generated. Substantial research interest has focused on these dynamics in the context of immune system perturbations, including in cancer [6–9], infection [10,11], autoimmune disorders [12,13], and therapeutic trials [8,14,15]. Observing changes in TCR populations not only uncovers cellular mechanisms driving disease, but can inform development of new diagnostics, biomarkers, and therapeutics involving T cells.

Less research has explored TCR dynamics in healthy individuals. Previous studies found that some TCRs remain present in individuals over decades [16,17], but these long-term studies may not directly relate to shorter-term events, such as diseases or treatments. Interpreting TCR dynamics when the immune system is challenged would be more straightforward if we had a clear picture of TCR dynamics in healthy individuals.

To help develop this picture, we report immunosequencing of peripheral TCR β chain (TCRβ) repertoires of three individuals at eight time points over one year. We focused on the TCRβ chain because, unlike the α chain, only one β chain can be expressed on each T cell [18], the β chain contains greater sequence diversity [19], and it more frequently interacts with presented antigens during recognition [20]. These factors suggest that TCRβ sequences should be sufficient to track individual T cells and their clones. Our analysis revealed overall individuality and temporal stability of the TCRβ pool. We also uncovered a set of temporally persistent TCRβs, which were more abundant, and shared across more people, than transitory TCRβs.

Results

**T cell receptor repertoires show individuality and stability through time**

To characterize the dynamics of T cell receptors in healthy individuals, we deeply sequenced the TCRβ locus of all T cells from peripheral-blood mononuclear cells (PBMCs) isolated from three healthy adults (for schematic of experimental design, see Figure 1a). We sampled each individual at eight time points over one year (Figure 1a). For three intermediate time points, we also sequenced flow-sorted naive and memory T cells from PBMCs (see Methods). Our deep sequencing effort generated ~21 million (+/− 6 million SD) sequencing reads and ~250,000 (+/− 100,000 SD) unique, productive TCRβs—which we defined as a unique combination of a V segment, CDR3 amino acid sequence, and J segment [21]—per sample. These values and other summary statistics per sample appear in Table S1. Most TCRβs had abundances near $10^{-6}$ (Figure S1), and rarefaction curves indicate that all samples were well saturated (Figure S2). This saturation indicates that our sequencing captured the full diversity of TCRβs in our samples, although our blood samples cannot capture the full diversity of the TCRβ repertoire (see Discussion).

We first examined whether previously observed differences among individuals were stable through time [7,22]. Looking at shared TCRβs (Jaccard index) among samples, we indeed found
that samples of PBMCs or memory T cells taken from the same individual shared more TCRβs than samples taken from different individuals (Figure 1b), and this pattern was consistent over one year. In adults, memory T cells are thought to make up 60–90% of circulating T cells [23,24], which aligns with the agreement between these two T cell sample types. In contrast, TCRβs from naive T cells did not cluster cohesively by individual (Figure 1b). As naive T cells have not yet recognized a corresponding antigen, this lack of cohesion might suggest one of two possibilities: (1) that before antigen recognition and proliferation, TCRβ repertoires are not specific to individuals or (2) the naive T repertoire is simply too diverse or too dynamic for individuality to manifest. We thus conclude that at the depth of sequencing and sampling of this study, individuality results from an individual’s unique antigen exposure and T cell activation history, which shape memory and total T cell repertoires.

We next examined patterns across samples from the same individual to understand TCR dynamics in healthy individuals. We observed only a minority of TCRβs shared among samples from month to month; indeed, samples of PBMCs at different months from the same individual typically shared only 11% of TCRβs (+/- 3.6% SD, range 5–18%) (Figure 1b).

Two factors likely played a role in the observed turnover of TCRβ repertoires: (1) changes in TCRβ abundances in the blood across time and (2) inherent undersampling of such a diverse system (see Discussion). Surveying peripheral blood immune repertoires undersamples at multiple points, including blood drawing, nucleic acid extraction, library construction, and sequencing. The resulting undersampling likely explained much of the low overlap of TCRβs among samples but simultaneously highlighted the significance of TCRβs shared across time points. To verify that patterns we observed were not artifacts of undersampling, we also analyzed a subset of high-abundance TCRβs (those ranked in the top 1% by abundance, see Methods, Additional File 1), which are less likely to be affected. In these TCRβs, we observed typical sharing of 63% (+/- 13.8% SD, range 35–88%) of TCRβs in PBMC samples across time (Figure S3a). PBMC and memory T cell samples (but not naive T cell samples) still clearly clustered by individual when only these TCRβs were considered (Figure S3a).

The frequencies of high-abundance TCRβs from each individual were largely consistent over time (Figure 1c). We found that abundances of the same TCRβs correlated within individuals over the span of a month (Figure 1d, S3b) and a year (Figure 1e, S3c). This correlation was particularly strong for abundant TCRβs (Figure S3b–c) whereas rare TCRβs varied more. This correlation held true in naive and memory T cell subpopulations, sampled across a month (Figure 1f–g). In contrast, correlation was much weaker among abundances of TCRβs shared across individuals (Figure 1h, S3d), again highlighting the individuality of each repertoire. We found that the proportion of shared TCRβs (Jaccard index) tended to decrease with longer time intervals passed between samples, although with a notable reversion in Individual 02 (Figure S4). We observed stable diversity (Figure 1i, S3e), clonality (Figure 1j, S3f), and V and J usage (Figure S5, S6; Table S2, S3) within individuals over time.

In the absence of experimental intervention, we observed complex clonal dynamics in many TCRβs, including cohorts of TCRβs with closely correlated expansion patterns (Figure S7). To avoid artifacts from undersampling, we looked for such cohorts of correlating receptors only in
high-abundance TCRβs (see Methods). In all individuals, many high-abundance TCRβs appeared together only at a single time point. We also found cohorts of high-abundance TCRβs that correlated across time points (Figure S7). Some of these cohorts included TCRβs that fell across a range of abundances (Figure S7a-b), while other cohorts were made up of TCRβs with nearly identical abundances (Figure S7c). Correlating TCRβs were not obviously sequencing artifacts (Table S4, Methods). These cohorts of closely correlated TCRβs indicate that even in healthy individuals whose overall TCR repertoire appears stable, underlying dynamics remain.

Taken together, these results revealed a diverse system, which nevertheless displayed consistent, unifying features differentiating individuals, plus longitudinal dynamics that suggested continual immune processes.

A persistent TCRβ repertoire contains elevated proportions of clonal, highly public TCRβs

During our analysis, we discovered a subset of TCRβs that was present across all eight PBMC samples from a single individual, a subset we called “persistent” TCRβs (Figure 2a). While approximately 90% of unique TCRβs observed over all of an individual’s PBMC samples occurred in only one sample, 0.3–0.8% of TCRβs occurred at all eight time points (Figure 2a). When considering individual samples, this pattern translated to 1–5% of TCRβs observed in each sample were persistent receptors (Table S5). When we considered only high-abundance TCRβs, the frequency of persistent TCRβ increased substantially (Figure S8a).

We hypothesized that these persistent TCRβs might be selected for and maintained by the immune system, perhaps to respond to continual antigen exposures or other chronic immunological needs.

In our data, we found multiple signatures of immunological selection acting on persistent TCRβs. The members of this persistent subset tended to have a higher mean abundance than TCRβs observed at fewer time points (Figure 2b, Table S6). We also observed that the number of unique nucleotide sequences encoding each TCRβ’s CDR3 amino acid sequence was generally higher for persistent TCRβs (Figure 2c, Table S7). This pattern of greater nucleotide redundancy varied across individuals and region of the CDR3 sequence (Figure S9a), but TCRβs with the highest nucleotide redundancy were reliably persistent (Figure S9b). Furthermore, we discovered that TCRβs occurring at more time points, including persistent TCRβs, shared larger proportions of TCRβs also associated with memory T cells (Figure 2d). Remarkably, 98% of persistent TCRβs also occurred in memory T cells, suggesting that almost all persistent T cell clones had previously encountered and responded to their corresponding antigens. We found a similar pattern in naive T cells, although the overall overlap was lower (50%), indicating that persistent TCRβs were also enriched in the naive compartment (Figure 2e). Persistent TCRβs did not show altered CDR3 lengths or VJ usage (Figure S10-S12). Like alpha diversity and clonality, the cumulative abundance of TCRβs present in different numbers of samples appeared stable over time and specific to individuals (Figure 2f). Surprisingly, although persistent TCRβs constituted less than 1% of all unique TCRβs, they accounted for 10–35% of the total abundance of TCRβs in any given sample (Figure 2f), further evidence that
these T cell clones had expanded. We observed similar patterns when analyzing only high-abundance TCRβs (Figure S8).

Taken together, these characteristics—persistence across time, higher abundance, redundant nucleotide sequences, and overlap with memory T cells—suggest immunological selection for persistent TCRβs. We therefore investigated whether persistent TCRβs coexisted with TCRβs having very similar amino acid sequences. Previous studies have suggested that TCRβs with similar sequences likely respond to the same or similar antigens, and such coexistence may be evidence of immunological selection [25,26].

To explore this idea, we applied a network clustering algorithm based on Levenshtein edit distance between TCRβ CDR3 amino acid sequences in our data [25–27]. We represented antigen-specificity as a network graph of unique TCRβs, in which each edge connected a pair of TCRβs with putative shared specificity. We found that TCRβs having few edges—and thus few other TCRβ with putative shared antigen specificity—tended to occur in only one sample, while TCRβs with more edges included a higher frequency of TCRβs occurring in more than one sample (Figure 3a, p < 10^{-5} for all three individuals by a nonparametric permutation test). This pattern indicates that TCRβs occurring with other, similar TCRβs were more often maintained across time in the peripheral immune system.

We next examined the association between persistent TCRβs—those shared across time points—and “public” TCRβs—those shared across people. Public TCRs show many of the same signatures of immunological selection as persistent TCRβs, including higher abundance [28], overlap with memory T cells [28], and coexistence with TCRs with similar sequence similarity [25]. To identify public TCRβs, we compared our data with a similarly generated TCRβ dataset from a large cohort of 778 healthy individuals [21] (Additional File 2). We found that the most-shared (i.e., most-public) TCRβs from this large cohort had a larger proportion of persistent TCRβs from our three sampled individuals (Figure 4a–b, Table S8, p < 10^{-5} for all three individuals by a nonparametric permutation test). Private TCRβs—those occurring in few individuals—most often occurred at only a single time point in our analyses. Interestingly, TCRβs that occurred at many but not all time points (i.e., 3–5 time points) were on average the most-shared (Figure S14a), but persistent TCRβs were specifically enriched in highly public TCRβs—here defined as those shared by over 70% of subjects in the large cohort (Figure 4c, S14b). The three most public TCRβs (found in over 90% of the 778-individual cohort) were found to be in the persistent TCRβ repertoires of all three individuals and were diverse in structure (Figure 4d).

Public TCRs are thought to be products of genetic and biochemical biases in T cell receptor recombination [29,30] and also of convergent selection for TCRs that respond to frequently encountered antigens [21,32]. To better understand the effects of biases during TCRβ recombination on receptor persistence, we used IGoR to estimate the probability that each TCRβ was generated before immune selection [33]. Similar to previous studies [30], the probability that a given TCRβ was generated correlated closely with publicness (Figure S15a). In our time series data, TCRβs that occurred at multiple time points tended to have slightly higher generation probabilities than TCRβs only observed once (Figure S15b), but persistent TCRβs did not have
higher generation probabilities than other receptors observed in more than one time point. In addition, more abundant TCRβs (both persistent and nonpersistent) did not have higher generation probabilities (Figure S15c–d). These results suggest that, unlike public receptors, persistent receptors and their abundances do not appear to result from biases in TCR recombination. The contradiction that public and persistent receptors are associated but only public TCRβs appear to be generated by recombination bias is possible because despite their association, these two TCRβ subsets are largely independent. Although the most public receptors are overwhelmingly persistent (Figure 4), they represent a tiny fraction of the persistent receptors in each individual. Thus, although these two subsets of the TCR repertoire—persistent and public—overlap and share many characteristics, they are also distinct, suggesting that they may play complementary roles in adaptive immunity.

Discussion

Our analyses revealed both fluctuation and stability in the TCRβ repertoire of healthy individuals, providing a baseline framework for interpreting changes in the TCR repertoire. We identified a number of consistent repertoire characteristics (e.g., diversity, clonality), which are known to be affected by immunizations, clinical interventions, and changes in health status [7,14,34]. These patterns differed among individuals across time, highlighting the role played by genetics [like human leukocyte antigen (HLA) type] and history of antigen exposure in shaping the TCR repertoire. We did not obtain HLA-type information from these three subjects, so the relative contributions of HLA type versus individual history remains unknown.

We further discovered a subset of persistent TCRβs that bore signs of immune selection. Persistent TCRβs tended to be more abundant than nonpersistent receptors, although this distinction is to a certain extent confounded by the fact that high-abundance receptors are also more likely to be detected in a given sample. Nevertheless, this circular logic does not detract from the immune system’s maintenance of specific dominant TCRβs across time. We further found that persistent TCRβs had higher numbers of distinct nucleotide sequences encoding each TCRβ. TCR diversity is generated by somatic DNA recombination, so it is possible for the same TCR amino acid sequence to be generated from independent recombinations in different T cell clonal lineages. Thus, coexistence of multiple clonal lineages encoding the same TCRβ amino acid sequence may reflect selective pressures to maintain that TCRβ and its antigen specificity. Similarly, the presence of many TCRβs similar to persistent TCRβs—as identified by our network analysis—could also result from selection for receptors that recognize a set of related antigens [20,36]. Previous studies using network analyses also found that public TCRβs tend to occur with similar TCRβs [25], further suggesting that both public and persistent TCRβs are key drivers of lasting immunity. In addition to using TCRβ sequencing to track TCRβs that proliferate in response to intervention, we propose that the three dimensions explored in this paper—similarity with other receptors, publicness across individuals, and persistence through time—represent useful strategies for identifying biologically important TCRβs.

The presence of near-ubiquitous (present in >90% of individuals in a cohort of 778 individuals) and persistent TCRβs led us to speculate that these TCRβs might be responding to a set of common antigens repeatedly encountered by healthy people. These antigens could be associated
with self-antigens, chronic infections (e.g., Epstein-Barr virus), or possibly members of the human microbiota. In fact, the CDR3 sequence CASSPQETQYF has been previously been associated with the inflammatory skin disease psoriasis [37] and CASSLEETQYF has been implicated in responses to *Mycobacterium tuberculosis* [20] and cytomegalovirus [38].

In addition to persistent TCRβs, our analysis revealed many receptors with unstable, transient behavior. Many high-abundance TCRβs did not persist through time, with many occurring at only a single time point (Figure 2b, S8a). These TCRβs could well correspond to T cells that expanded during a temporary immune challenge but then did not persist in high abundance afterward. These dynamics might also reflect the migration of T cells to and from different tissues, which could manifest as fluctuating abundance in the blood. The presence of dynamically expanding or migrating TCRβs in apparently healthy individuals poses an important consideration for designing studies monitoring the immune system. Studies tracking TCR abundances in cross-sectional immune system sampling [7,14,34–36] may capture not only T cell clones responding to intervention, but also expanding clones inherent in the T cell dynamics of healthy individuals. Repeated sampling before and after intervention could minimize such false positives.

Current immunosequencing methods have limitations that should inform the interpretation of our results. Most important, given such a diverse system as the TCR repertoire, even large sequencing efforts like ours undersample. Although our sequencing appeared to saturate our samples (Figure S2), additional bottlenecks during library preparation and, particularly, blood drawing limit our ability to capture full TCRβ diversity. Previous studies exhaustively sequenced multiple libraries from multiple blood samples, but even these estimates are considered a lower limit of TCRβ diversity [42]. This detection limit could confound our identification of persistent TCRβs. Many of the TCRβs that did not occur in all samples were undoubtedly present but too rare for our analysis to capture. Thus, identification of a persistent TCR repertoire was subject to an abundance cutoff, whereby we focused on TCRs that persisted above the detection limit of sampling. To check that our conclusions were not heavily altered by undersampling, we analyzed high-abundance TCRβs and found similar overall patterns, so we infer that our main conclusions are likely robust despite this experimental limitation. In addition, our study included data from only three female individuals ages 18–45. The immune system varies across sex [43] and age [44], and although the patterns we describe are clear, larger longitudinal studies on the immune repertoire with greater patient characterization (particularly HLA type) and representation (e.g., including men and a range of ages) will better define how these patterns apply across populations.

**Conclusions**

To better understand healthy immune system dynamics in humans, we profiled the TCRβ repertoires from three individuals over one year. We found a system characterized by both fluctuation and stability and further discovered a novel subset of the TCRβ repertoire that might play a key role in immunity. As immune profiling in clinical trials becomes more prevalent, we hope our results will provide much-needed context for interpreting immunosequencing data, as well as for informing future trial designs.
Methods

Study design
We sought to study baseline dynamics and characteristics of the TCRβ repertoire in healthy individuals across time. We sampled blood from three individuals from eight time points over one year. We kept our sample size small so that we could perform extremely deep immune repertoire profiling on each sample, a choice that should be taken into consideration when interpreting our results.

Sample collection
Three healthy adult female volunteers ages 18–45 provided blood samples over of one year, with samples taken on a starting date and 1, 2, 3, 5, 6, 7, and 12 months after that date (Figure 1a). We sequenced TCRβ chains from approximately 1 million PBMCs from each sample. From the samples at 5, 6, and 7 months, we also sequenced TCRβ chains from sorted naive (CD3+, CD45RA+) and memory (CD3+, CD45RO+) T cells.

High-throughput TCRβ sequencing
We extracted genomic DNA from cell samples using a Qiagen DNeasy blood extraction kit (Qiagen, Gaithersburg, MD, USA). We sequenced CDR3 regions of rearranged TCRβ genes and defined these regions according to the international immunogenetics information system (IMGT) [45]. We amplified and sequenced TCRβ CDR3 regions using previously described protocols [2,46]. Briefly, we applied a multiplexed PCR method, using a mixture of 60 forward primers specific to TCR Vβ gene segments plus 13 reverse primers specific to TCR Jβ gene segments. We sequenced 87 base-pair reads on an Illumina HiSeq System and processed raw sequence data to remove errors in the primary sequence of each read. To collapse the TCRβ data into unique sequences, we used a nearest-neighbor algorithm—merging closely related sequences—which removed PCR and sequencing errors. By sequencing genomic DNA and not RNA, our approach more accurately reflected T cell abundances but also captured both expressed and unexpressed T cell receptors [19].

Data analysis
In our analyses, we focused on TCRβs containing no stop codons and mapping successfully to a V gene and J gene (Table S1). Relative abundances of these “productive” TCRβ sequences, however, took into account the abundances of nonproductive TCRβ sequences, as these sequences were still part of the greater TCRβ pool. We defined a TCRβ as a unique combination of V gene, J gene, and CDR3 amino acid sequence. We examined nucleotide redundancy of each TCRβ by counting the number of T cell clones—a unique combination of V gene, J gene, and CDR3 nucleotide sequence—encoding each TCRβ. We defined TCRβs whose abundances ranked in the top 1% for each sample as high-abundance TCRβs, and we analyzed these TCRβs in parallel with the full TCRβ repertoire as a check for artifacts of undersampling (Figure S5, S8).

We calculated Spearman’s and Pearson’s correlation coefficients for TCRβ abundances across samples using the Python package SciPy, considering only TCRβs that were shared among samples. We calculated alpha diversity (Shannon estimate = e^(-Shannon entropy)) and clonality (1 – Pielou’s evenness) using the Python package Scikit-bio 0.5.1. We calculated Levenshtein
distance using the Python package Python-Levenshtein 0.12.0 and analyzed the resulting network using the Python package NetworkX 1.9.1.

To look for TCRβs with similar temporal dynamics, we focused on TCRβs that occurred in the top 1% at least twice. These TCRβs likely represented T cell clones that had expanded. We then calculated Spearman’s and Pearson’s correlation coefficients for all high-abundance TCRβ pairs, filling in missing data with the median abundance of TCRβs from each sample. We used median abundance—instead of a pseudocount of 1 or half the minimum abundance detected—because the immense diversity of the TCRβ repertoire means that most detected TCRβs are likely similarly abundant as TCRβs that were not detected. We identified pairs of TCRβs that had high (>0.95) correlation. To identify cohorts of TCRβs that co-correlated, we represented TCRβs as nodes in a network, where nodes were connected by edges if the corresponding TCRβs were highly correlated. We then searched for the maximal network clique (a set of nodes where each node has an edge to all other nodes) using NetworkX. We visually inspected these TCRβ cohorts for evidence of sequencing error, which might have resulted in a high-abundance TCRβ that closely correlated with many low-abundance TCRβs with similar sequences (Table S4). To test the significance of TCRβ cohort size, we performed the same analysis on 1000 shuffled datasets. Each shuffled dataset randomly permuted sample labels (i.e., the sampling date) for each TCRβ within each individual.

To test the significance of persistent TCRβ enrichment in (a) public receptors (Figure 4) and (b) TCRβs that occurred with many similar receptors (Figure 3), we analyzed 10,000 shuffled datasets. For these permutations, we randomly permuted the number of time points at which each TCRβ was observed and repeated the analysis.

We estimated the probability of generation of each TCRβ before to immune selection using IGoR version 1.1.0 with the provided model parameters for the human TCRβ locus [33].

**List of abbreviations**
TCR: T cell receptor
TCRβ: T cell receptor beta chain
V: variable gene or region of the T cell receptor
D: diversity gene or region of the T cell receptor
J: joining gene or region of the T cell receptor
PBMC: peripheral-blood mononuclear cell
HLA: human leukocyte antigen

**Declarations**

**Ethics approval and consent to participate:** All procedures were conducted under Western Institutional Review Board (WIRB, https://www.wirb.com/) protocol ADAP-002 (“Immunology studies of normal healthy individuals”). Subject enrollment and study procedures were directed by Adaptive Biotechnologies. Written, informed consent was obtained from all study participants.
Consent for publication: not applicable

Availability of data and materials: The dataset supporting the conclusions of this article is available in the immuneACCESS portal of Adaptive Biotechnologies repository, https://clients.adaptivebiotech.com/pub/healthy-adult-time-course-TCRB.

Competing interests: AMS and ROE are employed by, and have equity ownership with, Adaptive Biotechnologies. HSR has equity ownership with Adaptive Biotechnologies. EJA is a consultant and research advisor of OpenBiome and Finch Therapeutics. The remaining authors have no competing interest to declare.

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Author contributions: ROE, AMS, and HSR conceptualized the experiment and generated the data. NDC and HSB analyzed the data with input from MEB and EJA. NDC made figures, and HSB wrote the first draft of the manuscript with input from NDC. All authors contributed to manuscript development.

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References and Notes:


FIGURES
Figure 1. The TCRβ repertoire displayed stability and individual-specific characteristics across time. (a) Experimental design of T cell sampling. (b) A heatmap of Jaccard indexes shows clear clustering of samples by individual. Samples of naive T cells clustered less by individual than did PBMC or memory T cell samples. Relative abundances of the 20 most abundant TCRβs (c)
appeared stable through time. TCRβ abundances in PBMCs correlated within an individual across time points, including across a month (d, shared TCRβs = 33601, Spearman rho = 0.55718, \( p < 10^{-6} \)), and a year (e, shared TCRβs = 25933, Spearman rho = 0.53810, \( p < 10^{-6} \)), as well as across a month in naive (f, shared TCRβs = 15873, Spearman rho = 0.37892, \( p < 10^{-6} \)) and memory T cells (g, shared TCRβs = 47866, Spearman rho = 0.64934, \( p < 10^{-6} \)). TCRβs correlated much less across individuals (h, shared TCRβs = 5014, Spearman rho = 0.28554, \( p < 10^{-6} \)).

Shannon alpha diversity estimate (i) and clonality (defined as \( 1 - \text{Pielou's evenness} \), j) of the TCRβ repertoire were consistent over time.

**Figure 2.** A subset of the TCRβ repertoire occurred across all time points—the persistent TCRβ repertoire. (a) The number of TCRβs observed at \( n \) time points. Persistent TCRβs tended to have (b) greater abundance (Mann-Whitney \( U \) test, statistic=26297052589.5, \( p < 10^{-308} \)) and (c) nucleotide sequence redundancy (Mann-Whitney \( U \) test, statistic=25851211348.0, \( p < 10^{-308} \)) than other receptors. Mann-Whitney \( U \) tests between groups are in Tables S6, S7. Persistent TCRβs had higher proportions of TCRβs in common with memory (d) and with naive (e) T cell
populations and constituted a stable and significant fraction of overall TCRβ abundance across time (f).

**Figure 3.** Persistent TCRβs were more functionally redundant. We created a network graph of TCRβs from each individual, drawing edges between TCRβs on the basis of sequence similarity (Levenshtein distances), which reflects antigen specificity. We then grouped TCRβs into decile bins based on the number of neighbors (similar TCRβs) of each TCRβ. In other words, TCRβs in the 0–10% bin had 0% to 10% of the maximum number of neighbors observed for any TCRβ—the fewest neighbors—while those in the 90–100% bin had near the maximum number of neighbors observed. For each decile bin, we then counted how many samples each TCRβ occurred in from our time series data. (a) Vertical histograms of these distributions indicate that TCRβs with few neighbors—and thus few similar observed TCRβs—tended to occur at only a single time point, while TCRβs with more neighbors—and thus higher numbers of similar TCRβs observed—tended to have a higher proportion of persistent TCRβs. (b) The number of TCRβs in each neighbor bin (Figure S13a).
Figure 4. Persistent TCRβs were enriched in highly public TCRβs. We identified public TCRβs occurring in 0–10%, 0–20%, . . . 90–100% of individuals in an independent, large cohort of similarly profiled subjects (N = 778). For each of these decile bins, we examined TCRβs shared across each of our three individuals’ time series data and tallied the number of time points at which we observed each TCRβ. (a) Vertical histograms of these distributions indicate that more-private TCRβs—TCRβs shared by few people—occurred most often at only a single time point, while more-public TCRβs tended to persist across time. (b) The number of TCRβs evaluated in each decile bin. The vast majority of receptors were not shared or were shared across few individuals (also see Figure S13b). (c) In all three individuals in this study, persistent TCRβs included greater numbers of highly public TCRβs—defined here as receptors shared by over 70% of subjects from the large cohort—than receptors that only occurred once (independent t-test, statistic=-4.508, p=0.01). Asterisks indicate p < 0.05. (d) The three most public TCRβs (in over 90% of 778 individuals) were also persistent in all three individuals.
Chapter 5

Dynamic colonization of microbial strains and their functions in inflammatory bowel disease patients receiving fecal transplant

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Supplementary Information is in Appendix D.
ABSTRACT

For fecal microbiota transplantation (FMT) to be successful in complex immune diseases like inflammatory bowel disease (IBD), it is assumed that therapeutic microbes and their beneficial functions and immune interactions must colonize the recipient and persist in sufficient quantity and for a long enough period of time to result in a clinical benefit. But few studies have comprehensively profiled both colonization and persistence of transferred microbes and their functions. We analyzed longitudinal microbiome samples from a randomized controlled trial of 12 patients with ulcerative colitis who received fecal transplant or a placebo for 12 weeks. We uncovered a range of competitive dynamics among donor and patient strains, showing that the transfer of microbes from donor to patient is far from static. Indeed, one patient experienced a dramatic loss of donor bacteria 10 weeks into the trial, coinciding with a bloom of pathogenic bacteria and worsening of clinical symptoms. We similarly evaluated transfer of microbial functions, including desired ones like butyrate production and unintended ones like antibiotic resistance. By profiling bacteria coated with immunoglobulin A (IgA), we identified IgA-coated bacteria associated with inflammation, and we found that microbial interactions with the host immune system can be transferred across people. This transfer of immune function is likely critical for gut microbiota therapeutics for immune-related diseases. Our results illustrate the potential for longitudinal data from fecal transplant recipients to inform clinical decisions and demonstrate therapeutic strategies to alter not only the composition of the gut microbiota, but also its metabolic and immune functions.
INTRODUCTION

Buoyed by early success in recurrent Clostridium difficile infections (1), researchers are exploring whether fecal microbiota transplantation (FMT)—the transfer of entire fecal microbial communities from a healthy donor to a sick patient—can treat other microbiome-associated conditions. One of the most promising candidates is inflammatory bowel disease (IBD), a chronic condition characterized by periods of exacerbation and remission, suggesting that long-term longitudinal dynamics are key to understanding and treating the disease (2). When compared with healthy individuals, patients suffering from either of two types of IBD (ulcerative colitis or Crohn’s disease) have distinct gut microbial communities (3, 4). Thus, it has been hypothesized that manipulation of the gut microbiota and its interactions with the gut immune system might improve patient symptoms. Clinical trials have demonstrated that fecal transplants have moderate efficacy in patients with ulcerative colitis, but the factors driving patient response or nonresponse remain unknown (5).

It is broadly believed that the therapeutic element of fecal microbiota transplants is microbes and their functions (6). Many commensal bacteria are thought to promote gut and immune health, for example by the production of butyrate, which plays metabolic (7), regulatory (8), and immune roles (9–12) in supporting the gut epithelium. But not all microbial functions are beneficial. Fecal transplant material is rigorously screened for pathogens, but the upsurge of antibiotic resistance has raised concerns that fecal transplants could transfer potentially negative microbial functions, including antibiotic resistance (13).

In addition to the functions of the microbes themselves, the microbes’ interactions with the gut immune system may also play key roles in disease progression or treatment. The host immune system interacts with gut bacteria by responding to bacterial metabolites (10, 12), sensing direct contact between the host epithelium and bacteria (14), as well as coating bacteria with immunoglobulin A (IgA)—the main antibody produced in the gut and other mucosal tissues (15). These interactions play a pivotal role in the formation and maintenance of the host immune system (16, 17). Since many microbiome-associated diseases—like IBD—are of immune origin, the immune function of the gut microbiota might be the most directly related to host health.

Despite excitement around applying fecal transplants to IBD, no studies have comprehensively evaluated (1) which microbes transfer and persist across hosts, (2) the microbial functions that come along with them, and (3) whether immune functions of gut bacteria also transfer from donor to recipient. Previous reports of fecal transplants in IBD patients observed variable colonization by bacterial taxa from donors to recipients, but did not categorize the functions and immune interactions that were also transferred (18–21). Furthermore, most of these studies used minimal sampling (e.g., single time points before and after fecal transplant) and so could not show how transferred bacteria and functions varied over time. Particularly in the case of chronic, inflammatory diseases like IBD, understanding the longitudinal dynamics of transferred microbes and functions would advance our ability to determine why fecal transplants work for some patients and not others and help pave the way for more targeted therapies.

We comprehensively profiled the colonization dynamics of microbes and functions in a randomized controlled clinical trial of 12 patients with mild to moderate ulcerative colitis receiving fecal transplant. By bringing together analysis of microbial taxa, strains, functions, and immune interaction in this clinical cohort, we shed light on the dynamics of microbial colonization in the context of a complex disease.
RESULTS

Study design

We recruited patients at the University of Vermont Medical Center (in review). We deliberately selected two donors with high stool butyrate content (in review). After a course of broad-spectrum antibiotics (ciprofloxacin and metronidazole for seven days), patients received colonoscopic delivery followed by 12 weeks of daily capsules, of either fecal transplant material (from one of our two donors) or a placebo (Figure 1a). We chose to couple antibiotics pretreatment with two transplant delivery methods to maximize patients’ exposure to donor material and increase the likelihood that donor bacteria would successfully colonize their new host. To test the effect of daily capsules alone on bacterial transfer, a subset of fecal transplant recipients (n = 4) received capsule fecal transplant material from an alternate donor during 4 weeks in the middle of the clinical trial, after which they returned to taking material from their original donor (Figure 1a). We collected by mail near-weekly preserved stool samples from these patients during the trial and at an 18-week follow-up. At four time points, we also collected fresh stool samples during clinical check-ins. We sequenced DNA from these stool samples at the Broad Institute (Cambridge, MA) using 16S rDNA sequencing and shotgun metagenomic sequencing, producing datasets comprising an average of ~250,000 16S rDNA sequences and ~30.5 million metagenomic DNA sequences per sample (Table S1). To identify the abundance of different amplicon sequence variants (ASVs, akin to a bacterial species), we processed the 16S rDNA sequences using Qiime2 (22) and DADA2 (23). To track the abundance of bacterial species, we processed the metagenomic sequences using Metaphlan2 (24).

Antibiotics destabilized the microbiome of IBD patients receiving a placebo

An auxiliary finding that emerged in parallel with our primary results on colonization was that antibiotics severely destabilized the gut microbial communities of patients receiving a placebo, resulting in large-scale microbial gain and loss in these patients. The magnitude of changes we observed was greater than expected in healthy subjects. Previous studies tracking recovery of gut microbiota after broad-spectrum antibiotics in healthy people observed perturbation followed by consistent recovery of microbial communities—loss of bacteria followed by regain of the same bacteria (Figure S1a) (25).

In contrast, patients in our cohort who received a placebo exhibited diverse trajectories in their microbiomes. We calculated average beta diversity between patient samples and donor samples using Bray-Curtis distance and 16S rDNA and metagenomic species datasets. Visualizing the differences between microbial communities using PCoA (Figure 1b, PCoA using UniFrac in Figure S1b), we found that many placebo-treated patients ended the trial with a microbial community composition very different from where they started (Figure 1c, S1c–d). To see whether placebo-treated patients recovered the same bacteria that they lost while taking antibiotics, we categorized bacteria in each patient’s samples by their putative sources, including those detected in the patient’s baseline samples (“Patient”) and those undetected (“Unknown”) (Figure 1d, S1e). This latter category potentially included newly colonized bacteria from the environment, as well as endogenous patient bacteria that were under our detection limit. Most patients receiving a placebo regained some endogenous bacteria lost during antibiotics. But,
surprisingly, many patients (Placebo B, Placebo V, Placebo T) ended the trial with more bacteria from unknown sources than from themselves, reflecting a remarkable turnover in their gut microbiota. Although individual patients lost variable sets of taxa, a number of taxa were lost by multiple patients, including butyrate-producing *Subdoligranulum*, *Faecalibacterium*, and *Alistipes*, and commensals *Bacteroides* and *Dorea* (Table S2, S3).

In addition, we found that antibiotics triggered a short-lived increase in specific antibiotic resistance genes across all patients (Methods, Figure 1e). Specifically, the abundance of quinolone resistance genes increased immediately after antibiotics, likely reflecting selection pressure from ciprofloxacin, a quinolone (Figure 1e). These increases were not maintained over time (Figure S1f); neither did we observe temporary or lasting increases in other classes of antibiotic resistance (Figure S1f) or antibiotic resistance overall (Figure S1g). Placebo-treated patients also had a greater burden of tetracycline and aminoglycoside resistance genes than did fecal transplant recipients during the clinical trial period, although these levels were not appreciably higher than in baseline samples (Figure S1i,j). Taken together, our results emphasize that disease context—like IBD—can greatly influence the gut microbiota’s ability to recover from and respond to antibiotics.

**Donor bacteria colonized patients receiving fecal transplants**

Global diversity metrics indicated robust transfer and persistent colonization of donor bacteria in patients who received a fecal transplant. From our PCoA analysis of beta diversity, we found that each patient’s samples tended to cluster, samples from both donors clustered together, and patient-patient differences drove most of the variance (Figure 1b, S1b,c). The gut microbiomes of fecal transplant recipients clearly shifted toward the communities of the donors during the trial, as indicated by the Bray-Curtis distance from donor samples, whereas those of placebo-treated patients did not (Figure 2a, S2a). This difference persisted for the ~150-day trial period, although not for every transplant patient.

Unlike previous studies, we did not observe greater alpha diversity in fecal transplant versus placebo recipients, reflecting our discovery of high turnover in placebo-treated patients. Shannon index and richness in 16S and metagenomic data were similar in both treatment groups over the study period (Figure 2b, S2b–d), as was the change in bacterial community from baseline samples—by Bray-Curtis distance (Figure 2c, S2e). These results contrast with multiple studies reporting increased diversity in fecal transplant recipients compared with placebo recipients in diseases like *C. difficile* infection (26, 27), further exemplifying how fecal transplants can have varied effects in different diseases.

**Transferred microbial taxa exhibited varied dynamics in fecal transplant recipients**

Through frequent sampling, we profiled not only the microbial taxa that colonized transplant recipients but also their downstream dynamics, both of which likely underpin clinical response. As previously reported (18), different patients varied in their colonization rates—that is, the number and frequency of donor bacteria that successfully transferred from donor to patient (Figure 3a, Figure S3. Methods). At the resolution of bacterial species or ASVs, the proportion of bacteria transferred from the donor varied between 15% and 85% of the patient’s microbiome after fecal transplant (Figure 3a, S3). These transferred bacteria spanned phylogenetic diversity,
and almost all donor bacteria from Donor am—whose stool was transplanted into four recipients—were found to colonize at least one patient (Table S4).

Our longitudinal sampling further demonstrated that patients vary greatly in their ability to maintain colonized bacteria over time (Figure 3). Most patients showed a period of initial colonization after fecal transplant therapy began, followed by maintenance of transferred bacteria during the daily capsule delivery period and for months thereafter (Figure 3a). In contrast, patient FMT A had robust colonization of donor bacteria early in treatment—in fact more so than any other patient—but later lost many of these bacteria (Figure 3a). These colonized-then-lost bacteria included a number of ASVs of the genera *Bacteroides*, *Faecalibacterium*, *Ruminococcus*, and many others (Table S3). We categorized transferred ASVs in each patient as persistent or temporary colonizers and found that different patients had different frequencies of these two types (Figure 3b). Patient FMT A clearly had mostly temporary colonizers, but even patients with largely persistent colonization had taxa that only colonized temporarily.

Patient FMT A provides an intriguing clinical case as the sharp decrease in transferred donor bacteria coincided with a bloom of *E. coli* and associated virulence factors in the patient’s gut (Figure 3d). In fact, the data suggested that the loss of donor bacteria may have preceded the bloom. Furthermore, these changes appeared to track clinical outcomes. The patient reported feeling better during the early stages of treatment (week 4)—as measured by a standardized, clinically validated assessment tool (IBDQ) (28)—but later reported worsening of symptoms—a flare at week 12—which required administration of steroids (prednisone). Although we can only speculate whether the *E. coli* bloom or symptomatic change was a cause or effect of losing donor bacteria, this case exemplifies the variability of bacterial persistence after fecal transplant and suggests that monitoring microbial persistence may help inform clinical decisions.

The balance of conspecific donor, patient, and environmental strains fluctuate between dominance and parity

We then sought to profile the dynamics of individual strains within bacterial species to understand how conspecific strains from the donor, patient, and environment compete and coexist in treated patients. Previous reports have demonstrated that recipient and donor strains of the same bacterial species can coexist within fecal transplant recipients. Our longitudinal data allow us ask how the dynamics of this coexistence unfold. In particular, we asked whether donor strains could dominate over patient strains—which may have a competitive advantage as the endogenous strain—and whether the competitive balance of strains changed over time.

To resolve the longitudinal dynamics of bacterial strains, we used two complementary strategies to evaluate the contributions of different strains to the resulting bacterial community: a flexible genome approach (high specificity, lower sensitivity) and a single-nucleotide polymorphism approach using StrainFinder (medium specificity, medium sensitivity) (29). We focused on species with sufficient coverage for robust analysis (Figure 4a). Our flexible genome approach used read coverage of flexible genomic regions to identify strains with matching gene content (30) and by using full genome information achieves high strain specificity. This approach can positively identify matches of dominant strains (e.g., strain A versus strain A), but it cannot identify matches of mixtures of strains (e.g., strain A versus strains A and B). Thus a “mismatch” is considered ambiguous, since the two samples might contain entirely distinct strains or a mix of strains, resulting in lower sensitivity. Sample comparisons therefore had three
different outcomes: strain match (green in Figure 4), ambiguous (grey), and insufficient abundance or read coverage (red).

Using this flexible genome approach, we first confirmed that our methodology provided intuitively reasonable results by testing a number of control comparisons (Supplementary Information, Figure S4). We then compared samples from fecal transplant recipients with the donor’s samples and identified many matches between donor samples and patient samples after transplant, indicating that the dominant strain in a given patient sample was the same as in the donor (Figure 4a–d, S4c). We observed strain dynamics that fell into patterns determined by two factors: colonization (none, temporary, and persistent) and dominance (none, temporary, and persistent). These factors resulted in six observed patterns, and we provide toy examples of these patterns in Figure 4a:

1. Persistent colonization and dominance. The donor strain colonized after fecal transplant, came to dominate the strain community, and persisted as the dominant strain throughout the trial.
2. Temporary colonization and dominance. The same as #1, but all strains were subsequently lost.
3. Persistent colonization and temporary dominance. The same as #1, but at later time points, another strain from the patient or from an unknown source rose to an equal or greater abundance as the donor strain.
4. Persistent colonization without dominance. The donor strain colonized and persisted in the patient but never dominated the patient’s microbial community.
5. Temporary colonization without dominance.
6. No colonization.

We observed examples of each of these patterns, outlining the range of competitive dynamics between strains that can unfold after fecal transplant. Many Bacteroides species were successful in durably and dominantly colonizing (pattern #1), including B. vulgatus (patients E, W, F), B. dorei (patients E, W), and B. uniformis (patient E). The same bacteria in other patients durably colonized but only temporarily dominated the patient community (pattern #2), including B. vulgatus in patient P, B. dorei in patients P and F, and uniformis in patients W and P. One patient (N) appeared to be more resistant to colonization by donor Bacteroides species, and nearly always had a mix of strains (patterns #4 or #6). We also observed temporary colonization and dominance for B. ovatus and B. caccae in a smaller number of patients (Figure S4c). In the case of F. prausnitzii—a commensal bacterium thought to be related to gut health and negatively correlated with IBD—some patients exhibited persistent colonization and dominance of a donor strain (pattern #1, patient E), others were only temporarily dominated by the donor strains (pattern #3, patients P, N), and still others adopted strains that did not come exclusively from the donor but also unknown sources.

In cases of ambiguous matches, our flexible genome analysis could not define the contributions of individual strains from different sources, leaving some competitive dynamics undefined. For example, a donor strain of B. dorei temporarily dominated the community of Patient F, but later strain matching gave ambiguous results, which could mean that the donor strain disappeared from the community or that it coexisted with another strain. Consequently, to observe individual contributions of strains in mixed communities, our second approach reconstructed SNP haplotypes using StrainFinder, allowing us to deconvolute the contributions
of strains from the donor, recipient, and unknown sources over time. Although the sensitivity of StrainFinder allows us to quantify individual strains, its dependence on marker genes makes it less specific than our flexible genome approach (29). Because StrainFinder requires sufficient sequencing depth to properly model strains, we combined longitudinal samples into five time points (Methods, Figure 4).

We found that donor and patient strains frequently coexisted, even after temporary dominance by the donor strain. We first confirmed that the results from StrainFinder aligned with our flexible genome analysis, as shown for *F. prausnitzii* in FMT N and FMT E (Figure 4e). In the case of FMT A and FMT F, we observed that by the end of the clinical trial, strains of unknown origin largely displaced those from the donor. The dominant strains of some species (*F. prausnitzii* in FMT P, *B. dorei* and *B. vulgatus* in FMT N) were shared between the donor and patient, indicating that even high-resolution methods like StrainFinder depend on marker genes and cannot always resolve unique strains. Strains of *B. dorei* in FMT F and *B. uniformis* and *B. vulgatus* in FMT A showed temporary dominance by donor strains, with strains of unknown origin appearing in follow-up samples. We observed a similar variety of competitive dynamics in other abundant species (Figure S4f–i).

Taken together, these results demonstrate not only that donor and recipient strains can coexist (31), but also that the balance of this coexistence changes over time. In many cases, donor strains were able to outcompete endogenous patient strains, but this dominance was dynamic, as both patient strains and strains from unknown and possibly environmental sources often competed later in the trial.

*Capsule delivery from an alternate donor introduced limited novel taxa*

By looking at the subset of patients who took capsules from an alternate donor part-way through the study (Figure 1a), we were able to ask whether changing donor material resulted in additional colonization by new microbes. Although we were able to identify isolated evidence of novel bacteria colonizing from the alternate donor, the vast majority of newly colonized bacteria came from the original donor used for colonoscopic delivery and the first month of capsules (Figure S5). This limited colonization by alternate donor microbes suggests that capsule delivery to patients with intact microbial communities—as opposed to ones depleted by antibiotics—is an inefficient method for introducing new bacteria.

*Fecal transplants transferred beneficial functions that varied across time*

Beyond the microbes themselves, it is the functions they perform in the gut ecosystem that likely drives restoration of gut health. We thus tracked the colonization of functional genes implicated in maintaining health. Fecal transplant recipients showed increased richness in genes involved in complex carbohydrate metabolism, in comparison to placebo-treated patients, suggesting increased capacity to digest dietary polysaccharides (Figure 5a). On the other hand, fecal transplant recipients showed similar levels of butyrate biosynthesis and mucin degradation genes (Figure 5b–c). So although donors were chosen for high butyrate production—and this was reflected in a high diversity of butyrate biosynthesis genes (Figure 5c)—fecal transplant did not result in wholesale, persistent transfer of these genes, and transplant recipients had similar butyrate gene diversity compared to baseline and to placebo-treated patients. Two fecal transplant recipients—but no placebo-treated patients—temporarily reached comparably high
butyrate gene diversity, but these changes were temporary. Among fecal transplant recipients, we observed transfer and persistence of many genes involved in butyrate production and mucin degradation among fecal transplant recipients (Figure 5), including from health-associated commensals like *F. prausnitzii* and *Bacteroides* (Table S5) (32, 33). Thus, fecal transplants are effective in transferring beneficial microbial functions across hosts but may not result in overall increases the diversity of genes related to that function.

**Fecal transplants also transferred antibiotic resistance and virulence factors**

Not all microbial functions transferred to fecal transplant recipients are beneficial. Although, clinically, fecal transplants can effectively clear patients of antibiotic-resistant infections, the research community has raised concerns that fecal transplants could introduce novel resistance genes, with negative clinical effects (13). We observed the transfer from donors to patients of numerous antimicrobial resistance genes from donors to patients—including resistance to all major classes of antibiotics—and many of these genes were maintained for the full trial period (Figure 5d). Nevertheless, we found that the resulting burden of antibiotic resistance genes in fecal transplant recipients was not heavier than in our healthy donors (Figure S1g,h) and that transferred resistance genes were generally outnumbered by endogenous ones (Figure S6c). Thus, although the transfer of resistance genes is an unavoidable result of the complexity of this therapy, there remains no clinical evidence or bioinformatic indication that fecal transplants increase the overall risk of antibiotic resistance.

Similarly, we observed the unintended transfer of virulence factors. Despite a lower incidence of virulence factor genes in donors than in patients (Figure S6a,b), we observed colonization and persistence of such genes, and these genes made up a significant portion of the virulence factor pool (Figure 5e, S6d). Many patients exhibited an increase in the abundance of virulence factors during or shortly after antibiotics (Figure S6a,b). Two patients had an inordinate burden of virulence factors, including the patient FMT A who had a bloom of *E. coli*, and patient Placebo V (Figure S6f). Patient Placebo V’s health declined during the trial (Table S5), and this patient had one of the most dramatic turnovers in response to antibiotics (Figure 2), resulting in a microbiome dominated by newly acquired Proteobacteria and associated virulence factors (Figure S6e). In sum, although we observed the transfer and persistence of donor-derived virulence factors and antibiotic resistance genes, these transfers were modest and often outweighed by the endogenous microbial community’s virulence and resistance.

*IgA coating of gut microbes identified shared immune responses to commensal and IBD-associated bacteria*

In the context of IBD, interactions with the host immune system is perhaps the most important microbial function. Although it is reasonable to expect that transferring microbes would also transfer their endogenous metabolic capacities, it is much less certain whether transferred bacteria will elicit similar immune responses in a new host with a different immune system.

To understand host response to transferred gut bacteria, we used immunoglobulin A sequencing (IgA-seq) to profile bacteria coated with IgA antibodies (15). Secretory IgA is the primary antibody of mucosal surfaces, including the gastrointestinal, respiratory, and urinary tracts (34). Thought to act primarily by blocking proteins on the surface of invading pathogens, IgA has more recently been suggested to play a role in facilitating mucosal colonization by
commensal bacteria (35, 36). Although bacteria interact with the host immune system in many ways (e.g., via excreted metabolites, direct contact with the epithelium), we used IgA-seq as a proxy for bacterial immune function as it is one of the few immune functions that can be measured in vivo and high-throughput for bacteria in stool samples. To identify IgA-coated and uncoated bacteria, we used fluorescence-activated cell sorting (FACS) to separate these fractions of gut microbiota samples, and we 16S-sequenced the fractions to a median depth of 165,000 reads. We calculated IgA enrichment as the log-fold change in abundance of a bacterial ASV in each fraction.

We first asked whether overall IgA coating of bacteria differed among patients because if IgA coating of bacteria were the same across all patients and donors, then the likelihood of successful transfer of IgA coating would be very high. We found that IgA coating—the log-fold enrichment in the IgA coated fraction—of bacteria in two samples taken at different time points from one healthy donor had high agreement (Pearson r = 0.7, p < 1e-25) (Figure 6a), suggesting that IgA coating of bacteria in healthy individuals is stable across time; this result gave us confidence in our methodology. But although IgA coating in donor samples moderately correlated with mean IgA coating across all patients at baseline (Pearson r = 0.43, p < 1e-9, Figure 6b), the strength of correlation with IgA coating of individual patients varied considerably (Pearson r of 0.1–0.7, Figure S7a,b). This result indicated that IgA coating can vary greatly across patients and raised the prospect that transferred bacteria may not retain immune function. Some studies have suggested that IgA might target blooming or abundant bacteria to promote homeostasis (37, 38), but we did not observe a correlation between bacterial IgA coating of bacteria and abundance, variance, or bimodality (Fig S7c–e).

We then sought to identify bacteria that were strongly IgA coated or uncoated across all patients and samples, because these bacteria could trigger similar immune responses when transferred across hosts. Indeed, we found bacteria that were reliably and strongly IgA coated across patients and within patients across time (see Methods). These bacteria were a phylogenetically diverse group with organisms from all major phyla (Figure 6c, Table S6), including known commensals (e.g., Bacteroides) and many taxa that transferred from donors to fecal transplant recipients. We also identified Proteobacteria (including known opportunistic pathogens) and Ruminicoccus gnavus—as well as the closely related R. torques—as reliably IgA coated in patients and donors (Figure S78). Although we did not observe R. gnavus transfer from donors to patients, we found significant blooms of this bacterium (up to 40% relative abundance) in our patient cohort, while it was essentially absent in our donors (Figure 6d). This finding is in keeping with other research groups, who have reported that this bacterium blooms specifically in patients with IBD (39). These results further suggest that R. gnavus may play a role in immune dysregulation in IBD and warrants additional study.

Bacteria that were reliably uncoated by IgA across patients were less phylogenetically diverse and tended to represent known commensals. All of these bacteria were Firmicutes, including known butyrate producers like Faecalibacterium, Alistipes, Roseburia, Oscillibacter, Butyricicoccus, and other Lachnospiraceae species (Figure 6c, Table S6). On the basis of these results, we hypothesize that being uncoated by IgA may be more specific than being coated. Together, these data suggest that bacteria eliciting strong or negligible IgA responses across individuals likely play important roles in regulating host gut immunity and that transferring these bacteria can replicate microbial immune function in a new host.

*Strain-specificity revealed the transfer of host-immune function in fecal transplant*
We observed a final category of diverse bacteria that were variably IgA coated and uncoated across different individuals or even within the same individual across time (Figure 6c, S8, Table S6). If confirmed, such divergent immune effects across patients could complicate our approach to fecal transplant, which currently assumes the transfer of microbial functions from donors to patients. If the same microbe can trigger an IgA response in one person while suppressing it in another—which may lead to proinflammatory or anti-inflammatory responses—then fecal transplants may be less effective and less predictable at transferring immune function.

We hypothesized that variable IgA responses could stem from two processes: (1) divergent host immune responses—as mentioned above—or (2) strain specificity of IgA coating. In the second scenario, a given bacterium is variably IgA coated because each patient is responding to different strains of bacteria with identical 16S sequences, the basis for our IgA-seq data. Under this scenario, IgA coating is exquisitely strain-specific and transfer of the exact same strain would transfer a similar IgA response.

To establish the role of strain-specificity in explaining variably IgA-coated bacteria, we examined two subsets of bacteria in fecal transplant recipients: bacteria that were shared by donor and patient and bacteria that transferred from the donor to the patient after fecal transplant (Methods). Thus, for each bacterial 16S sequence, the first subset included a potential mix of donor and patient strains, while the second subset likely contained only one strain from the donor that then transferred to the patient. We found that the subset of strains transferred from the donor (exact strain matches) had greater correlations of IgA coating than bacteria shared between the donor and recipient (mixed strains) (Figure 6e); this pattern held across three patients who received fecal transplants from a single donor (Figure 6f). These results suggested that exactly matching strains triggered more-similar immune responses than mixed strains with identical 16S sequences, demonstrating that variable IgA coating of bacteria could in part be explained by strain-specificity. Although in one patient the correlation in IgA coating of exactly matching strains was still weak (Pearson r = 0.3), this finding further established that immune functions of transferred microbes can be broadly replicated in fecal transplant recipients, elevating the prospects of engineering the gut microbiota to modulate host immunity and disease.

**DISCUSSION**

*Disease context shapes gut microbiome recovery after antibiotics*

We found that administering broad-spectrum antibiotics destabilized the gut microbiota of placebo-treated IBD patients. Unlike healthy patients in other studies, our IBD patients readily lost and did not recover their original gut bacteria, exposing these patients to colonization by new bacteria, which in some cases ultimately outnumbered endogenous species. Previous work has reported that the microbiomes of IBD patients are more variable than those of healthy people (4), and our results establish that interventions like antibiotics can exacerbate this instability. Given that multiple clinical trials have studied the use of antibiotics to treat IBD (40–44), our results raise a red flag about potential unintended consequences of using antibiotics in patients with IBD or other microbiome-related diseases. Further assessment of different antibiotics’ effects on the gut microbiota of IBD patients would improve clinician’s ability to make informed clinical decisions about the risks of antibiotics.
Microbial strains exhibit a range of colonization dynamics

Our results illustrated a range of microbes and functions that can persistently colonize fecal transplant recipients. Only a small subset of rare bacteria appeared to never transfer (Table S5), suggesting that essentially all bacteria can be transferred between people. This finding reaffirms that the gut microbiota can be clinically engineered by transplanting whole gut microbial communities. It remains unknown whether more-targeted therapeutics using synthetic communities will show the same ability to colonize recipient hosts.

For fecal transplants or other targeted microbial therapeutics to have a clinical effect, colonized microbes must persist at sufficient abundance within the recipient. We observed a variety of fates of colonizing microbes in each patient over time—from wholesale colonization and persistence of new taxa to fleeting passage of individual strains. A significant fraction of microbes that colonized the recipient for multiple weeks later disappeared, suggesting that even though many bacteria can colonize a patient temporarily, competition, nutritional requirements, or immune system interactions may hamper persistence. This problem might be addressed by administering fecal transplants along with other treatments aimed at maintaining colonized bacteria (e.g., prebiotics) (38, 45, 46).

Coexisting conspecific strains also showed a range of competitive dynamics: in some patients donor strains dominated endogenous strains, while in others endogenous strains remained more abundant. Competitive dynamics like these may contribute to variable clinical responses to whole-gut-microbiota transplants and are likely to play an even greater role in more-targeted microbial therapeutics, whose efficacy hinges on the dynamics of a small number of strains.

In addition, in Patient FMT A offered an example of how patient health can impact the colonization of donor bacteria. After more than a month of stable colonization, this patient lost a large portion of transferred strains in a short period, which coincided with—or potentially preceded—a bloom in pathogenic bacteria and severe deterioration in symptoms. This dramatic decline warns us that continued patient monitoring may be needed to maintain treatment efficacy, particularly with chronic diseases like IBD. Patients like this who lose colonized bacteria from the donor could be retreated, restarting the clock on donor-strain persistence and intended clinical effect. Furthermore, although it remains unknown which of these shifts—loss of donor bacteria, bloom of pathogens, and deterioration of symptoms—came first, the progressive unfolding of these events raise the possibility that real-time tracking of patient microbiomes may enable early intervention and prevention of IBD flares.

Microbial and immune functions transfer across human hosts

We found that specific beneficial functions transferred from donors to patients and could also persist. Many gut microbiome studies have focused on the benefits of butyrate production, for example, and we were able to track the transfer of these genes from donor to patient. But even after receiving new butyrate genes from a donor, fecal transplant recipients did not show higher butyrate gene diversity compared with placebo-treated patients. This observation suggests that it may be difficult to increase overall genetic capacity for butyrate production via fecal transplants. Of course, the diversity of genes related to a function does not necessarily reflect the activity of those biochemical pathways. Instead, it may be more fruitful to focus on which butyrate
producing organisms are present (are some microbes more productive than others?) and which nutrients (e.g., dietary fibers) are available to those bacteria.

We found that fecal transplants can also transfer unintended functions (e.g., antibiotic resistance genes, virulence factors). To date, no evidence suggests that such unintended transfers have appreciable clinical effects (1, 47), but possibilities must be considered, particularly since antibiotic resistance can transfer between gut bacterial species (47, 48). Although it is probably impossible to purge an intact fecal community of all antibiotic resistance, targeted microbial therapeutics may be able to minimize or avoid it.

In the context of IBD, the function most critical to transfer and persist in the patient is the gut microbiota’s immune function. Our identification of numerous reliably and strongly IgA-coated or -uncoated bacteria across all patients and donors indicated retention of immune function across hosts. Strongly IgA-coated bacteria included IBD-associated bacteria (R. gnavus, E. coli), as well as known commensals (Bacteroides, Blautia)—a finding that complicates the frameworks of research suggesting that IgA-coated bacteria are largely pathogenic and inflammatory (15, 34). It may be that IgA coats any bacteria colonizing the gut mucosa, whether friend or foe. This speculation fits with recent reports of Bacteroides commensals using IgA to colonize the mucosa (35) and with our observation that reliably IgA-uncoated bacteria (Faecalibacterium, Roseburia) were luminal, carbohydrate-degrading taxa.

Furthermore, we establish that IgA coating of bacteria can be transferred across human hosts, suggesting that transferring gut microbes may be broadly effective in triggering specific and nonspecific IgA coating and immune pathways. In addition to bacteria that were reliably IgA-coated or -uncoated, many bacteria were variably IgA coated across patients, suggesting that potential host specificity of immune function could complicate clinical responses to fecal transplants. We found that this variability was in part due to the strain specificity of IgA coating: strains that transferred from donor to patient tended to have similar patterns of IgA coating. This specificity seems to contrast with previous reports of polyreactive IgA activity in the mouse small intestine (49). It is highly unlikely that this signal results from the IgA coating of bacteria in the daily capsules because of their small volume (one capsule per day) and the necessity for those bacteria to pass through the small and large intestine.

It is further possible that immune context of the donor may play a role in the transfer of IgA coating from donor to patient, either because of donor-specific immune responses or other donor-specific factors like diet or microbial community. For example, a particular bacteria strain might express different surface receptors depending on the nutrients in the host’s diet, which may then alter what would otherwise be identical immune interactions. We further speculate that it may be possible that an IgA-coated bacteria from the donor transferred into a patient may retain its IgA association, not because the patient innately coats that bacteria, but because the patient’s immune system learns the coating pattern from the donor’s IgA. In such a scenario, IgA coating and immune function may have an “inertia” when transferred between hosts. Additional study into the immune factors that generate transferable and variable IgA responses will deepen our ability to manipulate host immunity via the gut microbiota.

In summary, our study offers a first look at the dynamics of colonization and persistence of microbes, their metabolic functions, and their immune functions in IBD patients receiving fecal transplant. Our dense time series sampling revealed surprising complexity of microbial transfer and emphasized that for chronic diseases like IBD, continuing patient care may be necessary to maintain newly colonized bacteria. Our observations of broad transfer of microbes and their functions demonstrate the power of fecal transplant to alter a patient’s gut microbiome
and set the stage for developing targeted drugs that introduce and maintain specific microbes and functions to treat disease.

**METHODS**

**Clinical cohort and sample collection**

We collected samples from a clinical cohort described in (in review). Patients collected semiweekly stool samples at home or in the clinic, storing samples in RNA-later (ThermoFisher) and mailing them to a processing facility at OpenBiome in Somerville, MA. We collected fresh stool samples which were then stored in a glycerol buffer (1X PBX, 25% glycerol, 0.05% L-Cys) from baseline and four, twelve, and eighteen weeks after the initiation of fecal transplant during clinical evaluations at University of Vermont Medical Center.

**DNA extraction and sequencing**

We triple-washed RNA-later from samples and extracted DNA using a MoBio Powersoil DNA extraction kit. 16S rDNA libraries were prepared and sequenced by the Broad Institute Genomic Platform using the Earth Microbiome Project protocols and paired-end 250 base pair reads on an illumina Miseq (50). Shotgun metagenomic libraries were likewise prepared by the Broad Institute using Nextera protocols and sequenced on an illumina NextSeq.

**IgA-sequencing**

Samples were processed as described previously (15). We centrifuged glycerol-stored stool samples at 50 × g at 4°C for 15 min and then washed three times in 1 mL PBS/1% BSA at 8,000 × g for 5 minutes each. We collected the pre-sort fraction as 20 µL after resuspension prior to the final wash and stored at −80°C. We then resuspended the cell pellet in 25 µL of 20% Normal Rat Serum (Jackson ImmunoResearch) in PBS/1% BSA and incubated for 20 min on ice. After incubation, we added 25 µL 1:12.5 α-mouse-IgA-PE (eBioscience; clone mA-6E1) to each sample and incubated samples on ice for 30 minutes. Finally, we washed samples three times in 1 mL PBS/1% BSA, resuspended them in PBS/1% BSA, and transferred them to blue filter cap tubes (VWR 21008-948) for flow sorting. We sorted an average of 50,000 cells from the IgA-positive and IgA-negative bacteria into sterile microcentrifuge tubes on the BD FACSARia II at the MIT Koch Institute Flow Cytometry Core (Cambridge, MA). Samples were centrifuged, supernatant removed, and resuspended in a final volume of 10 uL sheath fluid. Samples were stored at −80°C until DNA library prep, in which 2 uL (~10,000 cells) was used directly as the template for PCR.

**Data analysis**

We analyzed 16S data using Qiime2 (22), DADA2 (23), and custom python scripts. We assigned taxonomic labels to 16S sequences using the SILVA database (51). We quantified the abundance of microbial species from shotgun metagenomic sequencing using metaphlan2 (24). To visualize changes in alpha and beta diversity, we calculated the mean values of samples within 5-day windows, and compared these values across treatments using a Student’s t-test. When assigning
ASVs and metagenomic species to sources, we labeled all bacteria observed in any of a patient’s baseline samples and in the donor sample as “Shared”, all other bacteria present in baseline samples as “Patient”, all bacteria absent from baseline but shared with the donor as “Donor”, and finally all others as “Unknown.” We defined “persistent” colonization of a bacteria (ASV or metagenomic species) as a bacteria that transferred exclusively from the donor that appeared in at least 3 samples post fecal transplant and remained present in at least one follow-up sample at 18 weeks after initial transplant. We defined “temporary” colonization similarly, except that these bacteria did not appear in any follow up samples. There are some limitations to assigning sources using this strategy. At 16S resolution, some common taxa (notably E. coli) are generally identical, such that many unique strains are identified as a single ASV. For example, in the case of E. coli, all transplant and placebo-treated patients and the donors shared a single ASV, and it is highly unlikely that every patient had the same strain. In these cases, our strategy might result in falsely identifying the source of the ASV. For this reason, we focus on the overall frequency of ASVs from different sources, instead of the abundance of each ASV, which minimizes the signal from highly abundant but potentially incorrectly identified ASVs.

To quantify the transfer of bacterial functions, we used shortbread (52) to quantify the abundances of genetic functions of interest, including butyrate biosynthesis (53), mucin degradation (54), glycoside hydrolase activity (55), antimicrobial resistance (56), and virulence factors (57). We quantified the abundance of quinolone resistance in baseline samples and in the 10 days immediately following the cessation of antibiotics and the administration of fecal transplant or placebo, and we compared these abundances using a Student’s paired t-test. We also calculated the area under the curve and compared these values using a Student’s paired t-test. We visualized the abundance of genetic functions in fecal transplant and placebo patients using the same 5-day windows as described above. We identified the sources of antibiotic resistance genes and virulence factors in the same fashion as bacteria.

To quantify the transfer of strains, we used two strategies: one based on flexible genome content and the other on single-nucleotide variants (SNVs). For the first strategy, we used a similar strategy as described previously (30). Briefly, we mapped metagenomic reads from each sample to reference genomes for each species using BWA (58) and quantified the number of reads mapping to each unique 1000bp segment of the reference sequence. To compare the strains in two samples, we then compared the read depths in each sample across all 1000bp segments. We identified a strain match as those comparisons for which no segment with a read depth greater than the median for that sample was entirely absent from the other sample. Comparisons that did not meet these criteria were called ambiguous. Comparisons where either sample had a median read depth less than 5 were considered absent due to insufficient abundance and read depth. To reconstruct the individual contributions of strain haplotypes, we used StrainFinder (29). To build the input alignments for strain finder, we used BWA (58) to align metagenomic reads from each sample to a database of AMPHORA genes (59)—a set of single-copy, universally carried bacterial genes—from various gut bacteria. We used samtools (60) to tally the nucleotide identities found at each position and used stringent filtering to remove reads with poor mapping quality, rare alleles, and sites with inordinate read depth. To provide greater depth of reads to StrainFinder’s maximum likelihood model, we combined reads from samples across the time series as described above. We only considered genomes with a median read depth over 50 in at least two samples. StrainFinder outputs the relative contributions of different strains to the abundance of a given species, so we normalized these values using the median read depth in each sample to better reflect the relative abundances of each strain across samples.
To understand host-immune interactions, we analyzed 16S data from IgA-seq using Deblur (61). We calculated IgA enrichment as the log2-fold change between the IgA coated and uncoated fractions. We observed that IgA enrichment values largely followed a normal distribution (Figure SX), thus, for each sample, we categorized strongly IgA coated or uncoated bacteria as those bacteria that were greater than or less than the mean +/- 1 standard deviation. To identify bacteria that were reliably IgA coated or uncoated across all samples or across all patients, we used a one sample t-test, with an FDR adjusted p value < 0.1. We constructed a phylogenetic tree of 16S sequences using FastTree (62) and visualized it using itol (63).

References and Notes


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**Author contributions.** NDC, JWC, LTTN, MBS, ZK, PLM, EJA designed the study; JWC and PLM coordinated patient recruitment, treatment, and sampling; MBS and ZK coordinated fecal microbiota transplant production and delivery to the clinic; NDC, JWC, LTTN, MBS, ZK coordinated sample collection; NDC, LTTN coordinated data generation; SMK performed IgA-seq experiments; NDC analysed the data; NDC wrote the first draft of the manuscript with substantive input from all authors.

**Data and materials availability.** We deposited raw sequence files of 16S, metagenomic, and IgA-sequencing results in NCBI’s SRA database (accession XXXX). Metadata is included as supplementary tables of this manuscript. Additional 16S ASV tables and metagenomic species tables as well as code used to generate the figures and analyses can be found at [https://github.com/nathanieldchu/uc_fmt](https://github.com/nathanieldchu/uc_fmt).

**Competing interests.** EJA is a co-founder and shareholder of Finch Therapeutics, a company that specializes in microbiome-targeted therapeutics. PLM is a Medical Director at Takeda Pharmaceutical Company.
Fig 1. Recovery from antibiotics in IBD patients involved substantial loss and introduction of microbial taxa. (a) Design of the clinical trial and sampling. * One placebo-treated patient had worsening symptoms and dropped out of the trial at 8 weeks. (b) PCoA on the basis of bray-curtis distance using 16S. Donor samples clustered on the left-hand side, while samples taken during antibiotics treatment tended to cluster to the right. (c) PCoA trajectories of placebo-treated patients indicated incomplete recovery from antibiotics in most patients. The PCoA space
is the same plot as in Figure 1b. The larger circles signify a baseline sample at the beginning of the clinical trial. (d) Tracking microbial sources revealed invasion of many novel bacteria after antibiotics as well as loss of many taxa. Each plot tracks the fraction of ASVs that were identified as coming from the patient—on the basis of the baseline samples—or from an unknown source—which could be bacteria that were below our limit of detection in baseline samples or bacteria from the environment. The red region of the figure indicates the course of antibiotics, while the green region indicates the course of capsule therapy (placebo or fecal transplant). (e) Many placebo and transplant recipients exhibited an increase in abundance of quinolone resistance genes in the ten days after the administration of antibiotics. Lines are colored by the change in resistance. Line color reflects the change, with red lines indicating an increase, while blue lines indicate no change or decrease. Also see Figure S1.
Fig 2. Fecal transplants resulted in global transfer and persistence of donor strains. (a) Time series of each patient's mean bray-curtis distance from the donor samples indicated a shift towards the donor communities in fecal transplant patients. Bold lines and confidence intervals (95%) reflect the mean across patients. Lines for each individual patient are in the background. Asterisks indicate significant difference between fecal transplant and placebo patients by a student’s t-test $p < 0.05$. Responders and non responders are as describe in (CITE). Regions of the graph colored as in Figure 1. (b) Alpha diversity (shannon index) of 16S profiles indicated little difference between fecal transplant and placebo recipients. (c) Similarly, the two treatment groups showed similar extents of community change when compared against baseline sample by bray-curtis distance. Also see Figure S2.
Fig 3. Longitudinal sampling of the microbiota revealed variable maintenance of donor bacteria. (a) Tracking bacterial sources identified bacteria transferred from donor to recipient as well as an invasion of bacteria of unknown origin. Each time-series plot indicates the fraction of total ASVs that were identified as from the patient, donor, shared between the two, or from an unknown source. Regions of the graph colored as in Figure 1. (b) Fecal transplant recipients had varying frequencies of persistent and temporary colonization of donor bacteria. (c) After an initial period of robust colonization, patient FMT A lost a majority of transferred donor bacteria; this loss coincided with a bloom of *E. coli* and a deterioration of clinical symptoms. Also see Figure S3.
a Example plot

Persistent colonization and dominance
Temporary colonization and dominance
Persistent colonization and temporary dominance
Persistent colonization without dominance
Temporary colonization without dominance
No colonization

b Bacteroides vulgatus

FMT F
FMT A
FMT P
FMT W
FMT E
FMT N

c Bacteroides uniformis

FMT F
FMT A
FMT P
FMT W
FMT E
FMT N

d Bacteroides dorei

FMT F
FMT A
FMT P
FMT W
FMT E
FMT N

e Faecalibacterium prausnitzii

FMT F
FMT A
FMT P
FMT W
FMT E
FMT N

f F. prausnitzii

FMT N
FMT E
FMT P
FMT A

B. dorei

FMT N
FMT E
FMT A
FMT P

h B. uniformis

FMT N
FMT E
FMT A
FMT P

i B. vulgatus

FMT N
FMT E
FMT A
Fig 4. Decolvoluting conspecific microbial strains revealed a range of competition dynamics. (a) Toy examples of plots for flexible genome analysis, which fell into six patterns determined by colonization and dominance (see Results). Green circles indicate a strain match between the sample and the donor sample, grey circles indicate ambiguous strain identity, and red circles indicate insufficient read coverage for analysis. The size of the circle reflects the median read depth across the genome for that sample. Flexible genome plots for (b) Bacteroides vulgatus, (c) B. uniformis, (d) B. dorei, and (e) F. prausnitzii. In cases of ambiguous strain identities, we analyzed the individual contributions of strain haplotypes using StrainFinder for (f) F. prausnitzii, (g) B. uniformis, (h) B. dorei, and (i) B. vulgatus. The y-axes represent the frequency of different strain haplotypes for a given species, normalized by the median read depth across all marker genes for that species (see Methods). Also see Figure S4.
Fig 5. Bacterial functions also transfer across human hosts. We tracked the incidence and transfer of bacterial functions using shortbred and curated databases. Fecal transplant recipients showed higher diversity of glycoside hydrolase genes (a), but not of butyrate biosynthesis genes (b) or mucin degradation genes (c). For each of these plots, we also show the area under the curve (AUC), where an asterisk indicates $p < 0.05$ by a student’s t-test. We also observed the transfer of (d) antibiotic resistance genes and (e) virulence factor genes from donors to recipients, although the incidence of these genes was not higher in fecal transplant recipients. Also see Figure S6.
Fig 6. IgA-coating of the gut microbiota reveals broad patterns of microbiota IgA coating and host-specific IgA responses. IgA enrichment of different bacteria is (a) highly correlated across different samples from one of our healthy donors and (b) even well correlated between the same donor and the patient baseline samples. (c) A phylogenetic tree containing bacteria that were identified as reliably IgA+ enriched (inner red circles), IgA− enriched (blue circles), or variably IgA+/− either across patients or within a patient across time (outer purple circles. Tree branches are colored by phylum. (d) Relative abundance of Ruminococcus gnavus in our patient cohort.
(e) Correlations of IgA enrichment in shared and transferred bacteria indicates stronger correlation in transferred bacteria. (f) This pattern was observed in all patients receiving Donor am. Also see figure S7.
Chapter 6

Conclusions

6.1 Limitations

Although diverse in nature and subject, the projects I have presented in this thesis all suffer from common limitations, which should color their interpretation and necessitate further studies to address. I group these limitations into two categories: samples sizes and street light effects.

6.1.2 Sample sizes wide and long

The challenges of obtaining sufficient sample size plagues virtually all research on human subjects, where ethics and logistics hinder subject recruitment. The dangers of insufficient sample sizes are superficially obvious: with fewer samples one has a greater risk of falsely claiming a pattern (Type I error) and, reciprocally, less statistical power to detect true patterns (Type II error). Such scientific errors, particularly in fields like medicine, can have profound effects on how science is interpreted with real-life consequences [1].

But even though we as a research community can universally acknowledge the dangers of insufficient sample size, that doesn’t necessarily tell us how to avoid it. I believe that sample size limitations can be thought of as falling along two axes: width and length.

*Width* refers to the number and coverage of your sampling. How many patients do you have in your study? What populations, sexes, ethnicities, lifestyles, or other groupings do those patients come from? Width is the most common type of sample size considered and addresses the question of whether patterns that you observe in your study can be generalized to larger populations. Of course, simply having a large number of study participants may not be good enough. A classic cautionary tale in medicine is the fact that many modern practices were built on studies of white male adults (how much of medicine is based on army research?) [2]. Even with thousands of participants, many of these conclusions may not translate to other populations, such as women or other ethnicities, who each have their distinct biological contexts.

*Length* refers to sampling from the same individuals through time. How many times do you measure your outcomes in your patients? For how long do you follow your patients to catch long-term effects? How much did you know about a patient’s medical history before they entered your study? Fundamentally, longitudinal sampling captures information about each individual’s trajectory through time, offering the opportunity to predict and understand diseases as a developmental process. Such sampling is often logistically difficult to obtain and has often been overlooked in comparison to the importance of sampling many people. But as medicine has matured as a field, we are beginning to appreciate the importance of time in disease development.
The birth of personalized medicine is one reflection of this appreciation, emphasizing the effects of each individual's unique physiology and medical history in shaping their development of disease and responses to treatment [4].

The projects in this thesis all suffer from limitations in the width and length of their samples sizes. In Chapter 2, I describe how the mobile element inhabiting the mutS gene was widespread among various clinically relevant bacteria, but only in fact tested the element’s excision in a small number of those other species. Without greater width of sampling across more bacteria containing this mobile element, it will remain unknown whether this element plays a similar hypermutator role in other bacteria as it does in *Vibrio splendidus*. In Chapter 3, I describe the use of PMA-seq to analyze the effects of processing on a small number of stool samples from the same healthy donor. Here again, we must temper our conclusions from this study because of insufficient width of sampling. More samples across many healthy donors would be necessary to confirm that the changes identified in this donor’s stool translate to other gut microbial communities, which will have entirely different compositions and characteristics. In Chapter 4, we address a critical limitation of many previous immunosequencing studies by sampling across time. Nevertheless, this came at the cost of width (N = 3), and further studies on a larger number of individuals are critical to confirm our conclusions in a broader sense. Furthermore, even our longitudinal sampling scheme had only monthly time points, meaning that it remains unknown how variable the immune repertoire is on shorter scales of days or weeks. Similarly, in Chapter 5 we tackled the lack of longitudinal data in fecal transplant studies, but again at the expense of width. In part because of the rigorous design of the study (randomized and controlled), we only recruited 6 patients who received fecal microbiota transplant and 12 patients overall. While we observed a remarkable variety of responses to fecal transplant, understanding what factors drive these variations in response will require much larger studies with a greater number of patients.

Despite the perhaps inevitable conclusion that bigger is always better, I am optimistic about the ability for these studies to contribute fundamental and translatable knowledge to the field. Researchers have to balance the nature of the questions asked with the resources and time available to recruit and process research participants. Not all studies can have 10,000 patients, nor do all studies need 10,000 patients. For example, our study of fecal transplants in patients with inflammatory bowel disease required significant investment from the patients over a four month period. For this reason, patient recruitment was extremely difficult. But because of our effort and that of the patients, we now better understand the longitudinal outcomes of fecal microbiota transplants, which can help shape the design of future, more streamlined studies that could more easily capture a larger number of patients. We must resist the temptation that more is always better and instead ask ourselves “how much is enough?” What number and what kinds of patients do I need to answer my burning question?

### 6.1.1 Street lights

The streetlight effect is named for a joke, in which a police officer comes upon a man looking for his wallet in the illuminated ground underneath a streetlight. The police officer asks where the man lost his wallet, to which the man replies “over there in the park, but this is where the light is.”
Another cornerstone of the limitations of scientific research, the streetlight effect means that we can only find what we can see, that our ability to discover depends entirely on the tools we have to inquire. The studies described in this thesis do not escape from this limitation, with important implications. For example, both Chapter 3 and Chapter 5 focus on the bacteria in fecal microbiota transplants, and with good reason, since it is often believed that the bacteria are the driving force behind the therapeutic efficacy of these treatments [5,6]. But the bacteria are also the best studied and most easily measured component of the microbiome. Fungi [7], microeukaryotes [8], and other organisms also inhabit the gut microbiota, not to mention the mix of food, proteins, macromolecules, metabolites, and other abiotic matter that fill the gastrointestinal tract. In fact, there is weak evidence that fecal transplants may still cure C. difficile infections when bacteria are filtered out of the transplant material [9]. Such reports rightly insert a note of skepticism in our interpretation of gut microbiome research, since papers so often ignore other aspects of the gut community or host interactions. In Chapter 4, we assess the dynamics of individual T cell receptors, but largely ignore the type of T cell those receptors are expressed by, which could have huge effects on that receptors role in the immune system. However, such paired receptor-immune function data is more difficult to generate, particularly at the scale we desired. Because of this, we have to carefully consider what questions our data can actually answer.

At the beginning of this thesis, I introduced how the advent of high-throughput science opened up a radically new scale at which we could quantify biological systems. But despite descriptors like “systematic”, “high-throughput”, and “deep”, these methods are still limited by the streetlight effect. Although powerful, they still measure specific aspects, and we would be well served to continue to limit our conclusions from these new tools to the questions that they can practically answer.

6.2 Extensions

Each of the chapters presented here stand on the shoulders of previous work and, in their turn, set the stage for additional studies.

In Chapter 2, the most obvious outstanding question is what physiological effects these mobile elements may have in bacteria other than V. splendidus. Although we appear to rule out the effect of hypermutation by nonsense mutation in a small number of strains, it remains possible that the action of this mobile element may serve to alter the activity of the mutS gene or its regulation, thus otherwise playing a part in modulating mutation rate. It also has yet to be tested whether any of the other hundreds of bacterial species containing these elements may have the same phenomena as we discovered in V. splendidus or yet still different mechanisms of altering mutation rates. Another remaining question is what role this mobile element plays in the evolution of natural populations, given that it was discovered in a lab settings under artificial selection pressure.

In Chapter 3, a clear follow up study is whether or not these changes caused by fecal material processing have an effect on clinical outcomes or bacterial colonization in fecal transplant patients, and, in fact, such a study is already underway in a clinical cohort of C. difficile patients. Although this clinical study is more relevant to human health, another potential avenue to evaluate the downstream effects of these treatments could be based on viability in laboratory culture, which separate viability from the additional factors of patient colonization. PMA-seq profiles the living portion of the bacterial community, but most of the bacteria most
severely affected by oxygen exposure were still present in the living fraction in appreciable amounts. If we ignore abundance, can we identify taxa that cannot survive even brief oxygen exposure? Such an observation would be extremely surprising, given that any gut bacteria so sensitive would have great difficulty in spreading across human hosts. Nevertheless, it is possible that in the context of fecal transplants, some portion of the community is lost, which could have therapeutic consequences.

In Chapter 4, our results left us wondering what role do persistent and public T cell receptors play in the immune system? Although many previous studies have observed that—against the odds—many individuals share public receptors [10], this newly identified subset of receptors further defines an even more intriguing subset. Why are these receptors so common and maintained at such a high abundance? Using yeast display libraries [11] and computational searches, one could reasonably identify the peptide antigens that trigger these T cells as well as their sources. Do these cells respond to common pathogens, commensal organisms, latent viruses, ubiquitous foods, or perhaps even endogenous human cells or proteins? Do patients with immune regulatory conditions also share these receptors? In addition, it is an open question how these receptors are maintained. In a simple model, these cells may be consistently exposed to their antigen across time and individuals, resulting in their continued proliferation. But it is possible that other immune mechanisms might be driving their proliferation as well. Although this hypothesis is quite speculative, identifying mechanisms to maintain certain immune receptors at high titers could pave the way for new immunotherapies. In any case, understanding both how and why these receptors are maintained would greatly deepen our understanding of adaptive immune maintenance.

Of the many channels of study left open by the project in Chapter 5, perhaps the three most promising are (1) to assess the relationship of colonization stability with clinical outcomes in a larger patient cohort, (2) to examine the similarities of IgA labeling from fecal transplant donors to patients in a larger patient cohort, and (3) to characterize the relationship between R. gnarus and the gut immune system. The first two proposed studies largely seek to establish the relevance of our results in a greater number of patients. But, as mentioned in the previous section, doing such an exhaustive study would be impractical for large numbers of patients. So what lessons can we take away from this study to shape the design and execution of a larger clinical trial? From our results, I take away that in an ideal case, 4 time points are necessary to capture the majority of the microbiome dynamics: baseline, 3–4 weeks after initial fecal transplant, the end of treatment at 12 weeks, and a follow up. Depending on the treatment schedule, the end of treatment sample might be skipped. For clinical assessments, I feel that IBDQ, calprotectin, lactoferrin, and—if possible—serologic biomarkers like C-reactive protein or cytokines would be valuable to collect at these same time points. More invasive colonoscopic assessments might be taken at fecal transplant delivery and at the end of treatment, like in our study. By sampling at these points, we capture a patient’s baseline, peak of transplant colonization, and longer-term stabilization. Hopefully, this 4-time point design would accommodate a larger number of patients.

One conclusion of our IgA results was that the rules governing IgA labeling gut bacteria are largely unknown and cannot fit into a simplistic pathogen-commensal model. By evaluating the changes in IgA labeling across many more fecal transplant recipients, we might come to understand which exact microbial strains are consistently labelled or unlabelled across individuals and which strains vary in their labeling in different hosts. What characterizes bacteria that are always labelled by IgA? Do all of these IgA’s target the same or similar peptide
antigens? What characterizes the bacteria that are variably labelled? How does this variation alter the microbiome’s activity in the gut? What role does the host immune system (like Human Leukocyte Antigen (HLA) type) play in whether or not these responses are conserved or different?

But while understanding these broad patterns may set the stage for gut mucosal immunity, deeper studies into specific interactions would greatly strengthen our ability to make mechanistic conclusions. The interactions of *Ruminococcus gnavus* and the immune systems is a particularly promising area. We found that the bacterium is reliably IgA labelled in both a healthy donor and our patient cohort, and like previous work, we find that it is both enriched in inflammatory bowel disease patients and exhibits bloom dynamics [12]. Previous work has suggested that there are multiple subclades of this species, only some of which are associated with inflammatory bowel disease [12]. Thus, a number of questions remain. What effect does IgA labelling have on *R. gnavus*? Does this bacteria inhabit the gut mucosa? If so, by what mechanism? Do IgA’s from different patients target the same proteins or peptides on *R. gnavus*? How does this bacteria’s role and behavior change between an inflamed gut in inflammatory bowel disease patients versus in a healthy gut in healthy subjects? Answering such questions would further our understanding of the workings of the gut immune system and be perhaps the first work to characterize how a single host-microbe interaction operates in the context of inflammatory bowel disease.

And it is this final line of inquiry—how does mechanism X affect disease Y—that provides a cardinal direction for any such follow up work. Although the studies outlined in this thesis largely sought to build foundational knowledge about the dynamics of the human-microbe interface, we pursued them with the goal of enabling new therapies and methods of clinical care. Fundamental science—science for science’s sake—must be pursued, but I believe in most cases fundamental question can almost always be asked through a translational lens. Although a simplified, laboratory-bound toy system might be the easiest to demonstrate a phenomena, often times with slightly more consideration of experimental design one can instead probe the same questions while using methods or biological systems with greater translational potential. To me, this is the best lesson I took from Eric and I will take from my PhD. The compromise between impact and interesting science, between discovery and application is almost always unnecessary. With some thought, soul searching, and a critical assessment of one’s own mission, one can find the areas where the love of science overlaps with a broader purpose. I move forward from my PhD armed with this knowledge and ready to step into this overlap.
Bibliography


Appendix A

Supplementary Information for Chapter 2

**Fig S1.** Hypersalinity selection experimental design. Each colony growth on a plate corresponded to ~24 generations, and each liquid medium culture corresponded to ~8 generations.

**Fig. S2.** A novel mobile element excises from the host chromosome. (a) Normalized likelihood
of binomial models with different transition frequencies given the mutation data. The null model is based on literature reports of average transition frequencies across multiple bacterial species. Lack of sequencing coverage indicated a 27-kb deletion shared by both hypermutator lineages. Reads mapping to this DNA region resulted from misalignment in genes with high similarity to other regions of the genome. (e) In V. splendidus 12B01, the orphaned start sequence to mutS—but not the mobile element-provided start sequence—matched the sequence of closely related isolates with no mobile element. (f) attP and attB sites of the mobile element revealed close homology. (g) qPCR results of the ratio of attB abundance during stationary and exponential growth (attB abundance during stationary growth divided by attB abundance during exponential growth) show that mobile element excision appears greater during stationary-phase growth. (h) Altered starburst morphology of hypermutator lineages grown on agar plates.

Fig. S3. Phylogeny of Vibrio hosts and mobile elements. (a) Phylogeny of genomes from
across the genus Vibrio, indicating in red those containing mobile elements within mutS. These elements were not contained within a monophyletic group. (b) Phylogeny of integrases from mobile elements within mutS. Elements are labeled by bacterial host genome and phylum and give evidence that these elements have been transferred horizontally. (c) In close relatives of 12B01, the phylogeny of proteins adjacent to the mobile element within mutS follow host phylogeny but not mobile-element phylogeny. For each gene, maximum likelihood trees show bootstrap support from 1,000 bootstraps as a percentage. The disagreement between trees built using host and mobile element genes indicates that only the mobile element was horizontally transferred between 12F01 and 13B01.

**Fig S4. Diagrams of mobile elements within mutS.** (a) Elements in E. norvegicus FF-162, E. coli 536, B. multivorans CF2, and P. putida F1. (b) The HTPMMQQ motif is part of the mismatch-binding region of MutS. The region appears to interact with the DNA backbone in crystal structures. Pink arrows indicate the amino acid motif in a 3D crystal structure of E. coli K-12 MutS complexed with a G-T mismatch.
**Fig S5.** Escherichia coli mutators also exhibit higher variance than nonmutator strains. Like Fig. 1c, colored bars represent the variance in the number of mutations gained during each time interval versus the Poisson model expectation. Because the sampling did not follow a regular generational interval, we split data before analysis into genomes that were sequenced 500, 5,000, and 10,000 generations apart. Nonmutators largely followed a Poisson model, but mutators exhibited much higher variance in mutation rate compared with the Poisson model expectation. This is an original figure and analysis using data from reference 40.

For files containing table data, please see the published tables online.

**Table S1.** Composition of SNP mutations accumulated across all experimental lineages.

**Table S2.** Mobile elements within mutS occur across Beta- and Gammaproteobacteria. Strains of Beta- and Gammaproteobacteria that have related integrases—identified by BLASTP—adjacent to the mutS gene. Strains that were manually confirmed to contain a putative mobile element within mutS are noted.

**Table S3.** Percent identity matrices for genes from close relatives of 12B01. This table includes the 16S, mutS, cinA, rec A, and the mobile element integrase genes from four closely related strains of *V. splendidus* with mobile elements within mutS.

**Table S4.** Genes with SNPs in multiple lineages after five rounds of salt selection.

**Table S5.** Strains and primers used in this study.
Appendix B

Supplementary Information for Chapter 3

Fig S1. Experimental design for oxygen exposure experiments.
Fig S2. PMA-seq H2O controls verify that PMA, not other PMA-seq processing steps, alters 16S sequencing results. (a) Beta diversity between H2O controls, standard 16S sequencing, and PMA-seq results. (b) Stacked bar plots and (c) individual bar plots of relative abundance of bacterial genera in anaerobic + cysteine and aerobic preparations from data generated using standard 16S sequencing, PMA-seq, and H2O controls.
Fig S3. Normalized abundances of bacterial taxa with increasing oxygen exposure. Stacked bar plots of relative abundances are normalized to total community size, which we estimated with qPCR.
Fig S4. Raw abundance data for oxygen exposure experiments. Data from two stool samples prepared with varying degrees of oxygen exposure and analyzed using standard 16S sequencing or PMA-seq. (a) Stacked bar plots and (b) individual bar plots of relative abundances of different bacterial genera observed with both sequencing methods from stool sample 1; (c) and (d) show results from stool sample 2.

Fig S5. Using the Poisson log-normal distribution to identify OTUs that changed significantly in relative abundance. Histograms of (a) Δ2 and (b) ΔF scores of OTUs calculated between technical replicates (cyan) and across oxygen, freeze-thaw, and lag time preparations (red). Fit lines are generalized normal distributions. Dotted blue lines indicate the 0.05 cutoff for the
distribution of $\Delta z$ and $\Delta F$ scores between technical replicates. In the case of oxygen exposure, the distribution of $\Delta z$ and $\Delta F$ scores between transplant preparations was much wider than the distribution of $\Delta z$ and $\Delta F$ scores between technical replicates, indicating pervasive shifts in abundance. In contracts, the same distributions for freeze-thaw and lag time preparations look very similar.

Fig S6. Individual OTUs within bacterial genera showed variable responses to oxygen exposure. Log-transformed relative abundances of the five most abundant OTUs from common bacterial taxa. Error bars represent standard error. Although OTUs within the genera *Faecalibacterium* and *Megamonas* responded similarly to oxygen, we observed different responses from OTUs within *Ruminococcus*, and *Oscillospira*. 
Fig S7. PMA-seq registers little alteration of the living bacterial community with more freeze-thaw cycles. (a) Stacked bar plots and (b) individual bar plots of the relative abundances of various bacterial genera with more freeze-thaw cycles, as identified by PMA-seq.
Fig S8. PMA-seq registers little alteration of the living bacterial community with longer lag times. (a) Stacked bar plots and (b) individual bar plots of the relative abundances of various bacterial genera with longer lag times, as identified by PMA-seq.
Fig S9. *Normalized abundances of bacterial taxa with more freeze-thaw cycles.* Stacked bar plots of relative abundances are normalized to total community size, which we estimated with qPCR.
Fig S10. *Normalized abundances of bacterial taxa with longer lag times.* Stacked bar plots of relative abundances are normalized to total community size, which we estimated with qPCR.
Fig S11. $\Delta z$ scores are largely unaffected by sequencing depth. Mean $\Delta z$ scores for each OTU plotted against the mean abundance for that OTU. Data shown are from six transplant preparation comparisons from two stool samples.
Fig S12. ΔF scores appear to be affected by sequencing depth. Mean ΔF scores for each OTU plotted against the mean abundance for that OTU. Data shown are from six transplant preparation comparisons from two stool samples.

For files containing table data, please see the published tables online.
Table S1. Taxonomic information of OTUs identified as differentially abundant between transplant preparations according to our Poisson log-normal analysis pipeline.
Table S2. Primers used in this study.

Data S1. Full OTU table with OTUs clustered at 97% similarity.
Data S2. OTU table of 77 high-confidence OTUs—ones present in all samples—clustered at 100% similarity.
Data S3. Sample metadata.
Appendix C

Supplementary Information for Chapter 4

Figure S1. Representative frequency rank plots for memory T cells, naive T cells, and all T cells from PBMCs from Individual 01. As expected, naive T cells had fewer abundant clones than PBMC or memory T cells. In all cases, the majority of TCRβs had abundances around $10^{-6}$. 
Figure S2. Rarefaction curves for each subject indicate that sample libraries were sequenced well past saturation.
**Figure S3.** Analyses examining only high-abundance TCRβs agree with results from full-repertoire analysis, suggesting that undersampling likely did not confound our results. (a) A heatmap of Jaccard indexes shows similar clustering of PBMC and memory T cell samples by individual and less clustering of naive T cell samples. Abundances of high-abundance TCRβs in PBMC samples correlated within an individual (individual 01) across time points, including across a month (b, shared TCRβs = 2057, Spearman $\rho = 0.66902, p < 10^{-6}$) and a year (c, shared TCRβs = 1390, Spearman $\rho = 0.59251, p < 10^{-6}$). High-abundance TCRβs did not appear to correlate across individuals, largely because of lack of shared TCRβs (d, shared TCRβs = 7, Spearman $\rho = 0.14286, p = 0.75995$). Shannon alpha diversity estimate (e) and clonality (defined as $1 - $Pielou’s evenness, f) of the TCRβ repertoire were consistent over time.
**Figure S4.** TCRβ repertoire overlap (Jaccard index) often decreases with increasing time between samples, except in Individual 02, where the final time point at one year past the first sample shared more TCRβs with the previous samples. Different colors depict changes over time relative to each sample. For example, the blue line depicts overlap between each sample after the first sample and the first sample, while the green line depicts overlap between each sample after the second sample and the second sample.
Figure S5. V gene usage across time and cell compartment in all three individuals. Each of nine plots represents each cell type in each individual. Within each plot, different colors represent different V genes, and each dot represents a comparison of the abundances of that V gene from one sample to another. Points that fall near a 1:1 ratio (indicated by the dotted line) are nearly identical in abundance between the two samples considered. These plots indicate that VJ gene usage was generally the same across time points, particularly in total and memory T cells. In naive cells, VJ gene usage varied more.
Figure S6. J gene usage across time and cell compartment in all three individuals. Plots are as in Figure S5, with similar findings.
Figure S7. Cohorts of TCRβs exhibit correlated dynamics over time. We found large cohorts of correlating TCRPs by Spearman (a) and Pearson (b) correlation. Although these TCRPs spanned a range of abundances, we did not observe any clear signs of correlation caused by sequencing or library preparation errors (Table S2). We also found smaller cohorts (c) of TCRPs with nearly identical abundances whose dynamics also correlated through time. The number of TCRPs found in all cohorts was significant ($p < 0.001$) in a random permutation test (see Methods). These TCRβ cohorts might be an artifact of sampling noise, or they may represent receptors involved in the same immune response.
Figure S8. Persistent high-abundance TCRβs exhibit similar patterns as overall persistent TCRβs. (a) High-abundance TCRβs had a greater prevalence of persistent TCRβs, although the exact values varied across individuals. Persistent high-abundance TCRβs also showed greater mean abundance (b) and nucleotide redundancy (c). Persistent high-abundance TCRβs also had higher proportions of TCRβs in common with memory (d) and naive (e) T cell populations and constituted a stable and significant fraction of overall TCRβ abundance across time (f).
a

Full CDR3 sequence
Trimmed by 3 nucleotides
Trimmed by 6 nucleotides
Trimmed by 9 nucleotides
Trimmed by 12 nucleotides

Individual 01

Nucleotide redundancy

Individual 02

Individual 03

Observed at n time points

b

Full CDR3 sequence
Trimmed by 3 nucleotides
Trimmed by 6 nucleotides
Trimmed by 9 nucleotides
Trimmed by 12 nucleotides

Nucleotide redundancy bin

Observed at n time points

TCRs

Individual 01

Individual 02

Individual 03
Figure S9. Nucleotide redundancy across individuals and with more stringent assignment of CDR3 sequence (figure supplements Figure 2c). (a) Each plot represents nucleotide redundancy for TCRβs that were observed in n samples. Rows represent plots for each individual. The leftmost column of plots comprises data from full CDR3 nucleotide sequences as identified by IMGT (as in Figure 2c): we observed that the pattern of increasing nucleotide redundancy in persistent TCRβs was not consistent across individuals. Each of the following columns plot data from CDR3 nucleotide sequences that were progressively trimmed on each end by 3, 6, 9, and 12 nucleotides. We trimmed these sequences because CDR3 sequences identified by IMGT generally capture a number of amino acids—usually one to four at each end of the sequence—that are derived from V and J genes. Nucleotide mutations in these leading and trailing ends are thus less likely to be of biological origin and more likely to be from sequencing error, since we do not expect nucleotides from the V or J genes to be altered during TCR recombination (except for deletions). From these plots, we can observe that nucleotide redundancy is generally stable over different lengths of trimming, suggesting that our data are not skewed by these potential sequencing errors. (b) To further examine the relationship between persistence and nucleotide redundancy, we grouped TCRβs into 10 bins according to nucleotide redundancy. Because nucleotide redundancy is extremely skewed—the vast majority of TCRβs are encoded by a single clonotype—we created these bins on a logarithmic scale: the first bin includes TCRβs with nucleotide redundancy values up to 1.6% of the maximum value for each individual; the second between 1.6% and 2.5% of the maximum value; and up to the 10th bin, which includes TCRβs with nucleotide redundancy values between 64% and 100% of the maximum value. For each of these TCRβ bins, we then plotted a histogram of the frequency of TCRβs that were observed at n time points. We observe a clear pattern across individuals and trimming lengths: TCRβs with greater nucleotide redundancy tend to occur at more time points, and the most redundant TCRβs are exclusively persistent receptors.
Figure S10. The persistent TCRβ repertoire exhibited little alteration of CDR3 lengths.
Figure S11. The persistent TCRβ repertoire does not exhibit altered V gene usage. These plots show V gene usage in TCRβs that occurred only once (x-axis) versus in persistent TCRβs (y axis). Each data point represents a single V gene. These values were closely correlated.
Figure S12. The persistent TCRβ repertoire does not exhibit altered J gene usage. Similar plots as in Figure S10 indicate that J gene usage is not greatly changed in persistent TCRβs.
Figure S13. Distributions of the number of neighbors and degree of sharing across people for all TCRβs and high-abundance TCRβs. Each plot is a distribution of all TCRβs from PBMC samples for each individual (a, c) or only high-abundance TCRβs (b, d). Plots (a) and (b) show the number of neighbors in a network based on Levenshtein distance and plots (c) and (d) show the number of subjects sharing a given receptor in a large, independent, and similarly profiles cohort.
a Individual 01

b TCRBs shared by
> 10% of cohort

TCRBs shared by
> 20% of cohort

TCRBs shared by
> 30% of cohort

TCRBs shared by
> 40% of cohort

TCRBs shared by
> 50% of cohort

TCRBs shared by
> 60% of cohort

TCRBs shared by
> 70% of cohort

TCRBs shared by
> 80% of cohort

TCRBs shared by
> 90% of cohort
**Figure S14.** Persistent TCRβs were rich in highly public TCRβs. (a) Over all TCRβs, receptors that occurred at an intermediate number of time points were on average most-shared across people, but these distributions are heavily skewed toward private receptors. (b) We focused on TCRβs that were shared by at least a certain percentage of individuals in the large (N = 778) cohort. We found that less public TCRβs were generally observed at few time points, while highly public TCRβs were predominately observed at all time eight points. These results were even more striking given that we observed ~100–1000-fold more TCRβs occurring at a single time point than at all time points.
Observed at n time points

Individual 01

Individual 02

Individual 03

Log10(probability of TCR generation)

Log10(probability of TCR generation)

Log10(average abundance)

Log10(average abundance)
Figure S15. Persistent and public receptors may result in part from TCR recombination biases. (a) As in previous studies, the probability that a given TCRβ was generated correlated closely with publicness in a cohort of 778 individuals. For each individual, only TCRβs occurring in both that individual and the cohort were considered. (number of TCRβs evaluated in individual 01 = 638091, Spearman rho = 0.51111, p < 10^{-6}; number of TCRβs evaluated in individual 02 = 338617, Spearman rho = 0.52231, p < 10^{-6}; number of TCRβs evaluated in individual 03 = 284990, Spearman rho = 0.51129, p < 10^{-6}). (b) TCRβs occurring at more time points tended to have higher generation probabilities, although persistent TCRβs did not have higher generation probabilities than other receptors observed at multiple time points. Letters indicate significant differences from all other groups by a Mann-Whitney U test (p < 0.001), while dagger (†) indicates groups that were not significantly different from multiple other groups. (c) Mean abundance of all TCRβs correlated significantly with generation probability but with a low correlation coefficient (individual 01: Spearman rho = 0.07884, p < 10^{-6}; individual 02: Spearman rho = 0.05300, p < 10^{-6}; individual 03: Spearman rho = 0.08208, p < 10^{-6}). (d) Mean abundance of persistent TCRβs did not correlate with generation probability (persistent TCRβs: number of TCRβs evaluated in individual 01 = 3448, Spearman rho = -0.08988, p < 10^{-6}; number of TCRβs evaluated in individual 02 = 1978, Spearman rho = -0.04341, p = 0.0537; number of TCRβs evaluated in individual 03 = 2965, Spearman rho = 0.04552, p = 0.01318)

Table S1. Overall TCRβ-sequencing statistics per sample: sequencing depth, productive TCRβ sequencing depth, fraction of productive TCRβ sequences, unique V genes identified, unique J genes identified, unique CDR3 sequences, unique TCRβs, unique TCRβ nucleotide sequences.

Table S2. V gene usage across subject and T cell population, expressed as both a fraction of all unique productive TCRβs and as a mean total abundance per sample.

Table S3. J gene usage across subject and T cell population, expressed as both a fraction of all unique productive TCRβs and as a mean total abundance per sample.

Table S4. Sequence and abundance information for the largest cohort of closely correlated TCRβs identified in each individual by Spearman’s or Pearson’s correlation.

Table S5. The fraction of TCRβs in each sample that occurred in 1–8 samples from that subject’s time series.

Table S6. Mann-Whitney U test statistics for mean abundance of TCRβs occurring in different numbers of samples during the time series.

Table S7. Mann-Whitney U test statistics for nucleotide redundancy of TCRβs occurring in different numbers of samples during the time series.

Table S8. The fraction of TCRβs in each sample that were shared to different degrees among subjects in a large, independent cohort.
Additional File 1. Count, frequency, V, J, and CDR3 amino acid sequence data for high abundance TCRβs in each sample. This is a tab-delimited, gzip-compressed file. The column “tcr” corresponds to a label that identifies a unique combination of CDR3 amino acid sequence, V gene, and J gene observed in this study. The column “count (templates/reads)” represents the counts of a given receptor’s DNA sequence in the sequencing data. The column “frequencyCount (%)” is the relative abundance of that tcr within each sample, accounting for productive and nonproductive receptors. The columns “vGeneName” and “jGeneName” are the IMGT assigned V and J genes. The column “aminoAcid” is the CDR3 amino acid sequence.

Additional File 2. Data for persistence across our time series and sharing across subjects in an independent, large cohort for TCRβs in this study. The columns “aminoAcid”, ‘vGeneName”, “jGeneName”, and “tcr” are the same as for Additional File 1. The column “n_cmv_public” is the number of subjects (out of 778) that shared that TCRβ. The columns “num_occ_sub1_pbmc”, “num_occ_sub2_pbmc”, and “num_occ_sub3_pbmc” are the number of time points at which a given receptor was observed in the PBMC samples for Individual 01, 02, and 03, respectively.
Appendix D

Supplementary Information for Chapter 5
Fig S1. Related to Figure 1: instability in the gut microbiome of IBD patients after antibiotics.
(a) Turnover in gut microbiome species in healthy patients administered a course of the broad-spectrum antibiotics vancomycin, gentamicin and meropenem. (b) PCoA of unweighted unifrac
distance of 16S. (c) PCoA of bray-curtis distances based on metagenomic species. (d) PCoA trajectories of placebo patients based on metagenomics species. (e) Source-plots of metagenomic species in placebo patients. (f) Abundance of antimicrobial resistance of varying classes across the time series. Cumulative abundance (g) and overall richness (h) of antimicrobial resistance genes in patients did not differ between placebo and fecal transplant patients. We did observe possible increased abundance of (i) tetracycline and (j) aminoglycoside antibiotic resistance genes in placebo-treated patients compared with fecal transplant recipients.

**Fig S2.** Related to Figure 2: Fecal microbiota transplants in IBD patients alter community composition without affecting diversity. (a) Mean bray-curtis distance from the donor samples based on metagenomic species. Longitudinal changes in alpha diversity based on (b) Shannon diversity of metagenomic species, (c) ASV richness, (d) metagenomics species richness. (e) Bray-curtis distance from baseline samples based on metagenomic species.
Fig S3. Related to Figure 3: longitudinal maintenance of transferred bacteria. Tracking bacterial sources identified bacterial species transferred from donor to recipient as well as an invasion of bacteria of unknown origin. Each time-series plot indicates the fraction of total metagenomic species that were identified as from the patient, donor, shared between the two, or from an unknown source. Regions of the graph colored as in Figure 1.
Fig S4. Related to Figure 4: strain level transfer of donor bacteria. Using the flexible genome approach, we confirmed that samples from the same donor registered a strain match, while samples from different donors did not. Shown are the read coverage of 1-kb windows of the reference genome for *Faecalibacterium prausnitzii* for (a) two samples from the same donor and (b) two samples from different donors. (c) Strain matches against donor samples for *Bacteroides ovatus*, *B. caccae*, *B. fragilis*, and *P. merdae* indicated further instances of transferred donors strains in fecal transplant recipients. To further evaluate our flexible genome method, we confirmed that (d) samples from placebo-treated patients did not match any of the donor samples.
and (e) samples from fecal transplant recipients who received an alternate donor did not match samples from that alternate donor except for isolated cases after the alternate donor period.

**Fig S5.** Capsule delivery from an alternate donor introduced limited novel taxa. Tracking of ASVs as in Figure 3 reveals that the transfer of unique taxa from the alternate donor is limited from daily capsules.
**Fig S6.** Related to Figure 5: transfer of functional capacities. (a) Log10 cumulative abundance of all virulence factors in each patient across the trial period. (b) Richness of all virulence factors across the trial period. (c) Cumulative abundance of antimicrobial resistance genes by source. (d) Cumulative abundance of virulence factors by source. (e) Cumulative abundance of Proteobacteria in Patient V during the trial period. (f) Cumulative abundance of virulence factors across the trial period for all patients.
Fig S7. Related to Figure 6: IgA coating of gut microbes. Some patients’ baseline samples had much greater correlation of IgA enrichment with a healthy donor than others. Line plots (a) and corresponding Pearson r correlation values (b) for each patient at baseline. IgA enrichment did not correlate with a bacterium’s (b) abundance, (c) variance, or (d) bimodality.

Not included due to size

Fig S8. Phylogenetic tree of bacteria showing the IgA enrichment across samples and patients. Phylogenetic tree was constructed on the basis of 16S sequences. Each column is a heatmap corresponding to each patient, indicating for a given patient and timepoint what.

TABLES

Table S1. Metadata for stool samples collected, including patient clinical data and sequencing depths for 16S and shotgun-metagenomic sequencing.

Table S2. Bacteria lost, gained, or maintained by placebo patients after the administration of antibiotics on the basis of 16S sequencing.

Table S3. Bacteria lost, gained, or maintained by placebo patients after the administration of antibiotics on the basis of metagenomic sequencing.
Table S4. Number of FMT patients receiving fecal material from Donor am (out of a total $n = 4$) that either (1) shared the ASV with the donor at baseline at after fecal transplant, (2) showed persistent colonization of the ASV from the donor, or (3) showed temporary colonization.

Table S5: Transferred genes and associated taxa of butyrate biosynthesis genes.

Table S6: Additional metadata for each patient in the trial.

Table S7: Taxa identified as reliably IgA-coated, IgA-uncoated, and variably IgA-coated or -uncoated