Title: Within-host evolution of the gut microbiome

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Abstract

Gut bacteria inhabit a complex environment that is shaped by interactions with their host and the other members of the community. While these ecological interactions have evolved over millions of years, mounting evidence suggests that gut commensals can evolve on much shorter timescales as well, by acquiring new mutations within individual hosts. In this review, we highlight recent progress in understanding the causes and consequences of short-term evolution in the mammalian gut, from experimental evolution in murine hosts to longitudinal tracking of human cohorts. We also discuss new opportunities for future progress by expanding the repertoire of focal species, hosts, and surrounding communities, and by combining deep-sequencing technologies with quantitative frameworks from population genetics.

Introduction

The microorganisms that inhabit a particular host, collectively known as the microbiota, are intimately intertwined with their environment and play an important role in influencing the health of their host. These host-associated communities are often noted for their high taxonomic diversity, particularly in the mammalian gut, where hundreds of species coexist with each other in close physical proximity [1]. Millions of years of evolution have shaped these symbiotic interactions [2], producing a diverse array of commensal gut species and vast amounts of strain-level variation at finer levels of genomic resolution [3-5]. Many commensal strains appear to be particularly well-adapted to the environment of their host species [6-10], suggesting that long-term evolution has played an important role in producing and maintaining this specificity [11-13]. While these long-term effects of evolution are widely appreciated, it has only recently become apparent that gut commensals can evolve on host-relevant timescales as well. Time-resolved sequencing has started to illuminate this process, with a growing number of examples, first in mice [14-22] and more recently in humans [23-31], showing that genetic changes can sweep through resident populations of gut bacteria over years, months, and even days. This capacity for rapid evolution has underexplored relevance for the structure and function of the gut microbiota.

These observations of ongoing evolution of commensal gut bacteria may initially seem surprising. Many of these species have inhabited their mammalian hosts for millions of years [12,13,32], and are not thought to engage in the immunological arms races that are common in bacterial pathogens [33]. Under these conditions, any strongly beneficial mutations should have already had an opportunity to fix long ago. Larger microbial communities also have the ability to adapt to changing environments through purely ecological means (e.g., shifting the abundances of the resident species, or acquiring a new strain from outside the host [23,34]), which could foreclose opportunities for additional within-host evolution. However, there is a growing recognition that some changes in the host environment (e.g., dietary shifts, the presence or absence of a particular community member) can create new opportunities for local adaptation that are not negated by shifts in the abundance of the resident strains. In these cases, mutations can create novel genotypes that are better adapted to the altered environment than their parent genotype, often involving subtle changes in metabolic capabilities [16-18,20,21]. The fitness advantages of these mutations are typically small by physiological standards (e.g., ≤10% change in growth rate), but these small advantages are more than sufficient to drive large shifts in frequency within a host over hundreds of bacterial generations (10-100 days). Given the

large population sizes in the mammalian gut (>10^{11} cells), even a miniscule mutation rate for these locally adaptive mutations (>10^{-10} per generation) will generate multiple such mutations within a host each day.

This ongoing evolution can have important functional consequences. Some genetic variants have been observed to alter metabolic phenotypes [15,17,18,21,35], the breakdown of drugs [36,37], the spread of antibiotic resistance phenotypes [38], or colonization resistance against pathogens [39]. Genetic modifications can also alter ecological interactions between species [40,41], driving broader shifts in the taxonomic composition of the host community. These dynamics could have important implications for probiotic therapies (e.g., fecal microbiome transplants), since they suggest that the ecological interactions between resident strains could strongly depend on their personalized history of co-evolution. More broadly, many microbiome experiments involving a controlled environmental shift typically focus on changes in taxonomic composition and gene expression, which are intrinsically dependent on the immune system, diet, and biophysical aspects of the host environment. Even though all of these factors are selection pressures, the potential for genetic adaptation over the same experimental timeframe is often ignored. Host factors that have previously been associated with microbiota

dysbiosis (inflammation, obesity, behavior, and circadian rhythm) may also be influenced by the accumulation of new mutations during the experiment. In this manner, the capacity for rapid bacterial evolution could have far-ranging implications for how the microbiome influences host physiology.

Despite the potential importance of these effects, the causes and consequences of within-host evolution in the gut microbiota are only starting to be explored. In this review, we highlight some of the key open questions, as well as new opportunities for progress using tools from genetics, sequencing, and systems biology.

What factors determine the evolutionary selection pressures within the mammalian gut?

Gut bacteria evolve in a complex environment, which is shaped by interactions with their host as well as other members of their local community. Understanding how these factors contribute to the evolutionary selection pressures within the gut has been a major focus of recent research (Fig.1).

Experimental evolution in murine hosts has been a powerful tool for addressing these questions. Much of this work has utilized *Escherichia coli* as a "focal"

112 species [15,20-22,43], due to its genetic tractability and ease of isolation. By
113 sequencing evolved strains from one or more host populations, the targets of
114 selection can be inferred from parallel mutations that repeatedly occur in the
115 same genes or pathways (Fig. 2). These experiments have shown that *E. coli*
116 evolution is remarkably predictable across hosts with the same diet and genetic
117 background [15,21]. However, both the number and types of mutations can vary
118 dramatically under different host conditions. For example, *E. coli* accumulate
119 fewer mutations in immunocompromised mice compared to wild-type mice, and
120 the fitness of reconstructed mutants differs between the two host genotypes [14].
121 The targets of adaptation can also vary with the age of the host [16,44], shifting
122 from metabolic functions in young mice to stress-related functions in older mice,
123 which could reflect their higher levels of gut inflammation [44]. These findings
124 suggest that some of the selection pressures in the mouse gut are shaped by
125 interactions with the immune system, either directly (through host-microbe
126 interactions) or indirectly (through altered microbiota composition).
127
128 Extrinsic host factors like diet can also shape the evolution of gut commensals.
129 The model human commensal *Bacteroides thetaiotaomicron* acquires different
130 mutations in mice with a diet high in plant polysaccharides and fiber versus a
131 diet high in fat and simple sugars: the latter selects for mutants with enhanced

ability to consume host-derived glycans [17], and that had increased fitness when plant polysaccharides and fiber are absent [45]. Moreover, weekly alternations between diets leads to oscillating frequencies of diet-selected mutations [17,46], indicating that even transient fluctuations in available nutrients can present a strong selection pressure.

In addition to host factors, other members of the microbiota can play an important role in shaping the selection pressures experienced by a given focal species. The mutations acquired by *E. coli* can be altered by co-colonization with a single additional gut species (*Blautia coccoides*), shifting from mutations that increase *E. coli*'s ability to compete for amino acids to those involving anaerobic respiration [15]. Evolution is also affected by intra-species interactions: while *E. coli* evolve via *de novo* mutations in many mouse evolution experiments [15,16,21,22], the presence of a resident mouse *E. coli* strain can shift the evolution of invading strains toward horizontal acquisition of prophage elements from the resident [47,48]. These observations echo behavior of the human gut microbiota, for which horizontal gene transfer has been observed both within [23,29,49,50][and between [31,50,51] resident gut species.

Conceptual and quantitative frameworks for interpreting short-term evolution

in the gut

With this recent proliferation of experimental data, there is a growing need for modeling approaches that can synthesize these diverse observations of microbiota evolution into a common conceptual framework.

Two contrasting models are often invoked to explain the rapid evolution of commensal gut bacteria. The first (known as "niche filling" [52]) proposes that evolution is mainly driven by mutations that allow species to exploit underutilized niches, which could arise from mismatches between bacteria and hosts (e.g., when human gut commensals are evolved in mice) or from the absence of normal competitors (e.g., during monocolonization). This model predicts that as more and better adapted species are added to a community, the space of open niches shrinks, producing fewer avenues for beneficial mutations [53,54]. However, an alternative view (known as "diversity begets diversity") holds that more diverse communities provide more opportunities for adaptation to the functions of other community members, e.g. by exploiting metabolic interactions [55] or by resisting interspecies competition such as type VI killing [56] or phage [47,48,57].

Empirical support for these models is currently mixed. While a recent study of

rainwater pools found that evolution was slower in more diverse communities [54], previous observations in the mouse gut showed that *E. coli* strains acquire similar numbers of mutations in monocolonized mice as they do with a diverse microbiota [15,48]. Similarly, observational data from humans suggests that the frequency of within-host sweeps is largely flat (or slightly increasing) over the diversity ranges typical of healthy human gut microbiotas [58].

These results highlight the challenges of defining the tempo of evolution in different community contexts. The qualitative models introduced above are often too simplistic (and hence too flexible) to explain evolutionary dynamics across experiments in which many variables change at once. Even well-defined genetic quantities, like the number and types of mutations that are observed in sequenced isolates (Fig. 2), depend on basic parameters such as population size and mutation rate [59-61], which can vary across hosts and in different community contexts. Inspired by population genetics theory and *in vitro* evolution experiments, we argue that a useful approach is to focus on the distribution of fitness effects (DFE) of new mutations (Fig. 3A,B), which summarizes the spectrum of mutations available to an organism in its current environment before they are amplified by natural selection. While environments are often defined in terms of abiotic factors such as growth medium, for the

192 microbiota the concept of environment must be generalized to include both
193 intrinsic and extrinsic host factors (e.g., diet) as well as the composition of the
194 surrounding community (Fig. 3C,D).
195
196 Together, the population size and the DFE control both the rate of adaptation
197 and the number and types of mutations that reach high frequency within a
198 population (Fig. 3E). By enumerating the spectrum of adaptive mutations before
199 they are amplified by selection, the DFE provides a metric enabling quantitative
200 comparisons of the adaptive landscape across environmental contexts. For
201 example, the DFE can distinguish between scenarios in which the number of
202 adaptive pathways increases or decreases as the surrounding community
203 changes, as well as between scenarios in which the magnitude of fitness effects
204 changes but the total number of adaptive mutations remains constant. This
205 ability enables quantitative tests of the two qualitative hypotheses discussed
206 above. Furthermore, by considering the joint distribution of fitness effects (JDFE)
207 across multiple environments (Fig. 3F), this concept can be extended to predict
208 the fitness tradeoffs that are likely to arise during evolution to conditions [62].
209 Such measurements are critical for understanding the contingency of the
210 adaptive mutations that arise in different environmental contexts.
211

DFEs have been enormously useful for understanding and quantifying evolutionary dynamics in laboratory evolution experiments [63-65], but their applications to gut microbiota evolution have so far been limited – largely due to difficulties in sampling the requisite number of adaptive mutations. The parallel mutations observed in isolate or metagenomic sequencing constitute a small and biased slice of the DFE, since they have already been filtered by natural selection [61]. Genome-wide transposon insertion sequencing (TnSeq) approaches have emerged as a promising tool for measuring the DFE of all single gene knockout mutations *in vivo* [66-69]. While existing TnSeq studies have largely provided information regarding deleterious mutations (i.e., genes whose presence is beneficial), recent work has shown that these libraries can also be used to identify spontaneous beneficial mutations that accumulate in these populations over time [46]. Thus, TnSeq could provide a scalable approach for quantifying the spectrum of adaptive mutations *in vivo*, and how it varies across focal species, diets, and community backgrounds.

In addition to *de novo* mutations, resident populations can be outcompeted by other strains of the same species that invade from outside the host. These strain replacement events have been observed in mice [21] and humans [23,26,28,70-72], and depend on the migration rates between hosts, as well as the ability of the

invading strains to expand to high frequencies in their new environment. The JDFE concept can also be extended to enumerate the fitness of circulating strains within the global strain pool (and the potential tradeoffs they encounter in their transmissibility). Such measurements will be critical for understanding the competition between local adaptation and transmission across multiple host communities.

How does evolution influence ecological structure?

While much work has focused on how community context influences evolution, a key related question is how short-term evolution impacts microbiota structure and function. If the niches of different species are relatively fixed and disconnected, then evolution would be expected to have a minimal effect on ecological structure, and then primarily on closely related species. However, if beneficial mutations can alter ecological interactions between species (e.g., by acquiring a new pathway via horizontal gene transfer, selection of mutations in transcriptional regulators resulting in increased expression of certain metabolic pathways and increased consumption of specific nutrients, or evolving resistance to a phage that was previously limiting population size), then fixation of these mutations could change the relative abundances of other species in the community – and potentially alter future selection pressures as well.

While several studies have shown that specific genetic modifications of gut commensals can alter the relative abundances of other species [40,41,73], it is not known whether these variants are representative of the beneficial mutations that accumulate during within-host evolution. A recent meta-analysis of human gut metagenomes found that genetic changes within species are statistically associated with shifts in community composition over the same time intervals [58]. These shifts were primarily driven by the extinction of distantly related species, rather than expansion of the focal species itself. These observations are consistent with theoretical predictions from simple resource competition models, which suggest that small shifts in the resource uptake rates of a single species can produce large shifts in species abundances in communities with a high degree of metabolic overlap [58,74,75]. Future experiments are needed to establish the causal directions of these statistical associations, and to quantify the niche-altering effects of beneficial mutations more generally.

Roadmap for the future investigation of evolution in the gut

Expanding the repertoire of focal species

While the initial focus on *E. coli* was instrumental for identifying the selection pressures facing gut commensals, future work will need to focus on many other

272 species to understand which discoveries generalize across gut commensals and
273 which are specific. *Bacteroides* species provide a natural starting point, given their
274 genetic tractability [68,76-78] and their high abundance and prevalence within
275 the gut of Western individuals [79]. *B. thetaiotaomicron* is a generalist [80] that has
276 long served as a model commensal due to its ability to consume host-derived
277 glycans when its preferred nutrients (plant-derived complex polysaccharides) are
278 not present in the diet [45]. Recent experiments have started to explore how this
279 metabolic plasticity impacts how *B. theta* evolves with different host diets [17].
280
281 It will also be necessary to investigate other species with distinct lifestyles from
282 *B. thetaiotaomicron*. Other *Bacteroides* species engage in interspecies cooperation
283 through cross-feeding of extracellularly digested polysaccharides [40]. *Bacteroides*
284 *vulgatus* is a natural candidate for exploring how these cooperative interactions
285 impact evolution, due to its high abundance in the human gut [79], and its
286 relevance for human health as a potential pathobiont [81] and ability to protect
287 against *E. coli*-induced colitis [82]. Other *Bacteroides* species interact more
288 strongly with the immune system (e.g., *Bacteroides ovatus* [83] or *Bacteroides*
289 *fragilis* [84], which are both highly coated by IgA), and hence could be useful for
290 understanding of the interplay between immune system and gut commensal
291 evolution. The use of native mouse species like *Bacteroides caecimuris* could

address questions about the role of host mismatch in driving within-host evolution [85].

Despite their importance, *Bacteroides* species represent only a fraction of the genetic and functional diversity in the mammalian gut. Additional biology may be uncovered by studying the evolution of more phylogenetically distant gut commensals such as mucus degradation specialists like *Akkermansia muciniphila*, or butyrate producers like *Eubacterium rectale*, which are essential for proper maturation of the immune system [86]. *Enterococcus gallinarum*, a model gut pathobiont, evolves into two lineages within mice, specialized in colonization of either the gut lumen or mucosal niches [42]. The strain evolved for mucosal colonization through altered gene expression and cell-wall architetcture and exhibited increased ability to translocate and survive within the mesenteric lymph nodes and liver, with a trade-off of reduced transmissibility [42].

A comprehensive understanding of the evolutionary potential of a given species may also require distinguishing between finer genetic backgrounds. Recent work has shown that different mutations accumulate in laboratory *E. coli* strains compared with natural isolates [20], and that the DFEs of two *B. thetaiotaomicron* strains can systematically differ even when they co-colonize the same mice [46].

Resolving these genetic interactions will likely require evolution experiments using isolates from a broad range of hosts and/or host species, which is becoming increasingly feasible with modern strain collections [26].

Systematic modulation of the surrounding community

Further progress will also rely critically on our ability to systematically vary the biotic environment in which the focal species evolves. Comparing the evolution of focal species in monocolonized mice and simple synthetic communities has been a useful tool for uncovering host- and microbiota-dependent factors in adaptation [15]. These efforts will be facilitated by the development of larger defined communities that mimic the diversity, composition, and functionality of the full mammalian gut microbiota [85,87]. Combinatorial manipulation of these defined communities will be essential for understanding how the surrounding community influences evolutionary trajectories.

A thorough understanding of these effects will likely require orders of magnitude more experiments than are currently feasible with existing germ-free mouse setups. While living hosts will remain essential for disentangling the role of some host-dependent features (e.g., the adaptive immune system), *in vitro* evolution of synthetic gut communities provides a promising way to achieve the

required levels of replication while maintaining the complexity of the surrounding community. A future challenge is to determine to what extent *in vivo* conditions can be translated into a laboratory context; recent successes with *in vitro* passaging of stool suggest that certain rich media are reasonable mimics of the nutrient environment within a host [88]. Organoid systems and other animal models may serve as a bridge between *in vitro* and mouse evolution experiments. Insects such as fruit flies have lower diversity gut microbiotas with less complex nutrient supplies and more interspecies competition [89,90]. Hopefully, all of these models synergize to improve our understanding of general principles of evolution in the gut.

Tools for quantifying evolutionary dynamics and phenotypes

Advances in sequencing are enabling exploration of evolutionary dynamics at finer temporal resolution and at larger scale. Barcoding enables tracking of thousands of lineages across hundreds of time points from an experiment in a single sequencing run (Fig. 2B), which should facilitate the design of experiments to quantitatively evaluate the effects of host factors such as the immune system or diet, environmental factors such as housing, and community context on evolutionary dynamics. The main obstacle to barcoding is the requirement of high transformation efficiency in the species of interest, which has not yet been

352 achieved for many gut commensals.

353

354 While animal models provide a tractable system for controlled experiments, it is
355 likely that some aspects of within-host evolution may ultimately be host-
356 dependent, given differences in gut anatomy and the potential for long-term
357 adaptation between commensals and their hosts. The decreasing cost of
358 sequencing should permit longitudinal sampling of humans more densely and
359 over longer intervals to pin down evolutionary trajectories from metagenomics
360 and isolate sequencing. Cost reductions in long-read sequencing [91] will also
361 clarify the role that mobile genetic elements and other difficult-to-assemble
362 structural variants play in driving short-term evolution within hosts. A broad
363 range of existing longitudinal studies have already been processed for DNA
364 extraction for 16S rRNA sequencing; revisiting these studies with metagenomic
365 sequencing could serve to rapidly expand the sequencing database from which
366 to detect adaptive mutations.

367

368 Ultimately, the ability to successfully interpret the functional consequences of
369 mutations will require other means of interrogation to gather phenotypic
370 information associated with mutations. Advances in metabolomics [92] will
371 reveal changes to the gut environment associated with enhanced metabolic

activity. Quantifying the phenotypic landscape of gene knockout and overexpression libraries in gut commensals [66,67,93] will provide a baseline expectation for mutations in each gene. Genetic tools to reconstruct observed mutations in the species of interest will be critical to close the loop so that mutants can be studied *in vivo* and in competitive colonization experiments. Finally, a greater understanding of the biophysics of spatial structure in the gut [1,94,95] may be necessary to acquire a full picture of gut ecology and evolution.

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Figures

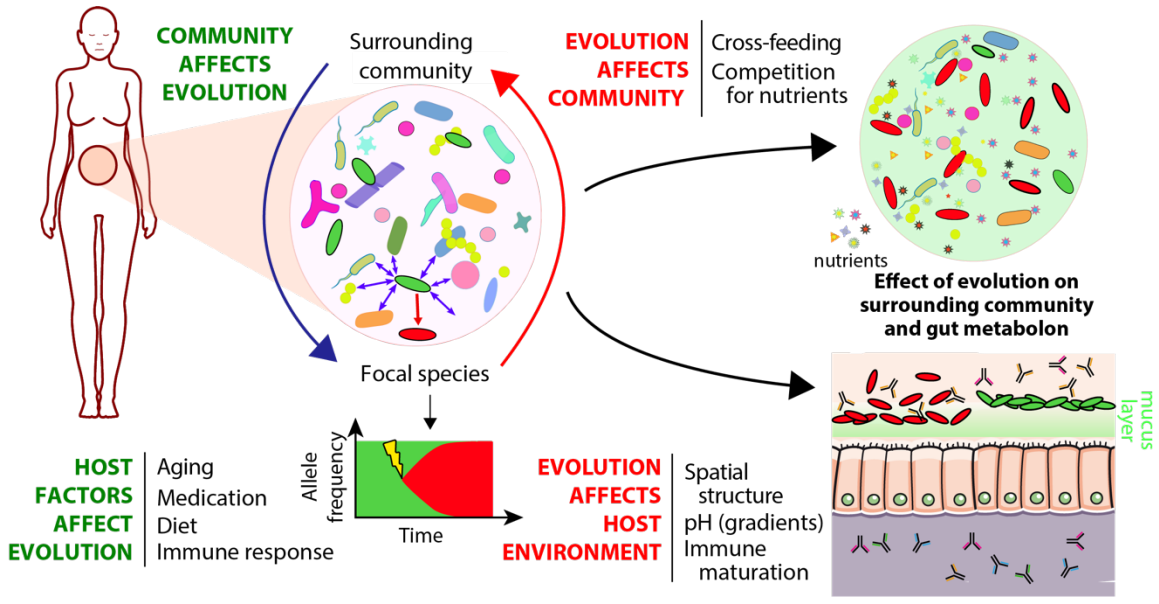


Figure 1: Connections between evolution and ecology in the mammalian gut. Host factors like aging, medication, diet, and immune status affect the gut microbiota through its composition. Focal species (green oval) can evolve due to selection pressures directly from the host factors or indirectly from the surrounding community. The evolved focal species (red oval) may differ from its ancestor through the ability to consume different nutrients or to survive and colonize different environments. Evolution of the focal species may in turn affect the surrounding community by perturbing the landscape of nutrient competition or providing nutrients through cross-feeding, and can affect the gut environment (e.g., by altering pH or colonizing distinct regions of the intestines).

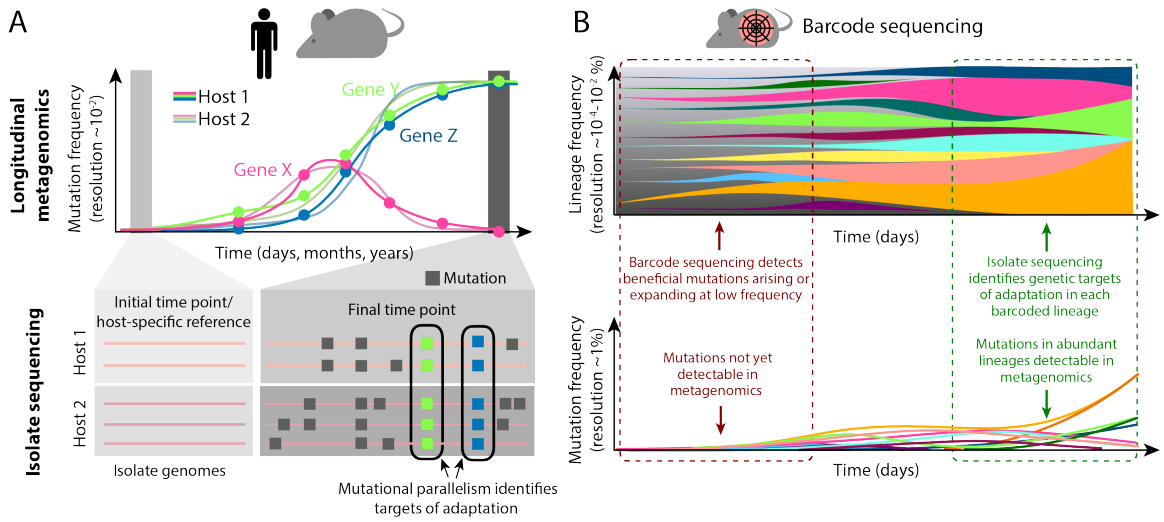
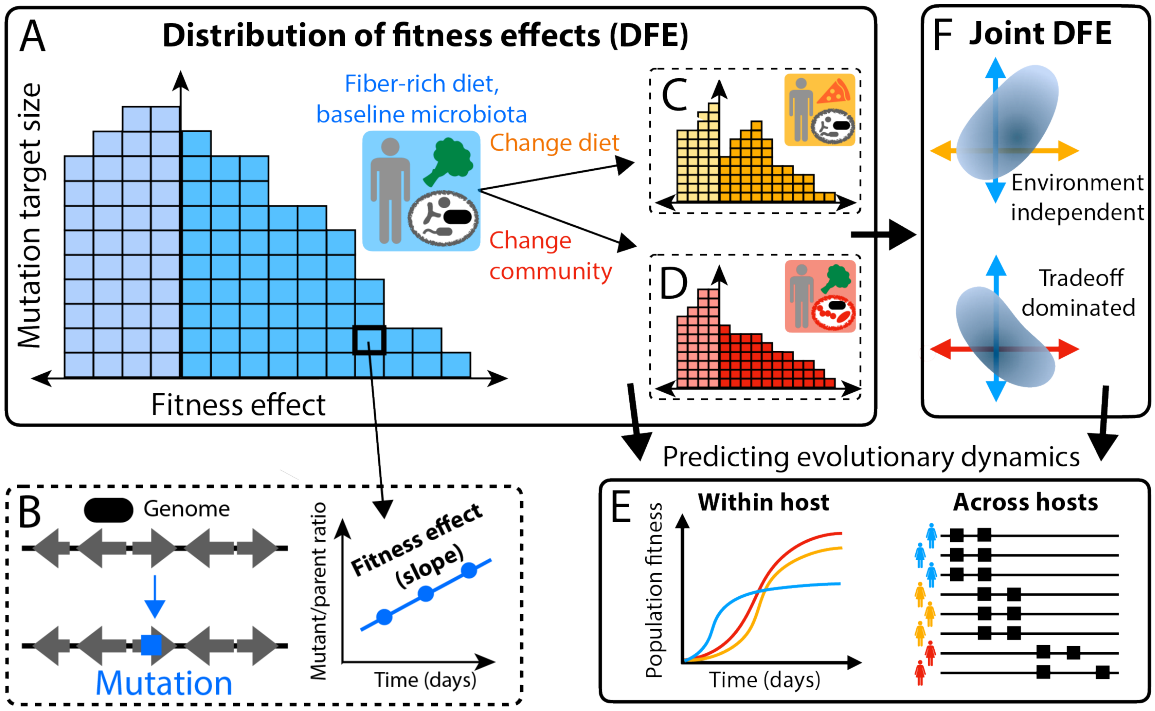


Figure 2: Sequencing approaches for quantifying within-host evolution of gut commensals.

A) Whole genome sequencing-based studies infer adaptive evolution by tracking individual mutations that rise to substantial frequencies within a host. Top: metagenomic sequencing approaches track the frequencies of (linked) mutations within a host over time, providing information about the total fitness benefits of their corresponding haplotypes. Bottom: isolate sequencing can provide information about the targets of adaptation, by observing parallel mutations across multiple independent lineages.

B) Barcode sequencing-based studies can simultaneously track tens of thousands of genetically tagged lineages within a single population with high frequency resolution, allowing high-throughput measurement of expanding lineages that acquire adaptive mutations.



414

415 **Figure 3: The distribution of fitness effects (DFE) provides a quantitative**

416 **framework for evaluating qualitative models of microbiota evolution.**

417 A) The DFE captures the spectrum of mutations available to a focal species in

418 a particular environment (e.g., a fiber-rich diet and a baseline microbiota).

419 B-D) Each tile in the DFE represents a mutation with a given target size and

420 fitness effect, which can be measured from the slope of the log ratio of

421 mutant-to-parent genotypes over time. Since the fitness effect of a

422 mutation may depend on host- or community-context, the same focal

423 species will have different DFEs across different environments, which

424 could include changes in host-extrinsic factors such as diet (C) or

425 differences in the surrounding microbial community (D).

E) The joint DFE (JDFE) generalizes the DFE across multiple environments, by enumerating the fitness effects of the same mutation across different environments.

F) The DFE and JDFE determine the evolutionary dynamics within a single host (e.g., the rate of fitness increase or the parallel mutations observed in sequenced isolates) as well the evolutionary tradeoffs (pleiotropy) of mutations in other host conditions.

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