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Evolution of commensal bacteria in the intestinal tract of mice

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Hundreds of different bacterial species inhabit our intestines and contribute to our health status, with significant loss of species diversity typically observed in disease conditions. Within each microbial species a great deal of diversity is hidden and such intra-specific variation is also key to the proper homeostasis between the host and its microbial inhabitants. Indeed, it is at this level that new mechanisms of antibiotic resistance emerge and pathogenic characteristics evolve. Yet, our knowledge on intra-species variation in the gut is still limited and an understanding of the evolutionary mechanisms acting on it is extremely reduced. Here we review recent work that has begun to reveal that adaptation of commensal bacteria to the mammalian intestine may be fast and highly repeatable, and that the time scales of evolutionary and ecological change can be very similar in these ecosystems.

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Introduction

"The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Élie Metchnikoff, The prolongation of life, 1910, pg 162) [1]

Microbes find shelter and resources inside the guts of their hosts. Hosts find genes, new traits and functions in the microbes they harbor. In humans, it is currently estimated that for each host cell there is at least one microbial cell [2], and that the number of microbial genes is greater than the number of host genes. High-throughput sequencing, particularly the use of 16S rRNA sequencing, has allowed for an unprecedented characterization of the gut microbiome, revealing that its composition is highly dynamic, both spatially and temporally. Starting at birth, microbes colonize hosts in a process involving ecological succession of species [3], which is characterized by large fluctuations in abundances and a high level of inter-host variation. Reaching adulthood the microbial species composition becomes more stable both within and between hosts. Importantly, the composition of the gut microbiota modulates the host's ability to resist pathogens [4] and its immune homeostasis [5•,6]. Moreover, the gut microbiota has multiple effects on peripheral organs, ranging from bile acid metabolism in the liver [7] to modulating behavior by affecting gene expression in the brain [8].

Recent advances in our understanding of gut microbial ecology and its relation to host health have been made. However, much less is known about evolutionary processes in the gut. Here we review work on how quickly and by what mechanisms evolutionary change may occur within a given bacterial species colonizing the intestine. We focus on mice to dissect key processes of microbe-microbe and host-microbe interactions, due to the accumulated knowledge of its physiology, genetics and behavior as a classical model organism. Furthermore, mice allow study of adaptation in the complex gut ecosystem under controlled conditions (*e.g.*, migration, diet, temperature). Such control allows unraveling the reproducibility of the adaptation pattern in the gut, in conditions where bacteria do not cause disease to the host. Evolution of commensal bacteria has received far less attention than the adaptation of pathogens. Nevertheless, it is important to study the evolution of commensals as this may be quite distinct from pathogens; for example, the fitness landscape of a pathogen may be marked by strong selection to avoid the host immune system. Another distinctive property of a pathogen's fitness landscape is a reduced number of interactions with other microbial species. Thus, while studying pathogen evolution is important for understanding disease, the contribution of commensal bacteria to host health indicates that studying their evolution maybe important for understanding how host health is maintained.

Richness of interactions in the gut

As a home for microbes, the host intestine constitutes an environment where commensal bacteria experience

multiple selective pressures. Healthy hosts typically maintain a rich and stable microbiota, yet how they do so is still as mysterious as it was in the time of Metchnikoff, who believed that a long, healthy life depended on the quality of the intestinal microbes [1]. As we review below, controlled experiments in mice and developments in mathematical modeling have recently been done to help determining key interactions that shape the temporal composition of the gut microbiota and its stability.

A key component of the environment of a given microbe is another microbe, thus microbe–microbe interactions are expected to be important in the gut. To understand the nature of such interactions, ingenious experiments where a stable microbiota ecosystem is perturbed and followed through time, have been performed in mice [9••,10]. As antibiotics cause considerable changes in the gut bacterial composition [11], they can be used as perturbations to obtain detailed temporal series data of microbiota composition (through 16S rRNA sequencing) as it recovers from the perturbation and achieves a new state of equilibrium. Assuming that a Lotka–Volterra Model (from classical ecological theory) governs microbe-microbe interactions and their dynamics, such data allows obtaining quantitative estimates of ecological interactions between groups of bacteria. Stein *et al.* [9••,10] were pioneers in this integrated design (Figure 1) to estimate interaction networks in the gut. Their analysis suggests that a network of negative and positive interactions underlies the gut microbiota composition. A similar conclusion was reached in a study where mice devoid of microbes (germ-free, GF) were colonized with the cecal contents of a conventionally-raised mouse. Following a great amount of within and between mice initial variation in community dynamics, a stable microbiota composition was achieved after three weeks. Importantly, the study revealed that among 136 possible pairwise interactions between the microbes, 67% were competitive (−/−), 16% parasitic (+/−), 12.5% ammensalistic (−/0), 3% commensal (+/0), 1.5% neutral (0/0) and not a single one involved mutualism (+/+) [12]. A possible consequence of intense competition may be to promote stability of the microbiota. Recent theory addressing what type of interactions underlie stability of a multispecies ecosystem of microbes, whose dynamics follow a Lotka–Volterra Model, predicts that many species are likely to stably coexist when the system is dominated by competition (negative pairwise interactions) [13••]. We note that the Lotka–Volterra Model only considers pairwise interactions and does not make explicit the precise mechanisms driving the interactions (such as possible metabolites that the species may exchange or specific limiting resources that may underlie competition between species) [14]. Such simplicity can have major drawbacks: for example, previous experiments have shown that for the simplest case where two bacterial strains grow in a chemostat with a single limiting resource, the Lotka–Volterra Model fails to make correct predictions on the dynamics of competition [15]. Conversely, when this model was applied to the temporal dynamics of the mouse gut microbiota, it was able to correctly identify a bacterial species that provides colonization resistance against infection by *Clostridium difficile* [4]. Thus, future work is needed to better understand the nature of microbe-microbe interactions in the gut.

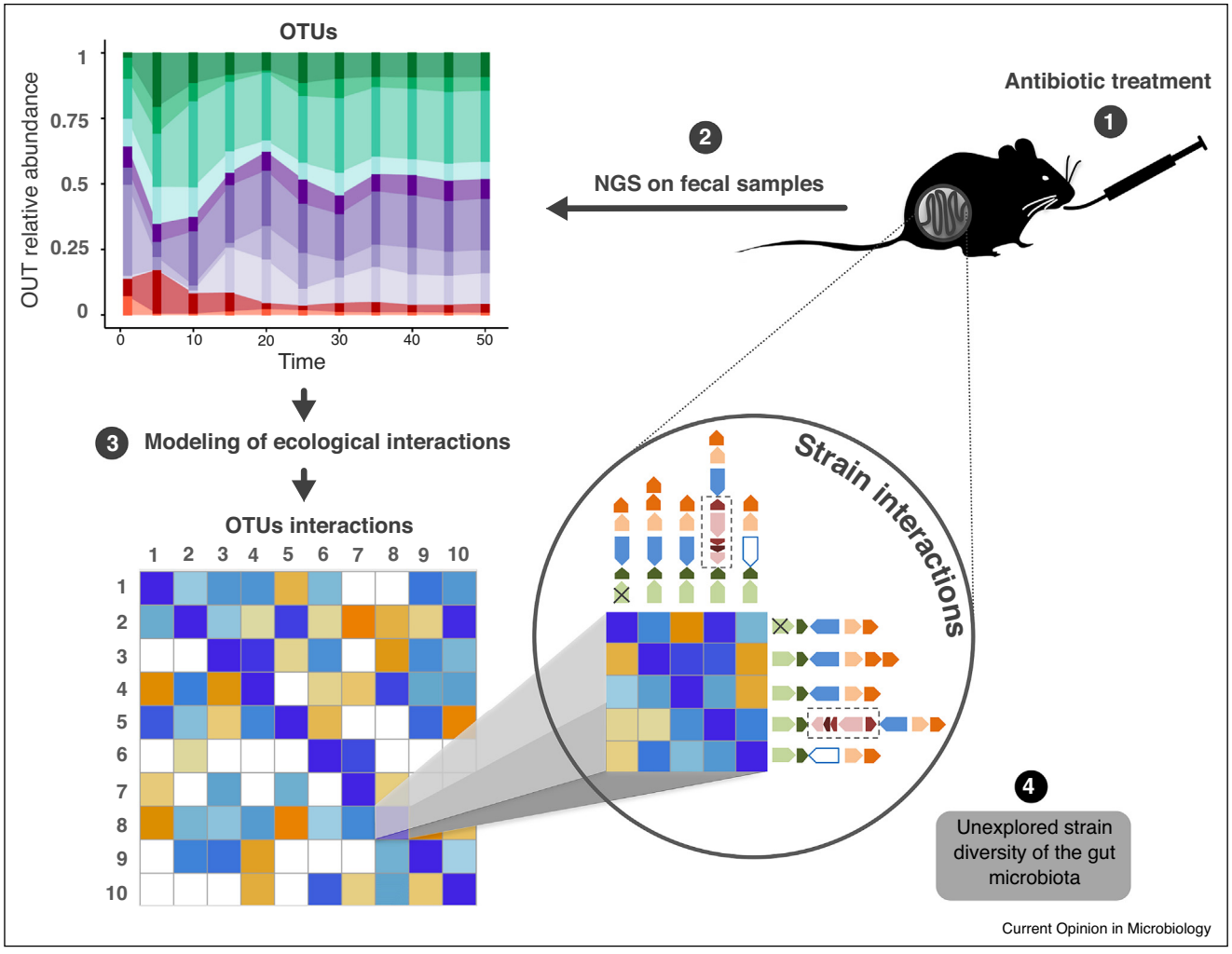
Host-nutrition and its gut microbes are also expected to interact, as the gut microbiota is known to be important for host digestion (*e.g.*, breakdown of complex carbohydrates). Several studies have demonstrated shifts in microbiota composition following dietary changes [16,17]. One example is the change from a low-fat/high-polysaccharide to a high-fat/high-sugar diet, with the latter diet leading to a strong increase in the proportion of Firmicutes relative to the Bacteroidetes phylum [17]. Importantly, a recent study has also shown that changes in diet can have trans-generational consequences for microbiota composition, with a low-fiber diet having a cumulative effect (across generations) that led to species loss. These missing microbial species could only be regained through fecal transplants from mice that had been fed with a high-fiber diet [18].

Interactions between the host immune system (IS) and gut commensals have also gained relevance. Both innate and adaptive immune responses have been shown to shape microbiota composition and determine the boundaries between the host and its microbes [19,20]. Reciprocally, commensals shape the host innate and adaptive immune responses, for example, GF mice have reduced levels of antimicrobial peptides [21] and colonic regulatory T cells [22], the latter being increased by the presence of specific microbes. Regarding the innate arm of the IS, some host antimicrobial peptides were shown to cause large fitness reductions to commensal strains of Proteobacteria, but not to strains of Bacteroidetes and Firmicutes, the latter belonging to a phylum typically found at high abundances in the gut [23]. Regarding adaptive IS, host immunoglobulins A (IgAs) were found to have diametrically opposed consequences for the fitness of different commensals. IgAs were shown to suppress the expansion of segmented filamentous bacteria [24], but also to promote the maintenance and diversification of certain Clostridia [5•]. These examples highlight the contribution of the host to generate and maintain a diverse gut microbiota.

Evolutionary change within species of the gut microbiota

Notwithstanding the ecological interactions mentioned above, the current vision of gut microbiota composition typically ignores an important characteristic of many bacterial strains: their capacity to rapidly evolve, either

Figure 1



Inference of microbe–microbe ecological interactions from time-series data after perturbation of the gut microbiota ecosystem.
The changes in relative abundance of operational taxonomic units (OTUs) over time after perturbation (such as diet alteration or antibiotics) allow estimation of ecological interactions. Here we represent a hypothetical example of a community dynamics returning to equilibrium. After an initial perturbation (indicated in 1), where the relative abundances suffer major alterations, the community stabilizes from day 35 onwards. The composition of the microbiota can be determined through 16S rRNA or whole metagenomic analysis of fecal material (indicated in 2). Following the dynamics upon perturbation and through the recovery period, it is possible to obtain a matrix of ecological interactions among the different OTUs (genus/family), under a Lotka–Volterra model as in Refs. [9••,10]. In this matrix (indicated in 3), blue shading represents negative interactions, yellow positive interactions and white lack of interaction; the intensity of the color is proportional to the strength of the interaction. This methodology ignores the hidden strain variation (including *de novo* emerging strains). These new variants differ from each other in at least one mutation (duplication, deletion, gene acquisition, gene inactivation and SNP; (indicated in 4)). Using a similar method as in Refs. [9••,10], it should be possible to estimate the network of interactions between strains.

by accumulating new adaptive mutations or by acquiring new genes (Figure 1). The gut microbiota should be a prime example of a system in which the ecological and evolutionary time scales may meet. The rate of evolutionary change depends primarily on population size, mutation rate and the effects on fitness of the mutations that spontaneously occur. The latter are determined by the strength of selection experienced in a given environment. Neutral mutations fix at the rate at which they emerge [25]. Deleterious mutations get eliminated, except if their effects are very small compared to the population size or if they hitchhike with beneficial variants to which they are linked. Advantageous mutations sweep to fixation with a probability proportional to their benefit [26]. In the gut, many bacterial species have population sizes composed of millions of cells, which are rapidly dividing to withstand the continuous flushing out of the intestine [27,28]. Given the strong and diverse selective pressures described in the previous section and typical estimates of bacterial genomic mutation rates

[29,30], a considerable amount of evolutionary change may be expected to occur.

Most of our understanding of bacterial evolution and adaptation has been gathered *in vitro*, under specific selective pressures (forward study of evolution), or by sampling extant genomes and inferring the processes that caused the observed differences (backward study of evolution). Ideally, and in the absence of a time machine, the forward and backward methods should be sufficiently complementary to allow an understanding of how bacteria evolve in nature. Remarkably, the power of experimental evolution (EE) to study evolution 'in real time' has been underexplored to better understand host–microbe associations [31]. In the context of the microbiota, we believe this approach can be very helpful in answering important questions such as: How fast do commensal gut bacteria evolve? Is strain diversification driven by selection or mostly the result of a neutral process? What is the typical effect of a new emerging mutation or a gene acquired by horizontal gene transfer?

Mutation and intense clonal competition in the gut

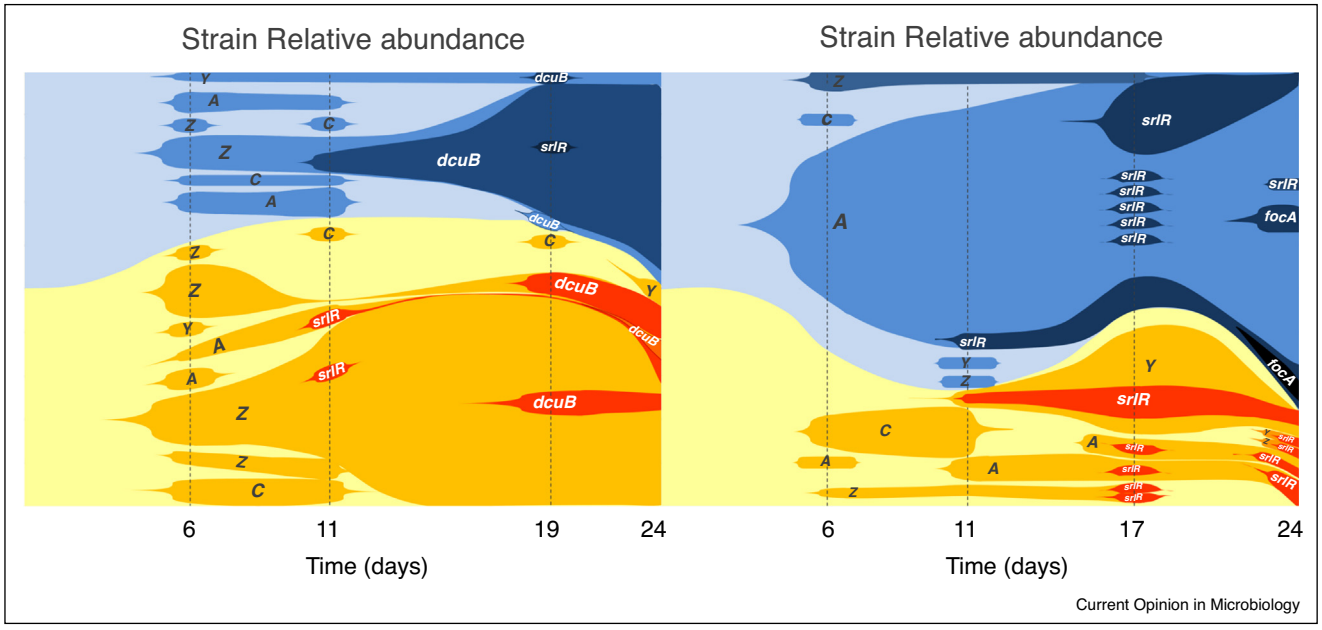
Microbes have been key to demonstrate the power of natural selection, especially in the context of disease (*e.g.*, evolution of drug resistance). In a healthy mammalian gut, under homeostasis, the action of natural selection on strain diversity has been less studied. While a simple assumption would be that commensal gut bacteria could be seating on a Fisherian fitness peak, and little adaptation should occur, the complexity of the gut environment, and the variation of gut microbiota composition across hosts and along time, points otherwise. There, selection may be influenced by spatial heterogeneity (*e.g.*, nutritional, oxygen, pH, bile salts and other gradients), environmental variation (*e.g.*, diet changes), tradeoffs within a given host [32] and when transmitting across species [33], phage predation [34], specific host and bacterial genetic backgrounds [35,36], and migration from the external environment [37]. EE has proven important for studying the relative role of mutation, selection and drift in bacteria colonizing the gut. Using the simplest possible system, a GF host (mice) that is then colonized with a single bacterial species, Giraud *et al.* [38] observed the independent emergence of strains with high mutation rates (mutators). Mutators could spread due to their increased ability to rapidly generate new adaptive variants during colonization of GF mice. In another colonization study, but now with conventional mice which have a complex gut microbiota, Barroso-Batista *et al.* [39••] followed the sequential accumulation of beneficial mutations in two fluorescently-labeled isogenic *E. coli* lineages sampled from feces. The timing and change in frequency of the fluorescences, caused by the emergence of new alleles, allowed the authors to estimate a rate of adaptive mutations of $\sim 7 \times 10^{-7}$ per generation, with a mean effect of ~7%. Importantly, multiple clones, carrying mutations at different loci, emerged and competed during adaptation to the mouse gut in a process known as clonal interference (Figure 2). The strong fitness effects of the emerging mutations in a given host (ranging from 2% to 14% [40]) were further shown to provide equivalent benefits in new hosts, suggesting that *E. coli* experiences similar pressures when colonizing genetically identical mice that eat the same food. Consistent with this interpretation, the molecular path of evolution taken by the bacteria, when colonizing different hosts, was extremely similar—evolutionary parallelism [39••,40]. Moreover, an average of ~2 mutations per genome were found to accumulate after ~450 generations (24 days). These results indicate that the tempo and mode of short-term molecular evolution can be highly repeatable in a complex gut ecosystem. In a longer-term EE, using a different *E. coli* strain colonizing outbred mice with a complex microbiota, Lescat *et al.* [41•] found that after about a year (>6500 generations), ~6.3 mutations per clone had accumulated. Moreover, they also observed substantial levels of parallel molecular evolution, arguing for a major role for selection in the process of strain diversification.

In both the short- and the long-term *E. coli* colonization studies, some level of epistasis between the accumulated mutations was observed [40,41•], suggesting that the genetic basis of gut adaptation can be conditioned by the strains genetic background. This highlights not only the need to study other strains, but also to determine if the typical rate of evolution in true mouse commensal strains is as high as for the strains followed in these studies, which were originally isolated from human stool.

Host genetics have also been shown to affect the tempo and mode of adaptation of these bacteria to the mouse gut. When comparing wild-type with Rag2-/- mice, which are severely immunocompromised because they lack B and T cells, Barroso-Batista *et al.* [35] found that the dynamics of *E. coli* gut adaptation were slower and the effects of the emerging mutations more variable, an effect attributed to the different microbiota compositions in the two host genotypes.

EE also allows understanding the selective pressures experienced by bacteria in the gut environment. Nutritional optimization was found to be one of the main challenges bacteria face in the microbiota community. Mutants that can grow faster on sugars present in the mucus [42,43] and specialist clones [44,45] that are able to explore different niches have been shown to emerge during adaptation to the mouse gut. In accordance with these findings, several studies [46,47] exploring the mechanisms by which an "uncompromised microbiota prevents pathogen infections" (colonization resistance, [48]) support the hypothesis that strains with completely overlapping nutritional niches might not be able to

Figure 2



Evolutionary dynamics of *de novo* emerging strains: multiple adaptive mutations compete for increasing in frequency – clonal interference – during *E. coli* colonization of the mouse gut.
Examples of the emergence of strain variation within a lineage of *E. coli* colonizing two hosts (adapted from Barroso-Batista *et al.* [39••]). Muller plots where new adaptive mutations spread in an initial isogenic population of fluorescently-labeled *E. coli* (either blue or yellow), which was used to colonize the intestine of streptomycin-treated mice. A, Y, Z and C represent genes from the galactitol operon, whose inactivation was shown to be adaptive. Each of the distinct alleles is equally fit in the gut and therefore polymorphism can be maintained for several days. *srlR*, *dcuB* and *focA* represent secondary targets for adaptive mutations. The two Muller plots represent independent mice, showing that parallelism in the genetic targets of adaptation is extensive. The darker the tone of blue or yellow, the higher the number of mutations carried by a given clone. For example, in the right panel, the ancestral strain in the blue background first acquires a mutation in *gatA* (~day 6). By day 11 a small proportion of that population acquires a second mutation in *srlR*, which is then followed by a third mutation in *focA*, thus creating a triple mutant in 24 days.

co-exist in the same community. This is the basis of the 'nutrient-niche hypothesis', first enunciated by Freter (reviewed in Ref. [49]), which postulates that microbes can only persist in a complex community if they use at least one limiting nutrient better than all others. This hypothesis assumes that bacteria compete for a nutrient pool that is equally available throughout the gut. To account for the inherent spatial structure and species distribution in the gut, the 'restaurant hypothesis' was developed (reviewed in Ref. [36]). This states that the potential for long-term colonization by facultative anaerobes (such as *E. coli* and Salmonella) depends on their ability to acquire nutrients locally in mixed-species biofilms. This hypothesis, coupled with the results of evolution experiments showing fast strain diversification, possibly driven by competition for limiting nutrients, suggests that bacterial nutritional adaptation may alter colonization resistance.

Horizontal gene transfer in the gut

Bacterial strain diversification can result from horizontal gene transfer (HGT), which may occur among distantly-related bacteria or even inter-kingdom species [50–53]. However, its efficiency was shown to decrease exponentially with sequence divergence [54,55]. HGT takes place via three different mechanisms: conjugation-mediated plasmid exchange, phage-mediated transduction or natural transformation [56]. Given its high bacterial density, the mammalian gut is likely a hotspot for HGT when compared to other ecosystems [54,57]. In humans, the gut is predicted to be the body site with the highest number of horizontally acquired genes per microbe (average of 48.6 genes) [58]. Horizontally-acquired genetic material, which can constitute up to 20% of prokaryotic genomes [59–61], enables a quantum leap in gene diversification of particular members of the gut microbiota, potentially changing their own evolutionary fate and that of the whole community.

Important questions concerning the role of HGT in the evolution of gut microbes remain unanswered: What is the typical rate of each HGT mechanism in the gut? What is the typical fitness effect of an HGT event? The mouse as model of bacterial colonization has been used to elucidate these issues.

Conjugation within the microbiota can be common and occur in a short timeframe. A study using GF mice,

colonized with human feces, found that a natural plasmid could be transferred between *E. coli* strains at a frequency of 10^{-5} after 6 hours of colonization [62]. In another study, the *in vivo* conjugation frequency of transposon Tn1545 from *Enterococcus faecalis* to *Listeria monocytogenes* in the gut of gnotobiotic mice was 1.1×10^{-8} after 35 days [50]. In the context of gut inflammation, Stecher *et al.* [63] observed that blooms of infecting *Salmonella* cells and of resident commensal *E. coli* lead to extremely high rates of conjugation of the colicin-plasmid P2 (plasmid present in all *E. coli* cells within 4 days).

Although metagenomic data show that bacteria from the gut microbiota carry considerable numbers of temperate phages [64], direct measurements of the rate of phage-mediated HGT (transduction) within the mammalian intestine have been understudied. Using the classical system of *E. coli* and its best studied phage λ, De Paepe *et al.* [65•] have undertaken a well-designed quantitative study to determine the rate of prophage induction and the fitness effects of prophage integration into the bacteria colonizing the gut of GF mice. They showed that the gut is an excellent environment for phage to spread, and estimated a rate of prophage induction of *~2%* per generation, which is much higher than observed in laboratory conditions. Such high induction was also shown to cause considerable fitness costs to the infected bacteria.

Finally, transformation was observed to be common in the respiratory tract, where the naturally competent *Streptococcus pneumoniae* shows a transformation frequency of 10^{-2} after 2 days of colonization [66]. However, transformation appears to be relatively rare in the mammalian gut. This may be due to a low amount of free DNA for transformation, either due to DNA shielding by the gut contents [67] or to DNA-degrading enzymes [63]. More studies will be required to accurately assess transformation rates in the gut.

Importantly, the horizontally-transferred traits that are under selection as bacteria adapt in the mouse gut are poorly known. Modi *et al.* [64] found that, in addition to antibiotic-resistance genes, phage genomes are also enriched for multiple genes related to metabolism (*e.g.*, carbohydrate metabolism or glycan synthesis and metabolism) after antibiotic treatment. In agreement with what was found in EE studies (previous section), these data could suggest that nutritional adaptation is one of the key selective pressures in the mouse gut and that both mutational and HGT processes contribute to that adaptation.

Conclusions

The extent to which evolutionary change occurs in the gut and shapes the genetic structure of its microbiota is still largely unknown. The findings stemming from the few studies of evolution in mice suggest that a bacterial population in the gut could contain several evolving clones, differing by several mutations or horizontally-acquired gene(s) with high selective effects. If these observations turn out to be general, two implications emerge: (i) a complete account of the genetic diversity within microbial ecosystems inhabiting a host could be a difficult task, as intra-species variation may be both large and highly dynamic [65•]; (ii) the strong selection for nutritional optimization indicates it may be "*possible to adopt measures to modify the flora in our bodies*" [1], by precisely manipulating diet to control which evolving strains may be allowed to stay in the gut.

Rapid evolutionary change can be critical to community structure [66], and to the observed diversity of the mammalian microbiota. Future theory should therefore consider whether incorporating high adaptive mutation or HGT event rates into classical models of ecology may help explain one of the big mysteries of nature: how can large numbers of species be maintained in ecosystems?

Conflict of interest

None declared.

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