Evolution on the bright side of life: microorganisms and the evolution of mutualism

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Mutualistic interactions, where two interacting species have a net beneficial effect on each other’s fitness, play a crucial role in the survival and evolution of many species. Despite substantial empirical and theoretical work in past decades, the impact of these interactions on natural selection is not fully understood. In addition, mutualisms between microorganisms have been largely ignored, even though they are ecologically important and can be used as tools to bridge the gap between theory and empirical work. Here, I describe two problems with our current understanding of natural selection in mutualism and highlight the properties of microbial mutualisms that could help solve them. One problem is that bias and methodological problems have limited our understanding of the variety of mechanisms by which species may adapt to mutualism. Another problem is that it is rare for experiments testing coevolution in mutualism to address whether each species has adapted to evolutionary changes in its partner. These problems can be addressed with genome resequencing and time-shift experiments, techniques that are easier to perform in microorganisms. In addition, microbial mutualisms may inspire novel insights and hypotheses about natural selection in mutualism.

Keywords: coevolution; adaptation; natural selection; cross-feeding; syntrophy

Introduction

Mutualisms are interactions between species where each has a net positive impact on the fitness of the other. They affect the survival of practically all living organisms. In plants, mutualistic descendants of bacteria convert energy from the sun into chemical energy for plant cells. These plants are visited by insects that provide a means of efficient sexual reproduction in exchange for nectar, while microorganisms in the soil provide the roots with phosphorous in exchange for organic carbon. In animals and insects, mutualists aid in digestion and protection from antagonists and may even control behavior. For microorganisms, survival often depends on the beneficial exchange of metabolites within communities. Clearly, if we want to understand how any of these species evolves, we must understand mutualism as a source of selection that affects the dynamics and products of evolutionary change.

During the last several decades, many evolutionary biologists have addressed this challenge, producing a wealth of theoretical and empirical data on adaptations to mutualism. Much of this work has aimed at learning how mutualists minimize the likelihood that their partner will negatively affect their fitness by cheating, but the possibility of coevolution has also been studied. This research has relied heavily on several naturally occurring ancient mutualisms, where at least one of the partners was a multicellular eukaryote. As a result, the development of theory and its application has been biased toward problems and contexts that may be most important for a relatively narrow range of species. In addition, this approach has limited the experimental power that can be applied to understanding mutualism.

More recently, scientists have begun experimenting with mutualistic interactions between microorganisms. Diverse mutualisms have been engineered or evolved in the laboratory with...
the goal of understanding microbial physiology and metabolism and understanding how mutualisms can evolve and be stable.10,12,13,17,18 These systems can be outstanding tools for experimentation, yet they have rarely been used to address some of the most pressing questions about the selection environment in mutualism.

The goal of this review is to facilitate the integration of these two parallel approaches to understanding mutualism. I do this by first describing microbial mutualisms and explaining why it is important to understand their evolution. I then provide an overview of our current understanding of adaptation and coevolution in mutualism, highlighting topics needing further study owing to neglect or experimental limitations and providing some suggestions for further experiments. Throughout this review, I use microbiological examples wherever possible.

A primer on microbial mutualisms

Why study microbial mutualisms?
The most important reason to study microbial mutualisms is that the vast majority of living beings are microorganisms.19,20 If they are ignored, we cannot have a comprehensive theory of mutualism as an interaction that affects all of life. Instead, we will end up with a narrow theory of mutualism that only applies reliably to a small part of one domain of life, encouraging microbiologists to unnecessarily create a separate conceptual framework for understanding beneficial interactions between species.

There are also practical reasons to study the evolution of mutualistic interactions between microorganisms. Positive interactions, such as mutualism and commensalism, are thought to play a fundamental role in the functioning and flow of carbon through microbial communities.21–23 Because they can often evolve so quickly, evolutionary changes in microbial populations are likely to be a part of the ecological processes affected by these interactions.24–27 Thus, studying the evolution of microbial mutualisms may provide insight into numerous processes involving microbial communities. Such processes include behavior, immunity, and digestion in animals or plants, biogeochemical processes controlling the flux of greenhouse gasses like carbon dioxide and methane, and degradation of hazardous waste.3,5,28–33

Finally, microorganisms can be powerful tools for studying evolution because of the ease of genetic manipulation and genome sequencing, of controlling environmental variation in the laboratory, of storing evolutionary intermediates in a dormant state, and of manipulating population size to alter the impact of natural selection.34 Their use in evolution experiments has allowed scientists to address fundamental questions about the process of evolution,35,36 foraging theory,37–39 origins of multicellularity,40 coevolution in host–parasite interactions,41 and the evolution of cooperation.42–44 However, experimental evolution has only recently been applied to understanding mutualism.10,11,15

Mutualistic interactions between microorganisms

In mutualistic interactions, one species provides a resource to another in exchange for a second resource or service that is provided by its partner.1,45 In microbial mutualisms, this typically involves the provision of an essential metabolite in exchange for a different one or for a service, such as swimming or the removal of toxic by-products. For example, in the transition from aerobic to anaerobic zones in many freshwater lakes, two-thirds of the biomass is composed of aggregates of phototrophs surrounding a heterotroph that provides the service of swimming in exchange for photosynthate (Fig. 1A).46–48 In the ocean, a significant portion of methane is removed as a result of a mutualistic interaction between bacteria and archaea.49,50 The bacteria use the waste electrons generated by anaerobic methane-oxidizing archaea to obtain energy, which also serves to allow the archaea to gain energy from anaerobic methane oxidation.50

Microbiologists have studied the physiology and biochemistry of these complex microbial mutualisms for decades. Here, I focus on two broad categories of microbial mutualisms that have been the focus of more recent work aimed at understanding their ecology and evolution: cross-feeding and a specialized form of cross-feeding called syntrophy.

Cross-feeding. Most microorganisms are not capable of synthesizing all of the amino acids and other molecular precursors that are required to make copies of themselves. Instead, they rely on other species to make and secrete them or release
them through cell lysis. These interactions can be mutualistic if both species are providing resources to each other. Cross-feeding interactions can be pairwise, but in microbial communities, there may be complex networks of species releasing and using each other’s metabolites.22,23 Metabolites can be transferred through diffusion or through nanotubes that connect the cytoplasms of mutualists, or as a result of endosymbiosis of a bacteria by a protist (Fig. 1B and 1C).10,11,13,14,50–53

Variation in nutritional requirements and gene content across clones and species of microorganisms suggest that cross-feeding is common in most microbial communities and probably the cause of our inability to obtain pure cultures of microbes.23,54 Research has shown that cross-feeding can be a phenotypically plastic response to changes in the environment, or it can result from genetic changes. Numerous cross-feeding interactions can arise as a result of acclimation, a phenomenon that can be predicted by genome-scale models, although their stability may depend on the density of each species or removal of toxic by-products.54–58

Other species may be completely reliant on cross-feeding because of erosion of the functional genes required for metabolite production. Two hypotheses have been developed to explain the evolution of obligate cross-feeding. In the Black Queen hypothesis, the loss of genes is beneficial in an environment where nutrients are scarce, making DNA replication costly. Frequency-dependence fitness maintains metabolite producers.6,59 The farm-and-forage hypothesis predicts neutral erosion of metabolic genes in nutrient-rich environments where they are not needed. When resources are scarce, these mutants survive by overproducing a nutrient like organic carbon to stimulate the growth of producer populations.60 Empirical support exists for both of these hypotheses, but theory suggests that over-producing cross-fed nutrients and relying on other populations is inefficient relative to growing autonomously in most conditions.10,59,61

**Syntrophy.** Syntrophy is a term that literally means “feeding together.” Unlike other cross-feeding interactions, syntrophs feed together by working together to complete one energy-yielding reaction.62 In oxygen-free environments with limited availability of electron acceptors (molecules used to capture and remove electrons during respiration, such as O2, NO3−, SO42−, and oxidized metals), a special kind of cross-feeding interaction is responsible for reducing carbon completely to methane, allowing degradation processes at higher levels of the food chain to continue.7,62,63 In these interactions, one species produces a by-product that becomes toxic unless it is kept at very low concentrations. The second species consumes this toxic by-product because it is its only source of energy. The toxic by-product is essentially excess electrons that are removed from the cell via hydrogen, formate, or other molecules, and they are toxic to the producer because of the extremely low energy yield of the fermentation reaction.7,62,63

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**Figure 1.** Images of microbial mutualisms and putative adaptations. (A) A phototrophic consortia isolated from Lake Dagow. Phototrophs called green sulfur bacteria surround a flagellated, colorless heterotrophic species. Ultrastructural characterization of this pairing identified electron-coated hair-like structures as well as periplasmic tubules connecting the outer membranes of the two species (not shown). Reproduced from Ref. 46 with permission. (B) Tubes connecting mutants of *Escherichia coli* and *Acinetobacter bayli* that cross-feed amino acids. Reproduced from Ref. 51 with permission. (C) Light microscopy image of *D. vulgaris* (curved rod) and *M. maripaludis* (spheres) that have evolved together for over 3000 generations in upright, unshaken tubes in a syntrophic interaction. (D) A granule that was isolated from an anaerobic digestor that treats brewery wastewater and was hybridized with 16S rRNA-based probes for bacteria (green) and archaea (red). The granule has defined layers of microorganisms from several bacteria and archaea taxa that are breaking down organic waste materials in a diffuse syntrophic interaction. Image reproduced from Ref. 68 with permission.
Some species are obligate syntrophs that have limited or no options for surviving outside of the interaction, while others may grow syntrophically when electron acceptors have been used up and then revert to a more independent mode of metabolism when electron acceptors are available. Some syntrophs aggregate and produce wires to transfer electrons directly, while others interact in guilds where electrons are transferred in the form of hydrogen or formate through diffusion. Syntrophies play crucial roles in the functioning of anaerobic communities in digestors used to degrade solids in wastewater (Fig. 1D), in the rumen of cows, in lake sediments, and in rice paddies, and they are primary producers in methane seeps in the depths of the ocean.

**Adaptation to mutualism**

**Mechanisms for adaptation to mutualism and their application to microorganisms**

In by-product mutualisms, such as syntrophy, the resources being traded are waste materials that just happen to benefit another species in the community, so there are no fitness costs to participating in this interaction. In most other mutualisms, producing and delivering resources is thought to come at a cost to fitness. Mutualists that maximize the ratio between benefits and costs are expected to have higher fitness in comparison with competitors that do not, all else being equal. A schematic representation of a putative relationship between benefits, costs, and resource transfer for a mutualist A is presented in Figure 2 and explained below, along with examples of such adaptations in microbes.

In the schematic representation depicted in Figure 2, the costs incurred by A depend on how much resource it produces and provides to its partner B. Assuming a fixed cost to producing and delivering the resource, the total cost of mutualism is increasingly higher if A provides more resource to B (area of dark gray triangle increases with increasing quantity of resource produced by A). Similarly, the benefits it receives from mutualism depend on the quantity of resource provided by its partner. In panel A, the black dots and dotted lines indicate that species A gets enough resource from B to benefit from the mutualism despite the cost of providing resources to B. Stable resource exchange rates (e.g., Fig. 2A) in microbial mutualisms have been defined for several interactions through a combination of metabolic or population modeling and experimental tests of stability. In addition, showed that the costs and benefits of methionine production in a cross-feeding mutualism varied among alleles that were substituted in independently evolving populations. As expected, there was an inverse relationship between the cost of production and benefit to the growth of the community.

Mutualist A in Figure 2B can increase the fitness benefits it receives from mutualism in one of the following ways. First, it can become more efficient at using, producing, or transferring resources. This kind of adaptation would change the relationship between the quantity of resources transferred and the benefits or costs received, causing the shape of the cost and benefit triangles to change. Efficiency improvements in resource transfer may decrease costs for both species and hence enhance their fitness. Changes in the use or production of resources by A could have indirect effects on the fitness of B if it results in more individuals of A that are producing benefits to B. Second, mutualist A could invest more in the mutualism, providing more resource to B so that it can get more resources from a larger population of B (Fig. 2C).

In microbial mutualisms, improvements in resource transfer or use have been observed in the lab within the first few hundred generations of evolution for several newly formed mutualisms. In some cases, new structures evolved that allow metabolite transfer to occur without loss of material through diffusion. For example, when two species of Geobacter were forced to cooperate through the exchange of electrons, they adapted by producing wires coated in cytochromes that pass electrons directly from one species to the next. In a cross-feeding mutualism, metabolites were transferred through tubes connecting the cytoplasms of two species.

However, new structures for resource transfer were not required to improve the productivity of some microbial mutualisms. In the first 300 generations of evolution of the syntrophy I study, changes in both Desulfovibrio vulgaris and Methanococcus maripaludis contributed to substantial increases in the number of cells produced from a fixed amount of a single limiting resource and resulted in substantial increases in growth of the community.

By 1000 generations, the communities grew about...
Figure 2. A conceptual diagram of the relationship between costs, benefits, and adaptation for hypothetical species A. Species A can provide a quantity of resources to its partner B, between zero and the maximum amount that is physically possible to produce (dark gray arrow). Similarly, B can provide a quantity of resource to A that varies between none and a physiological maximum (light gray). The dark and light gray triangles indicate the rate at which the costs (dark gray) and benefits (light gray) increase for species A as a function of the quantity of resource exchanged. Black dots indicate the actual quantity of resources provided or received by A, and the associated dotted lines mark the size of the cost or benefit for A at the indicated points. (A) Species A obtains more benefits from mutualism with B than it pays in costs. (B) Species A has adapted by becoming more efficient at producing the resource it provides to B. This decreases the physiological cost per resource, causing the dark gray triangle to be smaller. As a result, the benefit/cost ratio in panel B is higher than in panel A. (C) A provides the same amount of resource as in panel B, but its benefits are higher because it has stimulated the growth of the species B population. (D) Species A has maximized its benefit/cost ratio by providing less resource to B.

three times faster than the ancestor, a change that was correlated in *D. vulgaris* with loss of its ability to respire sulfate, a trait that has a substantial impact on survival outside syntrophy and may impede performance in syntrophy.18,77

Another way for mutualist A in Figure 2 to adapt would be to alter the rate of resource exchange between the partners so that it provides less while taking more, as depicted in Figure 2D. If this adaptation increases the fitness of A while simultaneously reducing the fitness of B, it may be referred to as cheating.8,71,78 Left unchecked, such cheaters could potentially destabilize the mutualism, causing it to go extinct.79 Finally, mutualist A could adapt by developing a mechanism to protect itself from cheating by B. Several putative defenses against cheating have been described theoretically,71,80 and some have been empirically tested in diverse species, including microorganisms.10,81–84

Laboratory experiments with cross-feeding microorganisms have repeatedly documented selection for cheating within mutualism and characterized the impact of exploiters on pairs of mutualists.10,84,85 However, most of this work has focused on identifying mechanisms for suppressing the effects of cheaters, often focusing on the impact of spatial structure and aggregation.10,15,84 This research has shown that spatial structure and aggregation keep cooperative pairings together for multiple generations, allowing them to flourish through partner-fidelity feedback.10,15,84 In partner-fidelity feedback, mutualists that are more cooperative will tend to be more productive and have higher fitness because they are providing more resources.
to each other compared with less-cooperative pairings. These higher levels of resource provisioning increase the fitness of the interacting partner more (e.g., Fig. 2C), allowing them to flourish and provide even more benefits in return.\(^{71}\)

Spatial structure also affects the distribution of resources that are being exchanged by mutualists and the potential impact of competition and exploitation on their stability.\(^{61,69,75,85–87}\) Cross-feeding can be inhibited by spatial structure if metabolites must diffuse too far between species.\(^{61,75,87}\) In addition, the extent to which an exploiter can invade a population depends on whether cross-fed metabolites are universally shared or whether the producer privatizes them by keeping some or most of the metabolites for itself.\(^{87}\)

Finally, research with microbial mutualisms has revealed novel mechanisms for suppression of cheating.\(^{12}\) Theory predicts that mutualists can become immune to cheating as a result of adapting to cheaters during the early stages of their evolution, although it does not describe exactly what these adaptations are.\(^{88}\) An example of such immunization was observed by Waite and Shou\(^{12}\) when they engineered yeast to cross-feed and then propagated them in conditions where no other known mechanism of resistance to cheating could occur. As the populations containing cheaters and cooperators adapted, they acquired new mutations. Cooperators dominated some populations, driving cheaters extinct, because they acquired new beneficial mutations before the cheaters, giving them a fitness advantage.\(^{12}\)

The examples described above imply that there is only one mechanism by which any given trait might affect fitness in mutualism. In reality, a trait could affect fitness in mutualism by multiple mechanisms (Fig. 2), depending on the context in which the species are evolving.\(^{89,90}\) For example, the wires and tubes produced by microorganisms that allow direct metabolite transfer between species are assumed to have been selected to increase the efficiency of chemical transfer (although neither the fitness effects nor the efficiency of transfer have been tested), but they could also have other effects.\(^{13,51}\) For example, wires produced by one species could be used to force a partner genotype to interact with it, even though that genotype would have higher fitness with another partner. Alternatively, like aggregation or spatial structure, wires and tubes may help keep genotypes together for multiple generations, enhancing partner-fidelity feedback.\(^{70}\) In addition, while it seems unlikely for wires and tubes, the possibility that a trait has no effect on fitness, or is a by-product of the construction of another trait, must always be considered when studying adaptation.\(^{90}\) Distinguishing between these possibilities requires measuring the effects of the trait on the fitness of both partners as well as testing what the trait can do.

**Challenges affecting the study of adaptation to mutualism**

Despite an abundance of theory and the existence of traits thought to be adaptations for mutualism, we are far from having a comprehensive understanding of how mutualism causes selection in various circumstances. Here, I highlight two problems with the current approach to understanding adaptation to mutualism and how they may be addressed in microbial mutualisms.

The first problem with the current approach is bias in the choice of traits tested as putative adaptations. Because of the complexity of organisms, it is difficult to examine every feature simultaneously, so biologists must make an educated guess about features likely to be under selection and those that are not. This process can result in bias about what kind of adaptations are studied. For example, in recent years, there has been a nearly singular focus on how mutualists defend themselves from cheaters, with very little work addressing other mechanisms of adaptation that are outlined in Figure 2. The reasons for this are unclear. Perhaps there has been an underlying assumption that the most commonly studied mutualisms have been evolving for so long that both species have optimized resource production and transfer efficiency. This may be true, but recent research suggests that fitness can continue increasing for longer than 60,000 generations, even for a simple organism evolving on a single limiting resource in a constant abiotic environment with no other species to interact with.\(^{91}\) Does one expect mutualists to have a constant optimum and to somehow reach it faster? Alternatively, perhaps researchers have assumed that cheating would provide the greatest fitness advantage in mutualism because the cheater pays no cost, and as a result cheating dominates mutualism evolution and should be the primary focus of studies.
of adaptation. Recent research has questioned the validity of this assumption for legumes and nitrogen-fixing symbionts. Although selection on cheating has been demonstrated in one community of these mutualists, a broader analysis of data on these interactions suggests that cheating is rare and not a major cause of selection.

The second problem is that empirical studies of adaptation to mutualism have not consistently linked what a trait can do with how and why it actually affects fitness. As a result, the validity of the most prominent examples of cheating in mutualism have been called into question. As Jones et al. explain, cheaters do not simply provide less resource to a partner. They must be able to procure enough from their partner to maintain a high benefit/cost ratio (i.e., in Fig. 2, the triangle for resources received must remain large) at the expense of their partner’s fitness. In addition, to affect the stability of the association, cheaters must arise from within the mutualism. Some commonly described examples of cheating in mutualism, however, have fallen short of these criteria. Some have been descriptions of outside species that exploit a mutualism. In other cases, cheaters were identified solely by their inability to provide resources to a partner without the tests of fitness effects that would be critical for ruling out alternative explanations, such as the possibility that they are poor mutualists.

Research on microbial mutualisms can contribute to generating a broader, rigorously tested picture of the selection environment caused by mutualism in numerous ways. First, synthetic biology can be used to test specific hypotheses and assumptions about when selection on cheating or other adaptations is expected to be strongest. For example, mechanisms for excluding cheaters can be removed to see if cheaters are more likely to evolve and become prevalent than they would otherwise. Alternatively, the possibility of cheating could be removed to see if defenses against it quickly erode. These experiments could be performed with mutualisms of varying dependencies.

Second, evolution experiments with mutualisms can be combined with genome sequencing and genetic engineering to perform rigorous, open-ended tests of the effects of mutualism on adaptation. Genome sequencing provides an unbiased picture of all the mutations (potential adaptations) substituted during evolution. The fitness effects of all or a random subset of these mutations could be tested by removing or moving each mutation into the unevolved ancestor and then comparing the fitness of both partners. Such experiments may provide an estimate of which kinds of adaptations are most commonly selected: those that decrease the fitness of a partner or those that do not.

**Coevolution of mutualism**

In the previous section, I explained how a mutualist could adapt to its interaction by maximizing the ratio of benefits to costs. When one species adapts to mutualism, how does that affect its partner’s evolution? One possibility is that adaptation in one species affects the fitness of its partner, but it does not change the relationship between its partner’s phenotype and fitness. For example, a methanogen adapting to syntrophy may become more efficient at converting energy from the interaction into biomass, increasing its abundance and capacity to remove hydrogen, and thereby benefiting both partners’ fitnesses. Such a change may affect all genotypes in the fermenter population equally regardless of their phenotype. Alternatively, some fermenter phenotypes may be better able to profit from faster growth of the methanogen, giving them a competitive advantage in the presence of the evolved methanogen, resulting in evolution in the fermenter population. In this second scenario, evolution of the methanogen changes the relationships between phenotype and fitness in the fermenter population, causing the fermenter population to evolve. If partners adapt to each other’s adaptations repeatedly, then they are coevolving.

There are several lines of evidence suggesting mutualisms coevolve, but few definitive tests demonstrating it, especially in interactions between microorganisms. Perhaps the most convincing evidence for coevolution comes from the existence in ancient mutualisms of complex structures and behaviors in each species that seem to match each other. There may be multiple alternative explanations for such observations, but phylogenies of yucca and yucca moths and of Mycorrhizae and plants have demonstrated reciprocal evolution of morphologies mediating the interaction of both species. Other researchers have demonstrated the potential for ongoing coevolution. In the interaction between Rhizobia and legumes, GXG interactions were observed, where the fitness of a
genotype in one species differs depending on the genotype of its interacting partner. Coevolution between flies and flowers was suggested by a correlation between fly proboscis length and floral tube length, and further supported by experiments estimating the fitness effects of floral tube length in the presence of varying fly proboscis sizes. Other scientists have claimed that coevolution was occurring based on codiversification or correlations between traits of interacting species, but this could result from two species adapting in parallel to similar changes in an environment. In microorganisms, intergenic epistasis and adaptation to a mutualist partner have been observed, but ongoing coevolution in mutualism has rarely been described.

This work suggests that coevolution can shape mutualisms and continue to affect their evolution, but it is far from providing a clear picture of when and how it will happen in diverse species. Achieving this will require more theoretical work focused on coevolution in mutualism, along with rigorous experiments that test those theories. Some of this can be achieved by using genomics to test for molecular dynamics consistent with coevolution in natural populations of mutualists. In microorganisms, time-shift experiments can be used to test whether and how coevolution is occurring at the phenotypic level. In a time-shift experiment, contemporary populations of one interacting species are tested against populations of their partners from the past and sometimes the future. Such experiments have been used successfully to test whether and how host–parasite interactions have coevolved in both natural and laboratory-based populations. Host–parasite time-shift experiments have been performed with microorganisms and with macroorganisms that have dormant stages. They have also been used to understand the coexistence of cross-feeders in experimental populations of Escherichia coli. Apart from these latter experiments as well as my own research on mutualism, time-shift experiments have rarely been applied to mutualism.

Below, a series of literal toy models are used to describe three mechanisms for coevolution in mutualism, their effects on evolutionary dynamics, and how they can be distinguished from one another and from coadaptation through time-shift experiments.

**Coevolution and natural selection in mutualism**

If a mutualist has coadapted with its partner but its partner has not evolved in response, its adaptations will have a similar effect on the fitness of all of the partners it has encountered during its evolution in mutualism. Thus, the pattern observed in a time-shift experiment will be a flat line, as depicted in Figure 3C. If a mutualist coevolved, however, then its fitness will vary depending on which partners it is paired with from the past or future (Figs. 3–5). The observed patterns will depend on the kind of selection that is being caused by coevolution, as described below.

**Coevolution in the early stages of adaptation to mutualism.** Populations may coevolve during the early stages of adaptation to a new mutualism, as each species is acquiring or repurposing traits that maximize fitness in the interaction. An example of this process is presented in the literal toy model that is depicted in Figure 3A. This mutualism consists of species A and B, which are each capable of consuming nutrients produced by their partner, similar to a cross-feeding interaction. The process of coevolution begins on the left side of the figure, where the species are capable of trading nutrients but have not yet adapted to this new interaction. Species A first adapts to the mutualism by acquiring a trait that allows it to make a rudimentary vehicle to transfer nutrients more quickly between partners. Now that this rudimentary vehicle exists, species B is able to acquire a new set of traits. The figure follows the evolution of this nascent association as each species acquires a new trait that might affect resource exchange via changes to the vehicle. During the process, the vehicle becomes increasingly complex as the species build upon one another’s adaptations over time. This process is similar to escalating coevolution. A key feature of escalating coevolution is that new adaptations are substituted successively as each species responds to changes in the other, resulting in correlated selective sweeps across species (Fig. 3B).

This scenario is analogous to any mutualism where each species appears to have acquired multiple adaptations affecting the interaction and where these evolutionary changes seem to affect each other. It could represent sequential acquisition, repurposing, and optimization of the numerous genes in
Natural selection in microbial mutualisms

Figure 3. A literal toy model of coevolution during the early stages of adaptation to mutualism. A vehicle that transfers resources between mutualists A and B is made progressively more complex as each species adapts to the impacts of the piece that was added by a partner. Numbers indicate populations that have evolved new traits during the evolutionary history of this association. (A) The process of coevolution occurring over time. (B) The underlying evolutionary dynamics. Selective sweeps occur whenever a population evolves a new piece for the car, but they can also occur for traits unrelated to mutualism (black lines). (C) The outcomes of a time-shift experiment if coadaptation has occurred. (D) Three alternative outcomes for a time-shift experiment with this mutualism.

Legumes and Rhizobia that allow the bacteria to fix nitrogen in nodules within a root instead of near the root’s surface.120 It could also apply to the evolutionary history of ancient associations, such as Mycorrhizal fungi and plants.105,121 Fungi first became endophytes in response to the presence of root exudates, then plants evolved recognition mechanisms to exclude pathogens, followed by the evolution of specialized plant and fungal cells.105 It could describe the initial stages of adaptation to a hypothetical cross-feeding interaction where one species might adapt by evolving the ability to make nanotubes. Subsequent evolutionary steps in such associations have yet to be documented, but a logical hypothesis might include changes in cell surface structures in the nanotube recipient, followed by changes to the shape or length of nanotubes made by the producer species.

When escalating coevolution is occurring through the processes modeled in Figure 3, the results of a time-shift experiment will depend on the relationships between the newly evolved traits as well as the sign of their effects on fitness. Population B1 (Fig. 3) can form an increasingly better car when it is paired with populations A, A1, and A1,2 from the evolutionary trajectory of species A. Assuming each new adaptation by A has a positive effect on the fitness of B, fitness in those pairings is expected to increase with each vehicle improvement (Fig. 3). This pattern can change when population B1 is paired with partners farther into its future, such as population A6. This population has adapted to pieces produced by descendents of B1, but which B1 has not yet evolved the capacity to produce. There are basically three potential effects of the pieces that genotype A6 produces. First, they may improve the utility of the vehicle despite the lack of supporting pieces from B, resulting in increasingly higher fitness for B1 in pairings with A populations that have more vehicle adaptations (Fig. 3D). Second, the pieces contributed by A6 may not be produced in the absence of the proper supporting pieces, so that the most advanced vehicle produced is B1/A1,2, regardless of which future A partner B1 is paired with. In this case, the fitness of B1 with future partners will be the same as it was with A1,2, making a straight line. Third, the extra pieces produced by A6 could ruin the function of the vehicle without the supporting pieces produced by future populations of B, causing the fitness of B1 to be lower with each partner that produces more pieces.

Coevolution in established interactions. Coevolution may continue to affect even the oldest
natural selection in microbial mutualisms

mutualistic interactions. Coevolving populations may substitute new variants in response to their partners’ adaptations or cycle between multiple existing variants, or coevolution may cause continuous purging of variants with unusual phenotypes. The toy model in Figure 4 depicts ongoing escalating selection. In this model, selection favors a tower that is taller than that of its interacting partner. Initially, the tower of species A is one brick high, while the tower of species B is two bricks tall. The two-brick tower of species B causes selection for a taller tower in species A, causing variant A1 to become common in the species A population. Species B then responds by evolving a taller tower, and this evolution of increasingly taller towers in both species continues. This process results in multiple correlated selective sweeps in species A and B, as shown in Figure 4B. In a time-shift experiment, pairing genotype B1 with all the evolutionary intermediates of species A, the fitness of B1 would be highest when paired with A and decrease steadily as it loses its tower height advantage.

Coevolution can also occur when the relative fitnesses of genotypes within a mutualist population are frequency dependent. Interactions between species can cause negative frequency-dependent fitness, where the fitness of a genotype is higher when it is rare. This situation can cause fluctuations in the abundance of genotypes in one or both species populations, especially if there are constraints on the available phenotypes. Such dynamics have been observed in host–parasite interactions where the host immune system selects for rare parasite variants. In mutualism, coevolution can cause fluctuating selection in two mutualist partners if there are conflicts between species about which variant has the largest positive impact on fitness. In Figure 5A, for example, blue or green genotypes of the circle species (A1 and A2) have higher relative fitness when their square mutualist partner population is of the opposite color. The square
Figure 5. A literal toy model illustrating how coevolution can result from negative frequency-dependent selection. (A) Asymmetry in partner preferences between species causes negative frequency-dependent selection in populations of mutualists A and B. In this example, the fitness of genotype 1 relative to genotype 2 of species A (blue or green circles, respectively) is higher whenever their square partner (species B) is of the opposing color. However, genotype 1 of species B has higher fitness when it has the same color as its partner. Blue arrows indicate how the populations of A and B have changed in response to the frequency of blue or green in each population on the left. (B) This frequency-dependent selection can result in fluctuating dynamics in both species. (C) In a time-shift experiment of this interaction, the fitness of genotype B1 would differ depending on whether or not it was paired with its most recent partner.

species B, however, has the opposite preference. This lack of congruence between the fitness interests of each species results in fluctuating selection in both species (Fig. 5).^{123}

Fitness conflicts like this may explain the prevalence of poor-quality mutualists or cheaters. For example, the blue and green circles (A1 and A2) could both be poor mutualists embedded within a population of high-quality mutualists (black circles, not shown). In this scenario, imagine the green square (B1) is able to avoid the green circle (A2) but not the blue circle (A1) (e.g., because of variation in molecular signals produced by A genotypes and recognized by B genotypes, as proposed in Ref. 96), so the blue circle (A1) has the advantage of acquiring more partners when the green square (B1) is common, but green square has less access to high-quality partners than its competitor (B2), because it cannot exclude low-quality partners. The blue square is at a similar disadvantage when the green square is common.

In microbial mutualisms, asymmetry in partner preferences could hypothetically arise for a few reasons. In syntrophy, some partners could have higher fitness with a soluble electron carrier like formate than with hydrogen, while it may be more optimal for the fermenting species to produce hydrogen. In cross-feeding mutualisms, the optimal rate at which a producer secretes a metabolite may not match what is optimal for a recipient, and vice versa. If genetic variation exists within populations for these features, then asymmetry in partner preferences could result. Physical interactions between microbial mutualists could also cause asymmetries in partner preference. Perhaps the optimal aggregate size or nanowire length or width for variants of one species is not optimal for that which is produced by the other. There could also be variation in the shape of molecules enabling attachment of appendages or cells, and the variant that attaches best may not be the variant that produces metabolite the best.

These descriptions of how coevolution can affect selection are simplified in two respects. First, with the exception of the frequency-dependent selection example, my descriptions have all been based on the assumption that evolution of one species has a positive effect on the other. However, mutualists...
may also adapt in such a way that they decrease their partner’s fitness. This situation could result in evolution of defenses in the partner and coevolution that resembles that observed in host–parasite interactions, causing escalating selection or fluctuating selection that can be detected with time-shift experiments.\textsuperscript{41,113} Time-shift experiment results for coevolution resembling the vehicle toy interaction described in Figure 3 could be especially complicated if some adaptations have the effect of decreasing a partner’s fitness while others improve it.

Second, my previous explanations are based on simple pairwise interactions, but several common features of natural communities are likely to affect these processes. Mutualistic interactions may commonly occur between guilds composed of functionally similar species, especially in microbial communities.\textsuperscript{6,127–129} It is theoretically possible for a species to coevolve with a guild, but the effects of coevolution may vary considerably among species within a guild.\textsuperscript{127} In addition, coevolutionary outcomes can be altered by predators, competitors, or mimics.\textsuperscript{85,130–133} Another reason coevolution may be more complicated is that mutualists likely also adapt to the abiotic environment or interactions with other species, and this may cause selective sweeps (Fig. 3B, black lines) that interrupt escalating selection.\textsuperscript{79,121,134} Finally, the relative abundance of these third parties and abiotic variables affecting the ecological success of mutualists often vary geographically, resulting in varying interactions and evolutionary outcomes across a species range.\textsuperscript{135}

**Conclusions and future directions**

Despite decades of progress on our understanding of mutualisms and how they evolve, we are still far from a comprehensive understanding of the impact of mutualism on adaptation and coevolution. Biologists have identified adaptations to mutualism that seem to fit theoretical expectations, but more must be identified and tested appropriately before the field can move from describing how mutualists can adapt to explaining how they will. In addition, while several models of coevolution in mutualism have been described, there are few examples where such models have been rigorously tested.

Throughout this review, I have described how microbial mutualisms could be used to address these gaps between theory and empirical research by providing in vivo representations of models.\textsuperscript{136} I suggested manipulating the possibilities for cheating or defenses against it in order to learn how each affects adaptation to mutualism. In addition, genome sequencing of evolved mutualists may provide an unbiased list of putative adaptations to mutualism, allowing researchers to gain a more comprehensive view of how mutualists adapt. I also suggested the use of time-shift experiments to test whether and how coevolution affects the evolution of mutualists. These suggestions are just a sampling of what researchers could do with microbial mutualisms to increase the breadth of our understanding of how mutualisms evolve. Realizing the full potential of microbial mutualisms as tools requires more scientists to use them. Those already studying microbes must also increase their focus on testing broader theories about mutualism.

The use of microorganisms as tools, however, is not the only way in which they can benefit the field as a whole. Phenomena occurring within microbial communities has already inspired new theories about how mutual dependencies evolve through the loss of traits, theories that may apply to other organisms.\textsuperscript{6,60} As improvements in technology help microbiologists to gain a better view of the inner workings of microbial communities, who knows what will be discovered and how it could shape our understanding of evolution in mutualism.

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**Competing interests**

The author declares no competing interests.

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