Measuring the Costs of Microbial Mutualism

Experiments involving freshly formed microbial mutualisms provide insights into processes affecting diverse microbial communities

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Nature abounds with mutually beneficial interactions between species, a subject that captivates many biologists. How does mutualism arise, and what is its impact on community processes? To address such questions, we are studying two simple forms of mutualism that are based on exchanges of metabolites between microorganisms. Studying these systems not only provides insights into the early stages of mutualism evolution, it also could reveal basic processes affecting diverse microbial communities.

Understanding cross-feeding interactions, in which one microorganism obtains nutrients from another, and their evolution will be critical for predicting or controlling microbial processes affecting human health, climate change, and cleanups of contaminated sites. Moreover, cross-feeding is a key element determining the survival of many species of interest. Knowing the ecological conditions favoring cross-feeding may be essential for isolating such species for further study and also for controlling their abundance and activity in natural environments. Further, laboratory experiments demonstrate that cross-feeding can evolve rapidly even from isogenic populations. When a natural community is perturbed, say, in a bioremediation attempt, new relationships between species may develop while metabolite fluxes change. Understanding the ecological and evolutionary conditions that favor new cross-feeding interactions will be necessary to interpret these changes.

Summary

• In mutualism, reciprocal altruism requires feedback and a means for directing benefits to cooperators.
• Experiments with cross-feeding microbial species provide examples of how differences in the cost of cooperation influence interactions that lead to improved mutualisms.
• After Desulfovibrio vulgaris and Methanococcus maripaludis are cocultured, samples of either co-adapted species can improve yields relative to samples from the ancestral coculture.
• Escherichia coli and Salmonella enterica serovar Typhimurium can rapidly evolve cross-feeding interactions, including secretion of metabolically costly compounds from cells of the latter species.
• Studying mutualisms in microbes could enhance our ability to engineer novel consortia and will improve our understanding of complex feedbacks between genetic variation and community ecology.

Costs, Benefits, and Stability of Mutualisms

Beneficial interactions between species arise for several reasons. In the case of by-product interactions, benefits to another species arise as byproducts of an organism’s actions (Fig. 1). For example, elephant dung benefits dung beetles. Such simple interactions
persist because they cost nothing to the provider. However, it is still possible that these interactions affect the evolution of each species. What of the adaptive dynamics that arise from novel byproduct mutualisms? How do species ratios fluctuate through time? What is the impact of adaptation on productivity and nutrient transfer through a community? Do partners sometimes become dependent on each other?

A second reason for beneficial interactions is reciprocity, or reciprocal altruism, where one organism provides benefits to another and receives something in return. This type of interaction, which can involve costly goods, raises questions about those costs. If it is only beneficial to aid a partner if the partner is aiding you, how does the cycle start? Furthermore, these interactions are prone to breakdown if cheaters invade, receiving benefits but not reciprocating.

Theory suggests that reciprocal altruism requires feedback and a means for directing benefits to cooperators. Spatially structured environments offer a way to keep benefits close to cooperators, rather than having those benefits simply diffusing to the population as a whole. The benefits an individual receives become tied to the help it provides its mutualistic partner, and, hence, cooperators receive more benefits and may out-compete nonhelping cheaters. Again there are questions: Does spatial structure adequately explain the criteria for the evolutionary origin of cooperation? When is reciprocity favored over other adaptive solutions? How are community properties influenced by cooperation?

**Cross-Feeding in Microbial Communities**

Microorganisms release a diverse array of molecules into the environment, including amino acids, fermentation products such as acetate, or electrons in the form of hydrogen or other molecules—making those nutrients available to neighboring organisms. Cross-feeding interactions affect most microbial communities. Cross-feeding interactions may be commensal, in which one species benefits but the other is unaffected. However, if both species benefit, the interaction is mutualistic. Mutualism may occur when the secreted byproduct inhibits the growth of the producer, or when both species use metabolites produced by one another.

Methanogenic archaea that depend on cross-feeding with fermentative microorganisms produce virtually all of the estimated 1 billion tons of global methane generated per year. In anoxic environments, cross-feeding bacterial communities first degrade complex carbon polymers primarily to hydrogen, carbon dioxide, and acetate through a series of fermentation reactions. Methanogens use these molecules to produce energy by reducing carbon dioxide to methane. Alternatively, other species of bacteria couple oxidation of these intermediates to reduction of another substance such as nitrate or sulfate through anaerobic respiration. In the absence of terminal electron acceptors, only fermentation and methanogenesis are favored, a process central to the global carbon cycle and to bioreactors that degrade municipal waste. Knowing more about cross-feeding and evolution should improve our ability to control microbial growth and to predict how natural and anthropogenic changes affect global processes.

Considering the diversity of molecules, species, physiological mechanisms, and environments in which cross-feeding occurs, how can we develop a framework that will allow us to predict and control cross-feeding and evolutionary outcomes, which depend in part on chance? One possibility is to focus on natural selection,
Hillesland: from Math and Music to Microbiology, Mutualism, and Knitting

Kristina Hillesland liked math as a child, but was more interested in understanding people, relationships, language and culture, philosophy, theology, and literature. Her interests shifted, however, after a high school teacher presented a series of broad scientific questions and encouraged the class to consider how information from fields such as biology, chemistry, and physics fit together. “At this point, science ceased to be a collection of facts about how things worked, and I was able to see it more from a philosophical perspective that was very exciting,” Hillesland says. “This didn’t quite make me decide to become a scientist, but it opened up the possibility that science was interesting in its own right, not just as a stepping stone to becoming a physician or engineer.”

Later, a microbiology professor required students to design and perform their own experiments. “This was my first experience doing research, and it made me consider becoming a scientist, although I didn’t know at the time how to do that,” Hillesland, 36, says. To be sure, her scientific career is still relatively short, as she is starting this fall as an assistant professor of biology in the science and technology program at the University of Washington, Bothell. Her research focus is on understanding interactions such as predation and mutualism between microbial species, and how these processes affect them.

“Evolutionary changes are an important part of that process, but very few microbiologists consider evolution,” she says. “I hope that by studying microorganisms that microbiologists care about, and studying their evolution in real time, as opposed to looking at what evolved in the ancient past, I can get more microbiologists to think about these issues and incorporate an understanding of evolutionary processes in their studies of microbial communities.”

Hillesland hopes her work will have practical applications in fields such as health, agriculture, and engineering. “The organisms I’m studying play important roles in communities responsible for processing human waste, and they produce significant amounts of methane, which is a greenhouse gas and potential biofuel,” she says. “I do not plan my research specifically to address these issues, but I hope that some of my findings will give engineers ideas that allow them to manipulate communities for these purposes someday.”

“I was very unsure about the greater meaning of the results,” she says, referring to her early experiments studying interactions between microbial species. “Many scientists study mutualism and cooperation from an evolutionary perspective, but they are often focused on ‘cheating,’ and the conditions that minimize its effects . . . There really weren’t many examples of work that was similar to my own. I felt like I was going out on a limb a little in asserting the evolutionary steps we would expect to occur. It was very gratifying when this work was accepted for publication and commented on by others. It gave me confidence in my own ability to define what is important without a famous or more seasoned scientist telling me the ‘right’ answer.”

Hillesland grew up in Pasco, an agricultural town in eastern Washington State, the third of five children. Her father, an engineer, taught her how cameras and diesel engines work, and why. “He taught me some calculus in 6th grade, but I didn’t really understand or remember it,” she says. “He liked to use napkins and a pencil to explain things, usually because it was directly after dinner. I always liked math and was excited that I was being taught these more advanced topics. When I was really young, I aspired to be the smartest person in the world.”

Dinner was an important family ritual. “We had wonderful conversations about religion, philosophy, politics, and other things,” she says. “I can remember that once we were all so caught up in the conversation, we had to literally go around the table and take turns voicing opinions and ideas one by one, just to make sure no one was left out. Other times we raised our hands. Everyone in my family was very creative, and our parents cultivated that by having readily available creative outlets for us at all times—a wall full of musical instruments, ready buckets of Play-Doh, and art supplies. I think creativity is an essential component of science and an important part of my success so far.”

Hillesland looks back on her childhood piano lessons as an important experience. “I am an impatient learner, and would tend to give up when it became difficult,” she says. “My piano teacher taught me how to set long-term goals, break complex problems up into smaller pieces, reward myself when I complete challenges, and to be accountable for my actions by recording them.”

Hillesland earned her B.S. in biology in 1998 from Pacific Lutheran University, and her Ph.D. in microbiology and molecular genetics in 2005 from Michigan State University. Until recently, she was a postdoctoral research associate at the University of Washington in Seattle. Her husband of nearly 13 years is a Google software engineer. They are expecting their first child, a boy, in late October. Hillesland, an avid knitter, now does some of her own designs. “One of my dreams is to someday have a pattern published in a major knitting magazine,” she says.

Marlene Cimons
Marlene Cimons lives and writes in Bethesda, Md.
which is a powerful force, especially in large populations. In controlled environments, natural selection often leads large populations of microorganisms repeatedly to similar phenotypes, even though the random timing of mutations may lead to other differences among independent populations. Do coevolving populations of microorganisms exhibit similar levels of parallel evolution? If so, can we identify key attributes that lead cross-feeders toward similar evolutionary outcomes and then use this information to predict their behavior? Recent experiments with two cross-feeding mutualisms provide examples of how differences in the cost of cooperation influence the conditions under which cross-feeding interactions lead to improvements.

**Experiments Evaluating Byproduct Mutualism**

We (Hillesland and Stahl) mixed the bacterium *Desulfovibrio vulgaris* and the archaeon *Methanococcus maripaludis* to study mutualism as the two species adapt to this new cross-feeding interaction. Because these two microbial species were isolated from different environments, this experimentally imposed mutualism is similar to a cross-feeding interaction that might arise when any two species begin exchanging byproducts naturally. Indeed, many species of *Desulfovibrio* engage in similar mutualisms with methanogens in lake sediments, bioreactors, and gut environments such as the rumen.

When we culture *D. vulgaris* with *M. maripaludis* on lactate in the absence of hydrogen, sulfate, or other electron acceptors, the two species can gain energy only by cooperating to degrade lactate to acetate and methane (Fig. 2).

We evaluated 24 independent cocultures of these two species. During the early stages of growth, the cocultures proved unstable (Fig. 3a), and two went extinct. The surviving 22 cocultures produce at least 30% more cellular material up to 80% faster after 300 generations of growth than did the ancestral coculture (Fig. 3b. black bars). We then separated the 300-generation-old *D. vulgaris* and *M. maripaludis*...
populations from each coculture and paired samples from each with its ancestral mutualist population to test the relative contributions of each species to productivity changes gained over the 300 generations in coculture. Either species from nearly every evolved coculture improved the yield of the coculture, relative to a fully ancestral coculture, but not to the same extent (Fig 3b).

Despite these parallel responses, the dynamics of hydrogen transfer affected *M. maripaludis* differently than it did *D. vulgaris*. We subjected the cocultures to two different environments. In one, constant mixing transferred hydrogen quickly throughout the tube. In the other, hydrogen was allowed to diffuse through the unmixed liquid medium and accumulate in the headspace, leaving only small quantities of hydrogen in liquid for *M. maripaludis* to absorb. The ancestral cocultures grew more slowly in this static environment than in the one that was well-mixed. However, cocultures that evolved in the static environment grew faster in static tubes than did those cocultures that were constantly mixed.

This result suggests that cells evolving in static tube cocultures acquire a trait that well-mixed cocultures did not acquire, such as the capacity to grow well even when hydrogen levels remain low. Experiments with cocultures of mixed ancestry suggest that *M. maripaludis* was the source of that adaptation. In the well-mixed environment, *D. vulgaris* improved the coculture growth rate, but many *M. maripaludis* populations did not. However, when they were grown without mixing, both species accelerate their coculture growth rates to similar extents (Fig 3b). We suspect that *M. maripaludis* cells either encountered elevated hydrogen levels adjacent to *Desulfovibrio* cells or adapted to extremely low levels of hydrogen in the static environment.

**How Mutualism May Evolve from Commensalism**

One of us, Harcombe, now at Harvard University, but formerly at the University of Texas at Austin with Jim Bull—studied mutualism between another pair of microorganisms, in this case *Salmonella enterica* serovar Typhimurium and an auxotrophic strain of *Escherichia coli* (Fig. 2b). When the two strains are grown together on lactose minimal media, the *Salmonella* strain relies on carbon byproducts excreted from *E. coli* because *Salmonella* cells cannot metabolize lactose directly. However, this particular *E. coli* strain grows poorly on its own because it is missing a gene needed for synthesizing methionine. Although together these two strains contain all the genes needed for carbon metabolism and amino acid synthesis, dividing those pathways into the two different species leads to very low overall productivity. In particular, growth remains slow because *S. enterica* serovar Typhimurium does not excrete methionine.

To test whether productivity could improve through mutualism, we grew these two strains on lactose plates. After only two transfers, distinct colonies grew from the mixed-species bacterial lawn (Fig 4a). We then used permissive plates to isolate bacteria of each species from those colonies, and these isolates were grown on lactose in monocultures and cocultures with ancestral partners.

Growth occurred only when *E. coli* was grown with the *S. enterica* serovar Typhimurium isolated from the colonies. Furthermore, those strains of *S. enterica* serovar Typhimurium were secreting high levels of methionine, which the *E. coli* strain required, according to
HPLC analysis. This ability to secrete methionine proves highly costly to *Salmonella*, decreasing its growth rate by almost 60% when *E. coli* cells are absent. These experiments show that novel cross-feeding interactions can rapidly evolve and may involve cooperative secretion of costly compounds.

We subsequently sought to identify environmental factors necessary for the evolution of this cooperative interaction. In general, selection for cooperation is thought to require feedback and a mechanism by which cooperators receive a disproportionate amount of the feedback benefits. On lactose plates both criteria are met. Indeed, if methionine-overproducing *S. enterica* serovar Typhimurium strains compete against nonproducers in the presence of *E. coli*, the overproducers rapidly outgrow the nonproducers.

However, on acetate plates the feedback mechanism is lost because both types of bacteria can consume the primary carbon source. In this case, costly cooperation is not favored, and *Salmonella* that do not excrete methionine out-compete the methionine overproducing *S. enterica* serovar Typhimurium. Similarly, in liquid media the second criterion is not met because the feedback of carbon byproducts is shared equally by all *Salmonella*. Because the cost of overproducing methionine is not balanced by increased access to a carbon source, cooperative genotypes are driven to extinction, leading the community to crash. Theory thus appears to encapsulate the environmental variables that are key for costly cross-feeding interactions to arise.

**Lessons Learned, Future Directions**

These two sets of cross-feeding experiments differ in terms of the species involved, the types of molecules being exchanged, and the environmental conditions under study. In both cases, however, the separate microbial populations encounter similar challenges while the particular pairs of microorganisms develop mutualisms (see box, p. 431).

Moreover, in both cases, the cross-feeding interaction was initially unstable but became dramatically more stable and productive. Further, in both cases, changes occurred relatively rapidly. These similar outcomes suggest that adaptive evolution may be a useful means for improving microbial consortia that are being engineered for industrial applications.

The main difference between the two sets of cross-feeding microbial pairs is in their respective responses to well-mixed versus heterogeneous environments. In both cases, the efficiency of the interaction depends on diffusion of metabolites between species, and mixing speeds that process. For the *Desulfovibrio-Methanothermobacterium* pair, ancestral cocultures grow faster in a well-mixed than in a heterogeneous environ-
ment, and the cocultures responded differently to these conditions.

The *Salmonella-Escherichia* pair, however, did not survive being well mixed. Why? Simply put, because of the high costs of this interaction. The *Desulfovibrio-Methanococcus* pairing comes without apparent cost, whereas overproducing methionine costs *Salmonella* greatly in terms of its ability to proliferate. In the absence of costs, traits that improve individual growth rates are also likely to improve the rate of community productivity, preventing “cheating” strategies from disrupting the system. This rule appears true even if partners mutually depend on one another. In contrast, when there is a cost to mutualistic interactions, stability may depend on spatially structured environments to promote directed reciprocity between cooperating species.

Mutualistic interactions are found across all levels of biology. Our studies suggest that the ability of one species to consume byproducts of another species can provide a cornerstone from which such pairs develop novel mutualisms. Though this idea is not new, our experiments are among the first to support that theory. Many questions remain, however. Will selection continue to improve community productivity? Can genome-scale metabolic models quantitatively predict optimal metabolite exchanges? What are some of the constraints on metabolic mutualisms? What does coevolution look like in mutually beneficial cross-feeding interactions?

Further studies of mutualisms in microbes could enhance our ability to engineer novel microbial consortia and will improve our understanding of the complex feedbacks between genetic variation and community ecology. Such information will help toward developing a deeper understanding of how complex microbial communities such as the human microbiome interact with their hosts.

**FIGURE 4**

Novel costly cooperation evolved. (A) Mutant cooperative *Salmonella* were evident because they allowed mixed-species colony growth on plates densely covered with the *E. coli* and *Salmonella*. The blue X-gal dye indicates that *E. coli* are able to grow only in the presence of cooperative *Salmonella*. (B) On agar plates cooperators spread in the *Salmonella* population, thereby increasing community productivity. (C) In shaken flasks cooperators decreased in frequency leading to community collapse. On the log density graphs, filled circles represent *E. coli* and empty circles *Salmonella*.

**SUGGESTED READING**


