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A Social Life for Discerning Microbes

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Microbes are not only extremely social but also extremely discerning about whom they socialize with. Recent research has uncovered some of the evolutionary explanations behind these feats of social sophistication in bacteria (Ackermann et al., 2008; Diggle et al., 2007) and, most recently, has provided insights into the molecular mechanisms of discrimination in yeast (Smukalla et al., 2008).

Cooperative acts ranging from minor help to marked self-sacrifice are seen in all corners of biology, from microbes to man. Given the presence of "freeloading cheaters," why is it that cooperative behaviors persist? Following the pioneering work of Hamilton (1964), a consensus has emerged that cooperative behaviors evolve and are maintained through a mix of self-interest and nepotism, which maximizes an individual's inclusive fitness, that is, the reproductive success of an individual and its close relatives. Cooperation may be self-interested if it directly benefits the actor as well as the recipients (for instance, increasing the success of one's own group). More extreme forms of cooperation—for example, altruistic cooperation where individuals experience a direct fitness cost in helping others—may be favored because the behavior helps recipients who are likely to share the altruistic gene (Hamilton, 1964). In other words, natural selection will cause a gene to spread if it confers an advantage on the individual in which the gene is present, as well as if it confers an advantage on other individuals with the same gene. But for this latter process to operate, altruistic acts must be preferentially directed toward other altruists. The most common scenario is that altruism is expressed blindly to neighbors, who will tend to be relatives (with an overrepresentation of similar genes) due simply to population viscosity (there is not complete mixing of individuals within the population) (Hamilton, 1964). A more complex scenario involves active processes of kin recognition and discrimination. However, this is not a perfect system as kin may still be "cheaters" that lack the vital gene for altruism.

Hamilton (1964) realized that one foolproof way to avoid wasting help on cheaters is for an individual to clearly display an altruistic or social gene and to recognize directly the same gene in others, rather than relying on proxy identifiers such as location or kinship. An individual social gene displays itself and enables recognition of the same gene in others, thus ensuring that help is only conferred on individuals expressing that gene. Richard Dawkins (1976) popularized this notion as the "green beard" gene: a gene that can be recognized externally because it confers a green beard on its carrier, ensuring that only green-bearded individuals are helped.

Hunting for Green Beard Genes

Green beard genes remained a plausible thought experiment until a remarkable empirical example was reported by Keller and Ross (1998): They discovered a gene cluster linked to the display and discrimination of identity in red fire ants. Next, Queller et al. (2003) identified an even tighter association between display, discrimination, and social traits in the first clear documentation of a single green beard gene in the slime mold *Dictyostelium discoideum.* When food is plentiful, *Dictyostelium* amoebae lead a single-celled, individualistic life of consumption and (asexual) reproduction. However, once starvation arrives they aggregate to form multicellular assemblies, replete with complex signaling mechanisms, division of labor, and individual sacrifice. The multicellular slugs differentiate into a sacrificial stalk, on top of which sit the lucky spores, which can then hitch a ride on invertebrates to potentially more favorable destinations. Queller et al. (2003) demonstrated that a single gene is responsible for holding nonstalk-forming "cheaters" at bay, by controlling entry to the spores. The *csaA* gene encodes a cell adhesion protein anchored in the cell membrane (the discernable green beard), which binds to homologous adhesion proteins (discrimination), building a multicellular aggregate preferentially of *csaA* carriers (targeted cooperation). This simple mechanism highlights the ability of microbes to harness molecular tricks to build sophisticated social behaviors (Foster et al., 2007), raising the possibility that these feats may be more common in microbes than previously suspected. Indeed, the bacterium *Proteus mirabilis* will only form motile swarms with those of the same strain. Although the molecular mechanism behind this recognition has not yet been fully deduced, a set of genes required for the recognition of "like" cells has been identified in *P. mirabilis* (Gibbs et al., 2008).

A Discriminating Yeast

The latest addition to the green beard gene stable, as Smukalla et al. (2008) report in this issue of *Cell*, appears in an unexpected microbe, the budding yeast *Saccharomyces cerevisiae*. Most strains of *S. cerevisiae* display an aggregating response to environmental stress called flocculation. Smukalla et al. (2008) now reveal that the expression of a single gene, *FLO1*, restores flocculation to a laboratory strain of *S. cerevisiae*, S288C, generating, at an individual cost, a social protection for S288C yeast within the aggregate (floc) against diverse environmental stresses such as ethanol and fungicides. But what about the potential for nonexpressing *flo1* cells to act as cheaters, exploiting the protection of established aggregates without paying the cost associated with *FLO1* expression? Smukalla et

al. (2008) go on to demonstrate that *FLO1* expression results in the targeting of help (aggregation) to other *FLO1*-expressing cells, another case of green beard-directed discrimination.

The molecular mechanism underlying the preferential attachment of *FLO1*-expressing yeast cells to like cells is similar to the role of *csaA* in *D. discoideum.* However, rather than binding to identical neighboring proteins as in the case of csaA, the adhesin encoded by *FLO1* binds to oligosaccharide chains on the surface of *S. cerevisiae* cells. Therefore, yeast decorated with Flo1 adhesins are more likely to bind together. Interestingly, although *FLO1*-expressing cells are still capable of some binding to cells that do not express *FLO1*, this low-frequency binding presents a surprising benefit to the cooperating *FLO1*-expressing cells near the surface of the aggregate. The Flo1 adhesins attract and bind a layer of cheater cells that coat the outside of the floc, so that these cheaters act as a first line of defense against environmental stress.

The overarching function of aggregate formation in yeast expressing *FLO1* and slime molds expressing *csaA* is similar, providing a self-chosen few with an escape from an unfavorable environment. Smukalla et al. (2008) show that, as for *D. discoideum*, *FLO1*-expressing yeast seem to regulate their aggregative behavior in response to their environmental state, including their local density (as it would be difficult to aggregate without many cells). This regulation may occur in direct response to environmental stresses such as ethanol, which may additionally serve as an indicator of density (ethanol is produced by yeast during fermentation). Interestingly, ethanol production may be under additional selective pressure by flocculating yeast because of its toxic properties, which may be more harmful to nonflocculating yeast. This suggests the possibility of cells specifically directing harm, rather tha just omitting cooperative acts, to individuals that do not share the same genes.

Who to Help, Who to Kill?

Classic green beard theory focuses on altruistic traits where the behavior imposes a net cost to the enactor and a net benefit to the recipient. However, the first empirical case of a green beard gene was an example of spite: red fire ants engaged in individually costly acts of antagonism against individuals lacking the green beard trait. Recent research also points to the widespread importance of green beard spite in microbes (Foster et al., 2007). For altruism or spite to evolve, Hamilton's rule $rb > c$ must be satisfied (Hamilton, 1964, 1970; see Box 1). By using spiteful green beard traits, microbes can actively target "unrelated" individuals that do not share the spiteful gene (Gardner et al., 2004).

Wherever microbial lineages compete for resources, a common outcome is the costly expression of secreted anticompetitor chemical weapons such as bacteriocins (Riley and Wertz, 2002). These compounds bind to specific receptors on recipient cells and act through numerous mechanisms, including the destruction of cell wall integrity and the inhibition of essential enzymes. Crucially, bacteriocins are tightly linked to specific immunity genes, meaning that individuals with a specific bacteriocin gene will also be immune to its effect (Riley and Wertz, 2002). This linkage creates a green beard gene complex that recognizes and punishes noncarriers. A number of recent papers have outlined a further example of microbial spite beyond the production of anticompetitor chemicals. Some of these studies found that bacteria carrying quiescent phage can use these "domesticated" viruses as a spiteful anticompetitor tool against bacteria that do not carry them (Brown et al., 2006; Joo et al., 2006). Other studies, including a recent report in *Nature* by Ackermann et al. (2008), have uncovered the potential ability of diverse pathogenic microbes to exploit the power of their hosts' immune systems to differentially kill competitors.

Pathogens can provoke an immune response that preferentially damages competitors. In contrast to specific targeting of certain individuals, the immunological "weapon" may be nonspecific (e.g., an inflammatory response). However, discrimination can still be engineered if the spiteful lineage is also better protected against the inflammatory reaction that they provoke (Brown et al., 2008). In their study, Ackermann et al. (2008) use a combination of mathematical modeling and in vivo experiments to shed light on the role that phenotypic noise plays within a population during self-destructive cooperative behaviors. As with all cases of self-destructive altruism (bee stings being a classic example), heterogeneous expression of the altruism gene within the population is crucial for the behavior to persist. If all individuals with the altruistic gene carried out the self-sacrificial altruistic act, there would be no surviving relatives carrying the altruism gene to reap the benefits. Ackermann and colleagues confirm in their model that altruistic acts directed toward individuals that share the same genes (high relatedness) in combination with gene expression heterogeneity (phenotypic noise) favor self-destructive cooperation. They further demonstrated that infection of the mouse gut by *Salmonella typhimurium* is mediated through mechanisms broadly consistent with their model. *S. typhimurium* uses heterogeneous expression of virulence factors (which could be stochastic but may be affected by the physiological state of the bacterium) to outcompete their competitors during colonization of the mammalian gut. Ackermann et al. report that a much greater proportion of the *S. typhimurium* population that invades the gut tissue expresses type III secretion system virulence factors (TTSS-1) than do the bacteria that remain in the gut, suggesting that TTSS-1 is required to invade gut tissue. Mortality of the TTSS-1-ex-

Box 1. Evolution of Altruism or Spite

For altruism or spite to evolve, Hamilton's rule of kin selection $rb > c$ (where r refers to the genetic relatedness between actor and recipient) must be satisfied (Hamilton, 1964, 1970). For an altruistic trait, c refers to the cost of the behavior to the actor, and b refers to the benefit to the recipient. In the context of altruism, this rule can only be satisfied if relatedness, r, is sufficiently positive, indicating that costly altruism can only be favored by selection if directed toward relatives. Whereas discrimination in choosing recipients is important to achieve sufficient relatedness for the evolution of altruism (although not essential as proximity to kin and "blind" altruism can suffice), discrimination is essential for the evolution of spite. Applying Hamilton's rule to a spiteful trait, c again refers to the cost to the actor ($c > 0$), but now b refers to the cost to the recipient ("negative benefit" where $b < 0$). Given $c > 0 > b$, Hamilton's rule can only be satisfied if r is negative, indicating that the evolution of spite requires targeting of those individuals who are less related to the actor than the average member of the population (Hamilton, 1970).

pressing bacteria that invade the gut tissue is very high, but their presence induces a host inflammatory response that kills more competing commensal bacteria than *S. typhimurium*. This act of self-destructive altruism by the gut tissue invaders therefore paves the way for invasion by their *Salmonella* brethren. (Ackermann et al., 2008).

Beard Diversity: Spite or Altruism in Action?

All of the above examples of microbial spite feature the concerted action of multiple genes that harm recipients and promote the survival of cells harboring them in the new environment engineered through the actions of these genes. Unlike a true green beard gene, these genes are at best a linked gene complex with opportunities for separation and recombination of toxin and resistance traits. This uncoupling of traits may provide opportunities for invasion by cheaters that retain the "tag" (toxin immunity) but do not carry out the costly social behavior (toxin production). However, a recent theory suggests that tag-based social behaviors can be readily maintained despite a reduced linkage between tags and behaviors if there is a diversity of tags or, to use the green beard analogy, many beard colors. As one tag-behavior pairing (e.g., altruism toward green beards) comes to dominate, the loose linkage between traits allows exploitation by cheaters (nonaltruistic green beards), who are in turn vulnerable to exploitation by rare new tag-behavior pairings (e.g., red beards and altruism toward red beards). Tags can thus become weak and potentially deceptive indicators of relatedness (compared to a classic single-gene green beard) but still function more reliably than spatial proximity alone (Jansen and van Baalen, 2006). This suggests that tag-based social systems are likely be associated with a diversity of tags (and associated directed altruism), where there is the potential for dissociation between tag and behavior. One example in the context of spiteful behaviors is that of soluble bacteriocins (pyocins) produced by the opportunistic pathogen *Pseudomonas aeruginosa*.

Intriguingly, this concept of tag diversity also may be applicable in the case of the yeast *FLO1* green beard gene. Unlike the *csaA* gene in *D. discoideum*, which shows little or no diversity within the species, *FLO1* in yeast is highly variable (Smukalla et al., 2008). Specifically, the number of tandem repeats within *FLO1* varies widely and correlates with the stickiness of the encoded adhesin—the more repeats, the greater the adhesive property. This diversity could be the result of broad variation in the environments encountered by the yeast, but it could also represent a discriminatory specialization in the binding between specific *FLO1* variants and specific cell-surface oligosaccharides. There is also the possibility that single-gene green beard traits could be "uncoupled" and exploited by cheaters. It is conceivable that due to the variation in *FLO1*, "green-bearded" cheaters—yeast expressing *FLO1* with a low number of tandem repeats—could arise. These cheaters would maintain their green beard identity at a low cost to fitness, thereby exploiting those yeast expressing *FLO1* alleles encoding adhesins with a greater number of tandem repeats. Thus, true green beards potentially can be exploited.

Public Signals and Discrimination

The roles of specificity, diversity, and discrimination take on a further twist when we consider that many acts of altruism or "public good" are regulated in a population density-dependent manner, typically with the production of extracellular altruistic acts being switched on at higher population densities. Bacteria are able to estimate cell density through a process called quorum sensing, where a collectively produced diffusible molecule is used as a proxy measure of cell density. Diggle et al. (2007) showed in the bacterium *Pseudomonas aeruginosa* that quorum-regulated public good traits (the production of extracellular digestive enzymes) are vulnerable to exploitation by a range of "signal" cheaters. In this study, cultured wild-type quorum-sensing *P. aeruginosa* competed against a range of isogenic mutant strains defective in either producing or responding to the quorum-sensing molecules in culture. The authors report that mutant strains that "opt out" of quorum sensing have a fitness advantage over wild-type quorum-sensing bacteria. In particular, the system is exploited by "signal blind" mutant bacteria that avoid contributing to the public good (the production of proteases to release nutrients from the special media used in the study) simply by ignoring the relevant signal to do so. Evolution of isogenic wild-type populations of *P. aeruginosa* in another in vitro study also supported this notion (Sandoz et al., 2007). After culturing wild-type *P. aeruginosa* for over 100 generations under conditions that require quorum sensing, a subpopulation of cells harboring mutations that inactivate the crucial quorum-sensing regulator *lasR* arose within the wild-type population (Sandoz et al., 2007). These spontaneous mutants that do not respond to the quorum-sensing signal consistently showed large fitness advantages over their wild-type ancestors, again suggesting that the metabolic burden imposed on cells by quorum sensing may make the system susceptible to the evolution of cheaters. The regulation of public good production by quorum sensing also raises the, as yet experimentally untested, possibility that cheaters purposefully overexpress a signal and therefore induce cooperation by gullible neighbors. Indeed, "super signalers" were observed in the in vitro *P. aeruginosa* experiments of Sandoz et al. (2007). This manipulative signaling may in turn lead to a recalibration of the signal response, which in theory could result in a potential stalemate between exuberant signalers and cynical receivers, a microbial version of the flashy signals of avian mating displays (Brown and Johnstone, 2001).

Although many quorum-sensing molecules are often nonspecific, with detection possible between species and even between kingdoms, doubt has been cast on the applicability of the term "signaling" in these contexts (Keller and Surette, 2006). Thus, only species-specific quorum-sensing molecules are considered here. The quorum-sensing molecules of *P. aeruginosa* are specific to this species, creating prime opportunities for social exploitation because any member of the species can potentially produce the signal for "public good" production in others while ignoring the signal themselves. One mechanism to reduce exploitation by signal cheaters is to make signals more specific. Rather than counting all cells, a well-tailored signal would count only those cells with the same signaling gene cluster, thus increasing the chance of changing behavior to maximize spread of the gene cluster without helping social cheaters. Patterns of quorum sensing in the bacterium *Staphylococcus aureus* offer intriguing hints

that specificity is indeed an important aspect of signal design in microbes. *S. aureus* bacteria control the expression of numerous virulence factors (for example, secreted exotoxins) in a density-dependent manner through the production and monitoring of autoinducing signal peptide (AIP) molecules. However, not all *S. aureus* AIPs are alike: Sequencing of the *agr* locus (controlling the Agr quorum-sensing system) reveals high genetic diversity across *Staphylococcus* species, with four distinct Agr types in *S. aureus* (Ji et al., 1997). Thus, Agr cells specifically measure and respond positively only to the densities of fellow cells of the same Agr type. Interestingly, this specificity itself has been exploited in a new way: Distinct variants interfere with each other during recognition, with AIP 1 blocking responses to AIP 2, and so on (Ji et al., 1997). Inhibition of quorum sensing in competing strains would seem detrimental because quorum sensing results in upregulation of toxins and degradative enzymes that may provide an advantage to all bacteria in the vicinity by producing nutrients and routes to new host tissues. However, inhibition of quorum sensing results in overexpression of bacterial attachment factors, keeping cells stuck to surfaces and preventing them from colonizing newly available host tissues. Clearly, optimal strategies for exploiting and interfering with quorum sensing will depend on the specific ecology of the bacteria.

Microbes have incredibly complex and sophisticated social lives that we are only beginning to understand. As a result, microbes present a unique opportunity to test social evolution theory and to garner clear support for the notion that individually costly cooperation is favored when individuals with the same genes can interact (through the process of kin selection). But is discrimination of social partners by microbes of more than just academic interest? We think it is for two reasons. First, manipulating the social life of pathogenic microbes could be a strategy for combating infection by antibiotic-resistant bacteria. Social cheaters engineered to be nonpathogenic could be introduced to infected hosts to weaken the population of pathogenic bacteria. Introducing spiteful producers of microbe-specific toxins would have a similar effect. Knowledge of the specificity of social interactions among microbes of course will be crucial. Second, given the low levels of recombination in many microbes, other genes are likely to be linked with social recognition genes. Thus, selection of particular social recognition genes could lead to correlated selection for other ecologically relevant traits, such as virulence and antibiotic resistance.

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