The origins of uncooperative rhizobia

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Mutualisms are thought to be destabilized by exploitative mutants that receive benefits from partners without reciprocation. Nonetheless, there is surprisingly little evidence for the spread of exploitation in mutualist populations. In particular mutualisms, non-beneficial partners are commonplace and this raises the question of whether exploitation is invading as an adaptive strategy. Here, we highlight the legume–rhizobium mutualism as a key test case. Rhizobial bacteria fix nitrogen in legume roots in exchange for carbon from their hosts. However, non-beneficial rhizobia are widespread, including non-fixing and non-nodulating strains. Recent research has shown that legumes can punish some uncooperative rhizobia and substantially reduce their fitness, but these sanctions must not be universally effective. Important questions about uncooperative rhizobia remain unresolved. (1) Is it adaptive for rhizobia to be uncooperative with hosts? (2) Do uncooperative rhizobia evolve from cooperative ancestors? (3) What are the mechanisms of rhizobial exploitation? We describe experimental approaches and testable hypotheses that address these gaps in our knowledge.

Mutualisms are cooperative interactions among species that are ubiquitous in nature (Herre et al. 1999). A potential impediment to the persistence of mutualisms is that individuals can evolve to selfishly take benefits from a partner without reciprocation (Soberon and Martinez del Rio 1985, Bronstein 2001, 2003, Yu 2001, Sachs et al. 2004). By definition, such exploiters gain fitness benefits at a fitness cost to the exploited partner (Sachs et al. 2004). While exploitation is predicted to spread in mutualist populations (Wright 1969, Soberon and Martinez Del Rio 1985, Herre et al. 1999, Bronstein 2001, 2003, Sachs et al. 2004) and potentially lead to the breakdown of mutualism, the empirical evidence for this is relatively scant (Sachs and Simms 2006). To invade a population of mutualists, a non-beneficial mutant must achieve a fitness advantage over cooperative genotypes. However, non-beneficial partners might often have poor fitness because they are maladapted to a current interaction (Thompson et al. 2002) or because they have been sanctioned (Bull and Rice 1991, Denison 2000). Surprisingly, studies of mutualism only rarely measure the fitness effects of an interaction in both partners (Kiers et al. 2003, 2006, Simms et al. 2006, Sachs and Wilcox 2006, Heath and Tiffin 2007), but this is required to test whether exploitation can spread as an adaptive strategy.

The legume–rhizobium interaction is an excellent model to examine exploitation in mutualist populations. Non-beneficial rhizobial partners are commonplace, and specific strategies and mechanisms of rhizobial exploitation have

been predicted (Denison 2000, Simms and Taylor 2002, Denison and Kiers 2004). Legumes can punish rhizobia that do not provide sufficient benefit (Denison 2000, Kiers et al. 2003, 2006, Simms et al. 2006), but the frequency of uncooperative rhizobia suggests that these sanctions are not universally effective. Here, we review the basic biology of the legume–rhizobium interaction, and two main types of uncooperative rhizobia; non-fixing and non-nodulating strains (Denison 2000, Simms and Taylor 2002, Denison and Kiers 2004). Subsequently, we suggest approaches to examine the origins and evolution of uncooperative rhizobia.

Basic biology and fitness effects of the legume–rhizobium mutualism

Several distantly related bacterial lineages form root-nodule mutualisms with legumes, most notably the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*, collectively called rhizobia (Sawada et al. 2003). Rhizobia infect the roots of legumes and differentiate into specialized endosymbiotic cells called bacteroids, which reduce atmospheric nitrogen in exchange for photosynthates provided by the plant (Sprent et al. 1987, Lodwig et al. 2003). Successful infection requires a compatible pairing of legume and rhizobial genotypes, though specificity varies widely in each partner (Denison 2000).

Rhizobial transmission among legumes is infectious (Sprent et al. 1987); rhizobia spread horizontally among host plants and not between parent plants and offspring (Parker 1999, Denison 2000, Simms and Taylor 2002). Furthermore, individual plants are commonly infected with multiple rhizobial genotypes (Dowling and Broughton 1986). Optimal virulence models, predict that: 1) horizontal transmission decouples the fitness interests of symbionts from their host, potentially allowing the spread of exploitative strains (Frank 1996), and 2) co-infection of hosts by unrelated symbionts promotes conflict over host resources, potentially to the detriment of the host (Frank 1996, Denison 2000, West et al. 2002, Simms and Taylor 2002, Sachs et al. 2004). An empirical test of virulence theory supports the hypothesis that increased rates of horizontal transmission can select for harmful symbionts over cooperative ones (Sachs and Wilcox 2006). In fact, many mutualisms in nature exhibit horizontal transmission (Herre et al. 1999), so exploitative mutants might commonly invade such mutualist populations.

Legumes receive significant fitness benefits from cooperative rhizobia: nodulation by effective rhizobia can improve legume growth by tenfold over infection by 'ineffective' strains (Burdon et al. 1999). Infecting rhizobia likely receive their most significant fitness benefit via release into the soil from senescing nodules (Kiers et al. 2003, Denison and Kiers 2004, Simms et al. 2006). The type of nodules a host produces can affect rhizobial reproduction (Denison 2000, Denison and Kiers 2004). Some legume species produce determinate nodules, in which bacteroids remain reproductively viable and can repopulate the soil; whereas other legumes produce indeterminate nodules, in which bacteroids terminally differentiate and soil populations are likely replenished by the subset of nodule rhizobia that remain undifferentiated (Denison 2000, Denison and Kiers 2004).

Strategies of uncooperative rhizobia

Rhizobia that offer negligible benefits to legumes appear to be widespread (Segovia et al. 1991, Sullivan et al. 1996, Quigley et al. 1997, Moawad et al. 1998, Burdon et al. 1999, Denton et al. 2000, Chen et al. 2002, Collins et al. 2002, Pongsilp et al. 2002), and have been described as 'cheating strains' (Simms and Taylor 2002), 'cheats' (Kiers et al. 2003), 'parasites' (Denison and Kiers 2004) and 'highly parasitic' (Thrall et al. 2007). In their review of selection pressures on rhizobia, Denison and Kiers (2004) focused on two potential strategies by which rhizobia could exploit hosts: 1) non-fixing strains that infect legumes but then offer negligible benefits to the host, and 2) non-nodulating strains that fail to form nodules, but reproduce in the rhizosphere (near root zone) of the host. Here, we explore additional aspects of these potential strategies.

Non-fixing rhizobia

Rhizobia that infect a legume, but then fix little or no nitrogen for their hosts are common (Quigley et al. 1997, Moawad et al. 1998, Burdon et al. 1999, Denton et al. 2000, Chen et al. 2002, Collins et al. 2002), but it is not

clear if these rhizobia are exhibiting an adaptive strategy. For instance, a nodulating rhizobium might also fail to provide a benefit because it is poorly matched (maladapted) to the host and consequently receiving little or no benefit from the infection. Rhizobia that offer significant growth benefits to one legume genotype are often poor mutualists when paired with other hosts (Expert et al. 1997, Parker 1999, Denison 2000, Murray et al. 2001, Heath and Tiffin 2007). Furthermore, if host responses deprive non-fixing rhizobia of fitness benefits, the non-fixing strategy could be disfavored by selection (Denison 2000, Simms and Taylor 2002, Denison and Kiers 2004).

Previous research has focused on two legume responses to non-beneficial infections, both of which are mechanisms of partner choice (Bull and Rice 1991, Simms and Taylor 2002, Sachs et al. 2004). One host response is pre-infection specificity, which can stop nodulation by some but not all non-beneficial rhizobial genotypes (Denison 2000, Perret et al. 2000, Simms and Taylor 2002). Once nodules have begun to develop, legume hosts can also respond by preferentially allocating resources to nodules that carry more effective strains (Singleton and Stockinger 1983, Singleton and Van Kessel 1987), causing nodules with non-beneficial rhizobia to be smaller and bear relatively few bacteria (Kiers et al. 2003, 2006, Simms et al. 2006). Post-infection responses to uncooperative rhizobia have been termed sanctions (Denison 2000, West et al. 2002, Kiers et al. 2003).

It is unlikely that all uncooperative rhizobia are adequately punished. First, only two legume species have been shown to exhibit sanctions; similar studies in other legumes have not uncovered such evidence (Atkins et al. 1984, Pate et al. 1984). Second, research on wild rhizobia (Abdalla 1992) and lab-generated mutants (Lodwig et al. 2003) has uncovered non-beneficial genotypes that attained higher nodule mass than beneficial strains. Finally, sanctions that occur at the nodule level are predicted to fail if multiple rhizobial genotypes commonly infect single nodules (Denison 2000, Simms and Taylor 2002, West et al. 2002, Denison and Kiers 2004). No study has investigated the fitness of rhizobia in multiply infected nodules, an important potential path by which rhizobia might bypass sanctions (Denison 2000, Simms and Taylor 2002, West et al. 2002). Conclusions about the adaptive status of rhizobia that fix little or no nitrogen must also be qualified because most non-beneficial infections that have been studied were lab-generated (Singleton and Van Kessel 1987, Lodwig et al. 2003, Kiers et al. 2003, 2006).

Non-nodulating rhizobia

Rhizobia that do not form nodules might avoid post infection sanctions by exploiting host resources that exist on or near roots, and bypassing infection entirely (Denison and Kiers 2004). Rhizobia can reproduce actively in soil and proliferate in the rhizospheres of some plants (Brockwell et al. 1987, Hagen et al. 1997, Hynes et al. 2001, Del Papa et al. 2003, Duodu et al. 2005), so the formation of nodules on legumes is only one potential life history for these bacteria (Denison and Kiers 2004, Duodu et al. 2005). In fact, non-symbiotic rhizobial strains

have been found to predominate numerically over symbiotic rhizobia when their abundances were directly compared (Segovia et al. 1991, Sullivan et al. 1996, Pongsilp et al. 2002). Some rhizobial genotypes appear specialized for a saprophytic lifestyle and persist on or near host roots while only rarely infecting them (Duodu et al. 2005). The fitness effects of rhizobia that inhabit – but do not infect – host roots are unknown. Non-nodulating rhizobia might provide indirect benefits to hosts, for instance by blocking infection of pathogenic bacteria, or their effects could be neutral. It is also possible that non-nodulating rhizobia harm legumes by blocking infection of beneficial rhizobia. For instance, mutant non-nodulating strains have been experimentally shown to reduce nodulation by beneficial rhizobia during initial infection (Singh and Ahmad 1991). However, it is not clear from these short-term experiments whether host fitness is ultimately depressed by the presence of non-nodulating rhizobia.

Unresolved questions and novel approaches

Is it adaptive for rhizobia to be uncooperative?

Studies are needed to compare the fitness of non-fixing and non-nodulating rhizobial strains with sympatric, beneficial rhizobia. We suggest five key elements for such investigations: (1) Study rhizobia that associate with small, fast growing legumes, to facilitate fitness assays of hosts and bacteria. (2) Isolate rhizobia from nodules and directly from host rhizospheres. The rhizosphere contains non-nodulating strains and might be enriched for non-fixing rhizobia, since sanctions are only known to occur after nodule formation. (3) Compare rhizobia within local populations. Cooperative and uncooperative rhizobia must be isolated from compatible hosts at small scales (meters as opposed to kilometers) to maximize the possibility of isolating recently diverged rhizobial lineages. (4) Estimate rhizobial fitness in isolation and in competition. Independent measures of rhizobial fitness (Kiers et al. 2003, 2006) should be coupled with co-inoculation assays that compete cooperative and uncooperative strains. It might be that non-beneficial rhizobia attain high fitness in isolation, but are unable to compete with other genotypes. (5) Conduct fitness comparisons with independent phylogenetic contrasts. To reduce the chance that the fitness comparison will be confounded with differences at other traits, non-nodulating and non-fixing strains should be compared to nodulating, beneficial rhizobia from the same clade.

Do uncooperative rhizobia commonly evolve from cooperative ancestors?

Certain lineages of mutualists have exploiter taxa nested within them (Sachs and Simms 2006), indicating that exploitation has evolved from a cooperative ancestor, but such a pattern has not yet been clearly documented in rhizobia. At a broad phylogenetic scale, some rhizobia have parasitic sister lineages, including *Afipia, Agrobacterium* and *Bartonella* (Sawada et al. 2003). However, while such taxa appear closely related to (and potentially nested within; Sawada et al. 2003) rhizobial lineages, few if any of these

bacteria are potential parasites of legumes. Indeed, some are animal parasites, with very different life histories. Data on the origins of naturally occurring non-nodulating rhizobia are also limited (Sullivan et al. 1996, Pongsilp et al. 2002), and it is unclear from these data whether non-nodulating rhizobia are nested in symbiotic lineages. Interestingly, the opposite scenario of nodulating rhizobia evolving from non-nodulating genotypes has been demonstrated. Sullivan et al. (1995) discovered that nodulating genotypes evolved rapidly in a non-nodulating population of rhizobia through horizontal gene transfer. A fascinating aspect of rhizobial genomes is that the loci that encode symbiotic cooperation are often clustered on plasmids or on symbiosis-islands that can be transmitted among individuals (Sullivan et al. 1995, 1996, Wernegreen and Riley 1999, Moulin et al. 2004).

Future studies are needed to investigate the origins of uncooperative rhizobia, and we suggest two elements for such investigations: (1) Use phylogenetic tests to resolve transitions to non-fixing and non-nodulating states (Huelsenbeck et al. 2003). To investigate these evolutionary shifts, rhizobial phylogenies should be reconstructed using vertically inherited markers (i.e. 'housekeeping' genes) that exhibit sufficient variation. Symbiosis loci will have less utility in this regard because they can be horizontally transferred, degraded or even lost (e.g. non-nodulating rhizobia) during these transitions. In a related plant-bacterial mutualism, ancient divergences between symbiotic and ineffective (non-nitrogen fixing) genotypes were found in actinobacterial *Frankia* that nodulate *Alnus glutinosa* (Wolters et al. 1997). (2) Examine the gene genealogies of multiple symbiosis loci to determine the extent to which lateral transfer of symbiosis-islands or plasmids is associated with shifts between mutualism and exploitation (Sachs and Simms 2006). Lateral transfer of symbiosis loci might be associated with such evolutionary shifts for two reasons. Firstly, multiple co-adapted alleles or loci are likely required for rhizobia to exploit hosts and wholesale transfer of the symbiosis loci is an efficient mechanism for bacterial genomes to gain suites of host-associated traits (Dobrindt et al. 2004). Furthermore, lateral transfer itself can promote selfishness, as it disassociates the fitness interests of the symbiosis island loci from the bacterial genome as well as the plant host. Since horizontal transmission can confound the phylogenetic resolution of these evolutionary reversals, well resolved phylogenies must be reconstructed that include beneficial and non-beneficial rhizobia using the gene genealogies of both 'housekeeping' as well as multiple loci from the laterally-transmissible portions of the rhizobial genomes. However, there are potential complications to this approach. These analyses could be hampered if intra-genomic recombination or horizontal transmission occurs at high rates. Moreover, if only a few loci are responsible for conferring uncooperative status, then correlations between genotype and phenotype would be difficult to uncover.

What are the mechanisms by which rhizobia exploit hosts?

Uncooperative rhizobial traits could evolve through multiple pathways, and mechanistic approaches should complement evolutionary studies. One prediction has been that

rhizobia exploit legumes by hoarding plant resources during infection (Denison 2000). Carbon compounds that rhizobia gain from legume hosts are usually channeled into the energetically expensive nitrogen fixation pathway (Trainer and Charles 2006), but non-fixing rhizobia might redirect carbon from the plant into bacterial storage compounds such as poly-ß-hydroxybutyrate (Denison 2000). Another potential mechanism of rhizobial exploitation has been uncovered by investigations of metabolic processes. Experiments by Lodwig et al. (2003) revealed amino acid cycling between *Rhizobium leguminosarum* and their pea hosts, in which *R. leguminosarum* shuts down ammonium assimilation and relies on the host for certain amino acids. Yet, a lab-created rhizobial mutant (RU1357) that cannot complete the cycle appears to exploit the plant host. The mutant synthesizes amino acids, but does not transfer them to the host pea. Hosts infected with RU1357 become nitrogen starved and grow poorly compared to peas infected with control stains while harboring more nodules and greater overall nodule mass than the controls (Lodwig et al. 2003). Further experiments are needed that directly estimate the fitness of the rhizobial mutant (i.e. release of viable rhizobia from nodules) relative to the wild type rhizobial ancestor and study natural variation at the locus. Finally, evolution of quorum sensing systems (QS) could drive shifts between rhizobial mutualism and exploitation. Rhizobia and bacterial plant pathogens employ similar QS systems to colonize and invade hosts (Soto et al. 2006) and QS systems are known to affect key symbiotic traits such as infection, specificity and nitrogen fixation (Gonzalez and Marketon 2003). Molecular studies that investigate the mechanisms of rhizobial exploitation could focus on any of the pathways described (i.e. PHB metabolism, amino acid cycling, quorum sensing) using two potential approaches. One would be to study natural variation at candidate loci among cooperative and uncooperative rhizobia and another approach would generate mutants at candidate loci to study the effects on rhizobial cooperation.

Conclusions and future directions

Recent phylogenetic analysis suggests that evolutionary transitions from mutualism to exploitation are rare in nature (Sachs and Simms 2006), which is surprising because the evolution of exploitation has often been thought to be an important impediment to the persistence of mutualisms (Soberon and Martinez del Rio 1985, Sachs et al. 2004). Only a handful of cases are documented in which mutualists have undergone an evolutionary reversal to exploit their partners (Sachs and Simms 2006). Two hypotheses can explain the rarity of such evolutionary transitions, each with different phylogenetic predictions. The constraint hypothesis suggests that cheating very seldom evolves because mutualist lineages exhibit genetic constraints that hinder the emergence of cheaters (Foster et al. 2004). Such constraints can arise by mutation accumulation in traits that are neutral in a mutualist lifestyle. For instance, in endosymbiotic mutualists, gene loss over time might ultimately constrain shifts to cheating (Moran and Wernegreen 2000). The transience hypothesis suggests that cheating traits emerge in populations, but do

not persist because the newly 'parasitic' interactions are unstable and prone to local extinction (Sachs and Simms 2006). Some researchers have suggested that most examples of exploitation come from individuals with no evolutionary history of mutualism (Bronstein 2001, 2003, Yu 2001), perhaps because the resulting tripartite interactions are more evolutionarily stable (Pellmyr and Leebens-Mack 2000, Sachs and Simms 2006). Detailed phylogenetic analyses that investigate mutualistic traits across multiple populations could resolve whether exploitation commonly, rarely, or never persists in mutualist lineages.

The legume–rhizobium mutualism provides an excellent system in which to empirically test mutualism theory. The interaction has been studied in great detail and across many taxa and exhibits features that are thought to promote exploitation. Nonetheless, it is unclear whether uncooperative rhizobia commonly evolve via adaptive strategies or are often maladapted. To better understand the evolution of exploitation, empirical work on the legume–rhizobium interaction (and other mutualisms) should focus effort on two main fronts. Studies should (1) examine the fitness of uncooperative mutants compared to cooperative members of the population and (2) firmly root investigations in a phylogenetic framework so that the origins and breakdown of mutualistic traits can begin to be resolved.

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