

Partner Choice in Nitrogen-Fixation Mutualisms of Legumes and Rhizobia¹

ELLEN L. SIMMS² AND D. LEE TAYLOR

Department of Integrative Biology, 3060 Valley Life Sciences Building #3140, University of California, Berkeley, California 94720-3140 and Plant Conservation Research Center, University of California Botanical Garden, 200 Centennial Drive #5045, Berkeley, California 94720-5045

SYNOPSIS. Mutualistic interactions are widespread and obligatory for many organisms, yet their evolutionary persistence in the face of cheating is theoretically puzzling. Nutrient-acquisition symbioses between plants and soil microbes are critically important to plant evolution and ecosystem function, yet we know almost nothing about the evolutionary dynamics and mechanisms of persistence of these ancient mutualisms. Partner-choice and partner-fidelity are mechanisms for dealing with cheaters, and can theoretically allow mutualisms to persist despite cheaters.

Many models of cooperative behavior assume pairwise interactions, while most plant-microbe nutrient-acquisition symbioses involve a single plant interacting with numerous microbes. Market models, in contrast, are well suited to mutualisms in which single plants attempt to conduct mutually beneficial resource exchange with multiple individuals. Market models assume that one partner chooses to trade with a subset of individuals selected from a market of potential partners. Hence, determining whether partner-choice occurs in plant-microbe mutualisms is critical to understanding the evolutionary persistence and dynamics of these symbioses. The nitrogen-fixation/carbon-fixation mutualism between leguminous plants and rhizobial bacteria is widespread, ancient, and important for ecosystem function and human nutrition. It also involves single plants interacting simultaneously with several to many bacterial partners, including ineffective (“cheating”) strains. We review the existing literature and find that this mutualism displays several elements of partner-choice, and may match the requirements of the market paradigm. We conclude by identifying profitable questions for future research.

INTRODUCTION: CHEATING AND THE STABILITY OF MUTUALISM

The evolutionary persistence of nutrient-acquisition-symbioses between plants and microbes has received little attention, which is unfortunate, because these mutualisms are widespread (Sprent and Sprent, 1990; Read, 1991), ancient and ecologically and environmentally important. Indeed, mycorrhizae may have facilitated the transition of plants to terrestrial habitats (Malloch *et al.*, 1980) and microbial symbioses remain critical determinants of global nutrient cycles, community structure and productivity (Vitousek and Walker, 1989; Rygielwicz and Andersen, 1994; Allen *et al.*, 1995; Bever *et al.*, 1997; van Der Heijden *et al.*, 1998; Klironomos *et al.*, 2000; Klironomos and Hart, 2001).

A substantial portion of the world's supply of organic nitrogen is fixed via the symbiosis between root-nodulating rhizobial bacteria and leguminous host plants (Postgate, 1998). This association is generally assumed to be mutualistic, but rhizobial strains vary in effectiveness (Burdon *et al.*, 1999) and ineffective bacteria are widespread, indicating that cheating may occur.

Evolutionary models suggest that mutualisms should not persist if cheating is unconstrained, yet the symbiosis between legumes and nitrogen fixing bacteria may have endured since the origins of the diverse

and ancient Leguminosae (Doyle, 1998; Terefeework *et al.*, 2000). Although many aspects of the interaction between agricultural legumes and N-fixing rhizobia are well understood at the physiological, cellular and molecular levels, how cheating bacteria might be constrained in this symbiosis is unknown.

We define cheating as accepting a benefit without reciprocating. For example, non-photosynthetic orchids are likely to be cheaters because they obtain nutrients from mycorrhizal fungi without providing carbon in return (Taylor and Bruns, 1997). Cheating has been the subject of several recent empirical studies, mostly concerning plant-pollinator interactions and intraspecific interactions in primates (*e.g.*, Noë, 1990; Bronstein, 1991; Noë *et al.*, 1991; Tyre and Addicott, 1993; Addicott and Tyre, 1995; Pellmyr *et al.*, 1996; Yu and Pierce, 1998; Addicott and Bao, 1999; Barrett *et al.*, 1999, 2000; Pellmyr and Leebens-Mack, 1999, 2000).

Theory suggests that partner-choice in a market of potential symbionts can constrain cheating, but only if the following conditions apply: 1. A range of partners is available, 2. There is a mechanism for effecting choice, and 3. The cost of evaluating partners is less than the benefit derived from choosing a good partner. As we describe below, individual legumes in crop situations are often nodulated by multiple bacterial strains that vary widely in effectiveness (Sprent and Sprent, 1990; Denison, 2000). Further, certain legumes control allocation to nitrogen fixation by restricting associations to particular phylogenetic groups of bacteria, limiting the number of bacterial infections, and adjusting levels of nodulation in response to the avail-

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² E-mail: esimms@socrates.berkeley.edu

ability of nitrogen from other sources. However, it is unclear whether these behaviors sufficiently punish cheating to maintain a mutualism. Further, it is unknown whether wild legumes choose bacterial partners from markets of potential symbionts, nor how partner-choice by plants might feed back to bacterial population structure.

THE BIOLOGY OF NITROGEN FIXATION IN LEGUMES

Origins of N-fixation

Nitrogen is extremely abundant, comprising about 79% of the atmosphere (Postgate, 1998). However, plants cannot convert atmospheric dinitrogen to useful organic forms, and mineral nitrogen is labile and of limited supply in soils. Consequently, when water is abundant, nitrogen commonly limits plant growth in terrestrial ecosystems (Tamm, 1991; Vitousek and Howarth, 1991; Berendse *et al.*, 1993; Vitousek *et al.*, 1997). However, a wide range of Bacteria and Archaea possess nitrogenase and are capable of reducing dinitrogen to organic forms (Postgate, 1998). A substantial portion of the world's supply of organic nitrogen is fixed via the symbiosis between rhizobial bacteria and leguminous host plants (Postgate, 1998).

Counter to earlier morphological hypotheses, molecular systematic studies have shown that plant families which form N-fixing root nodules with rhizobia or actinomycetes are relatively closely related (Soltis *et al.*, 1995). However, there are several non-fixing families and many non-fixing species within this single clade. These findings suggest either that symbiotic nitrogen fixation arose only once, and was subsequently lost many times, or that members of this clade were pre-adapted to nitrogen-fixation, allowing symbioses to evolve independently several to many times (Soltis *et al.*, 1995; Swenson, 1996; Doyle, 1998). Due to these uncertainties, the age of symbiotic nitrogen fixation is unknown, but may predate the origin of legumes in the Maastrichtian (late Cretaceous), some 65 million years before present (Herendeen *et al.*, 1992). Regardless, it is clear that symbiosis with legumes arose independently in two or more lineages of alpha Proteobacteria. The deepest divisions occur between the *Bradyrhizobium* group, which is closely related to *Blastobacter*, *Rhodopseudomonas*, etc., the *Mesorhizobium*–*Rhizobium* group, some members of which are closely related to *Agrobacterium* (Young and Haukka, 1996; Wang and Martinez-Romero, 2000), and the newly discovered *Methylobacterium nodulans* (Sy *et al.*, 2001). All the nodule-forming bacteria of legumes are loosely referred to as rhizobia. Currently, seven genera and at least 28 species of rhizobia are recognized (Wang and Martinez-Romero, 2000; Sy *et al.*, 2001).

Physiology and molecular biology of N-fixation

Due to the experimental tractability and agricultural importance of the legume-rhizobium symbiosis, molecular signaling between plants and bacteria and the ensuing development of symbiotic nodules have been

intensively studied in a few species, producing a wealth of information. As described briefly below, nodule formation involves several “handshaking” or “lock and key” steps, each of which provides opportunity for interaction specificity between plant and bacteria.

Legume roots secrete a variety of (iso)flavonoids which induce symbiotic genes in homologous bacteria (reviewed in Spaik, 1995; Long, 1996; Cohn *et al.*, 1998; Perret *et al.*, 2000). Bacterial recognition of the particular flavonoids produced by a host species provides the first opportunity for plant choice, as only the correct flavonoid/s induce symbiotic gene expression in a particular rhizobium strain. The ability of the bacteria to perceive a particular flavonoid signal is mediated in part by the transcriptional regulator NodD, which varies functionally among rhizobial strains. Among the most important NodD regulated responses to the correct flavonoid is the production of lipo-chitin oligosaccharide (LCO) “nod factors,” which in turn influence regulation of many plant genes (Spaik, 1995). Bacterial nod factors are composed of four to five beta 1–4 linked N-acetyl glucosamine units (a chitin backbone) and a fatty acid. Nod factors can vary in their fatty acids, the lengths of their sugar backbones, and the saturation of the acyl unit and decorations (glycosylation, sulfation, methylation) of the reducing and non-reducing ends of the backbone (Perret *et al.*, 2000). Only the correct nod factor/s induce root hair curling and infection thread formation (the first steps in nodule formation) in a particular legume species. Hence, the diversity of nod factors produced by rhizobia, and discrimination of these factors by plants, contribute the second level of specificity to the interaction and create an opportunity for partner choice by the plant (Perret *et al.*, 2000).

In a compatible interaction, the infection thread expands from the root hair to subtending cortical cells and fills with a glycoprotein matrix. Compatibility at this stage depends, in part, on recognition by the plant of particular polysaccharides on the bacterial cell wall. Rhizobia vary in polysaccharide composition of their cell walls, which provides another opportunity for plant choice of partners using the “lock and key” cascade that determines the degree of plant and bacterial specificity (Perret *et al.*, 2000). Compatible bacteria multiply and move into the root cortex as the nodule structure develops around them. Certain plant cells within the developing nodule then engulf rhizobial cells and surround them with the peribacteroid membrane. Within this structure, bacteria differentiate into bacteroids, change shape, and upregulate nitrogenase and the auxiliary enzymes required for dinitrogen reduction. However, bacterial release from the infection thread can be blocked in incompatible interactions, suggesting that the plant requires appropriate signals from the bacteria at this stage as well (Perret *et al.*, 2000). Host plant cells then provide energy in the form of dicarboxylic acids (malate and succinate) and ensure a low but steady oxygen flux via leghaemoglobin

regulation (Waters and Emerich, 2000). In an effective symbiosis, the bacteroids carry out nitrogen fixation and ship ammonia (possibly via an alanine shuttle) to the plant across the peribacteroid membrane (Waters and Emerich, 2000).

The fantastic advances in our understanding of plant-rhizobium signal transduction, cooperative nodule development, and the biochemistry of nitrogen fixation reveal a complex and elegant coevolutionary tapestry. They do not, however, clarify the selective forces that promote effective symbiosis and/or punish cheating in plants and rhizobia.

THEORIES OF MUTUALISM

With the shift from group-selection to individual-selection reasoning in the 1960s and 1970s (*e.g.*, Williams, 1966) came the realization that cooperation between individuals requires an evolutionary explanation. If the fitnesses of host and symbiont, or host and parasite, correlate strongly from one generation to the next (*i.e.*, there is frequent vertical transmission of the symbiont, but infrequent horizontal transmission), then increasing cooperation can be favored (Bull and Rice, 1991; Yamamura, 1993), but see (Frank, 1996*a, b*). However, strict vertical transmission has rarely been detected, while horizontal transmission occurs widely, in the major plant-microbe mutualisms—mycorrhizae (Smith and Read, 1997) and root-nodule N fixation (a vertically transmitted above-ground N-fixation symbiosis is described by Perkins and Peters, 1993). The degree to which dispersal of host and symbiont diaspores may be spatially co-constrained, and thus lead to genetic associations across multiple generations (imperfect vertical transmission), has not been adequately studied (Wilkinson, 1997).

Lotka-Volterra type models without vertical transmission, which posit reciprocal benefits with no cost, predict positive frequency-dependent selection favoring the most common host and symbiont genotypes (Law and Koptur, 1986), and lead to infinite population expansion. Incorporating costs produces more realistic outcomes (Boucher *et al.*, 1982). However, if benefiting a partner entails a cost, then cheating, *i.e.*, accepting a benefit without adequately reciprocating, yields the highest immediate gain.

In a model based on the “prisoner’s dilemma,” Trivers (1971) found that the immediate advantage inherent in cheating makes any costly cooperative behavior evolutionarily unstable. Since this seminal work, numerous models have been constructed to explain the evolutionary persistence of cooperation or mutualism in the absence of vertical transmission (Axelrod and Hamilton, 1981; Keeler, 1981; Eshel and Cavalli-Sforza, 1982; Soberon-Mainero and Martinez del Rio, 1985; Bull and Rice, 1991; Enquist and Leimar, 1993; Nowak and Sigmund, 1993; Frank, 1994, 1995; Noë and Hammerstein, 1994; Bendor and Swistak, 1995; Poulin and Vickery, 1995; Brembs, 1996; Doebeli and Knowlton, 1998; Genkai-Kato and Yamamura, 1998; Roberts, 1998; Schwartz and Hoeksema, 1998; Wahl

and Nowak, 1999*a, b*). These models fall into two major classes (Bull and Rice, 1991): partner-fidelity and partner-choice.

Partner fidelity

Partner-fidelity models, including those based on the prisoner’s dilemma, show that mutualism can be stable if pairs of individuals interact repeatedly and adjust their behavior in response to their partner’s behavior in the previous interaction (Axelrod and Hamilton, 1981). Thus, stability depends upon the ability of an individual to recognize the individual with which it interacted previously, maintain fidelity to that partner, remember the outcome of the previous interaction, and modify its behavior in accord with that outcome. Recent extensions of the model show that stability disappears when individuals are allowed to reactively adjust their level of investment, but that adding spatial structure can restore stability (although levels of altruism continually fluctuate) (Eshel and Cavalli-Sforza, 1982; Doebeli and Knowlton, 1998). These models may explain cooperation in certain interactions (*e.g.*, within singly infected nodules), but for two reasons are inadequate for explaining nutrient acquisition symbioses between plants and soil microbes. First, individual plants normally associate with many symbionts. Second, plant and microbe usually disperse independently (Duggar, 1935; Cass-Smith and Pittman, 1938; Erdman, 1943; Moodie and Vandacevye, 1943; Vincent, 1954; Alexander, 1984; Gallon and Chaplin, 1987; Bottomley, 1992; Genkai-Kato and Yamamura, 1998), so plant-microbe interactions are not iterated but must be renewed every generation.

Partner choice

Partner-choice models, in contrast, do not depend upon repeated interactions. Instead, individuals enter a “biological market” of potential “traders” and choose “trading partners” from among them (Noë and Hammerstein, 1994). Choice may be based either on receipt of honest signals indicating the relative qualities of potential partners or on active evaluation of partner quality through some sort of trial interaction. The quality of the commodity a partner offers in trade contributes to setting the value the other trader is willing to offer in exchange, and hence influences choice. As pointed out by Noë and Hammerstein (1995) conceptually similar models apply to a variety of contexts ranging from sexual selection to economics. Models of strategic behavior, costs and benefits, resource trade, biological markets, partner-choice, mate-choice, and sexual selection all utilize market paradigms to better understand how evolution may shape inter-individual interactions. Of course, models of human economics cannot be used directly, because evolution requires genetic variation among individuals, whilst economic models treat entire classes of traders as homogeneous (Noë and Hammerstein, 1995). Further, most of the biological models listed above analyze decision-making rather than explain the persistence of mutualism.

Nonetheless, an effective partner-choice mechanism would constrain cheating and thereby promote the stability of mutualisms (Bull and Rice, 1991).

A PARTNER-CHOICE VIEW OF NITROGEN FIXATION

Enzymatic reduction of dinitrogen is energetically expensive and rhizobia rarely, if ever, fix nitrogen while free-living (Postgate, 1998). The successful plant symbiont must pay the energetic price of the reduction reaction, conduct a complicated signal exchange with rhizobia, produce leghaemoglobin, and form a novel organ—the nodule. In an efficient functional symbiosis, the nutritional benefits of N-fixation presumably offset the costs associated with these traits. Numerous empirical laboratory and field studies in crop plants and some undomesticated legumes have documented decreasing nodulation rates with increasing soil nitrogen availability (e.g., Singleton and van Kessel, 1987; Caetano-Anolles and Gresshoff, 1991; Lang *et al.*, 1993; Rubio Arias *et al.*, 1999; Thomas *et al.*, 2000). This observation suggests that the relative benefits of nodulation decline with increasing abundance of reduced nitrogen, which plants can obtain directly from the soil. Legumes also restrict nodulation when inadequate supplies of other nutrients, especially phosphorus, limit plant growth (Caetano-Anolles and Gresshoff, 1991; Tsai *et al.*, 1993; Oliveira *et al.*, 1998; Taiwo *et al.*, 1999). The fact that plants reduce nodulation under conditions of high nitrogen or low phosphorus availability illustrates that plants do not permit unlimited infection by compatible rhizobia. In fact, in some legumes, the majority of root-hair infections by compatible rhizobia do not lead to nodule formation (Nutman, 1962). Moreover, susceptibility to further inoculation declines following an initial inoculation event (Nutman, 1962; Bhuvaneswari *et al.*, 1980; Bhuvaneswari *et al.*, 1981; Pierce and Bauer, 1983; Heron and Pueppke, 1987; Malik and Bauer, 1988; Takats, 1990). This so called “autoregulatory” response is systemic, and appears to be in part genetically controlled, as some “supernodulation” mutants are partially defective in autoregulation (Olsson *et al.*, 1989). These observations suggest that market principles, such as decreasing price with increasing supply, may apply to the exchange of nitrogen for photosynthate between legumes and rhizobia.

Market perspectives seem particularly well suited to understanding the evolutionary dynamics and persistence of legume-rhizobium and mycorrhizal symbiosis, because (1) individuals exchange commodities (plant-derived photosynthate for microbe-derived mineral nutrients) to their mutual benefit, (2) individuals in each “trading class” are functionally and phylogenetically diverse, (3) at least one trading partner (the plant) interacts simultaneously with diverse traders (microbes), (4) the exchange value of commodities is a source of conflict, and (5) at least one trader (the plant) appears to possess mechanisms that would allow it to evaluate and choose partners on the basis of symbiotic quality. As reviewed below, isolated pieces of evidence sug-

gest that elements of a biological market and partner-choice occur in the legume-rhizobium interaction. However, most evidence derives from agricultural systems whose characteristics may be biased by artificial selection. Further, even among agricultural systems, there are no examples in which all these assumptions have been evaluated. Recent advances in molecular ecology provide the tools for integrated analyses of coevolved native systems, which are needed to measure genetic and functional variation in rhizobia and understand evolutionary responses by plants to this variation. It is only when this suite of studies has been conducted that we will know whether biological market models are appropriate for plant-microbe mutualisms.

EVIDENCE FOR PARTNER CHOICE

Commodity exchange: mutual benefits

Rhizobia survive and reproduce outside of legume nodules, and legumes resist infection under some conditions. Hence, the symbiosis is not obligate for either partner. There is abundant evidence that host plants usually benefit from symbiotic nitrogen fixation; how bacteria may benefit is less clear.

It was once thought that all differentiated bacteroid cells are reproductively dead—that they die along with the host plant cell (Brock, 1988). More recent studies have shown that this is not always the case: in several legumes with determinate nodules, bacteroids can divide and multiply after release from the host cell (Tsien *et al.*, 1977; Sarath *et al.*, 1986). However, in several legumes with indeterminate nodules, including alfalfa (Lussenhop, 1993), and even some determinate species, bacteroids do not appear to retain viability (Sutton and Paterson, 1980, 1983).

In the latter cases, a paradox exists as to why moribund bacteroids carry out nitrogen fixation. The solution most likely involves increases in inclusive fitness via nitrogen fixation and consequent allocation of photosynthate to genetically identical vegetative bacterial cells inside or outside the nodule (Jimenez and Casades, 1989; Simms and Bever, 1998). The conditions under which benefit to kin outside the nodule could maintain nitrogen fixation are restrictive (Bever and Simms, 2000; West *et al.*, 2001, 2002). In particular, spatial structure of the bacterial population must ensure that benefits to vegetative kin are sufficiently greater than benefits to non-kin that they offset the loss of reproductive capability of bacteroids.

One mechanism that may increase benefits to relatives is found in strains whose bacteroids convert plant metabolites to rhizopines—compounds that cannot be re-assimilated by the plant nor used by unrelated soil bacteria (Murphy *et al.*, 1988). However, this mechanism for benefiting nodule-surface kin can itself persist only under certain conditions of spatial structure in the free-living rhizobium population (Simms and Bever, 1998). Rhizopine might also benefit vegetative kin within the persistent infection threads inside indeter-

minate nodules (Denison, 2000). Again, however, the evolutionary persistence of this trait depends critically on spatial structure of the vegetative kin. In this case, the persistence of the rhizopine trait would depend upon the frequency of nodules occupied by more than one rhizobium strain.

It is difficult to explain the elegant signaling between legumes and rhizobia unless these bacteria somehow benefit from residing within nodules. Indeed, there is abundant circumstantial evidence for such benefit. For example, soil populations of rhizobia increase following nodulation of a homologous host and increase again when the crop is mature and the nodules are senescing (Bushby, 1984; Moawad *et al.*, 1984; Kuykendall, 1989). Further, populations of specific rhizobia are often orders of magnitude higher under a compatible host than under a non-compatible legume (Kucey and Hynes, 1989). However, such observations do not indicate whether the population increases are due to nodule occupancy, or simply to general bacterial proliferation in the rhizosphere (Bottomley, 1992). One study documented high population densities specifically within senescing clover nodules (Chatel and Greenwood, 1973), but even this study does not document that population growth is greatest in effective symbiotic genotypes. Moreover, many rhizobia can persist in soil for years or decades in the absence of their homologous host (Kamicker and Brill, 1986). Determining the exact mechanisms by which bacteroids benefit from nitrogen fixation deserves considerable further effort.

Evidence of a market: functional and phylogenetic diversity

In a biological market, members of the rarer trading class should exercise stronger choice, while individuals in the more abundant trading class should offer more in trade at a lower price (Noë and Hammerstein, 1994). Soils with indigenous legumes usually support large populations of native rhizobia (Bottomley, 1992) and rhizobia generally outnumber their host plants. Further, their extreme size disparity dictates that individual bacterial cells are less likely to encounter multiple potential partners than are plants. In particular, after infection, plants might choose among bacterial strains through differential resource allocation whereas bacteria would need to leave the nodule to exercise choice. Hence, plants most likely represent the choosy trader.

Do individual plants have choices? Rhizobial populations in soil are genetically diverse, both in agricultural settings (Sprent and Sprent, 1990; Denison, 2000), and in many wild legumes (Pinero *et al.*, 1988; Laguerre *et al.*, 1993; Bottomley *et al.*, 1994; Hagen and Hamrick, 1996a, b; Hartmann *et al.*, 1998; Doignon-Bourcier *et al.*, 1999; McInroy *et al.*, 1999; Parker, 1999b, 2000; Sterner and Parker, 1999; Tan *et al.*, 1999; Wang *et al.*, 1999a, b; Parker and Lunk, 2000; Willems *et al.*, 2000). These populations are also functionally diverse in terms of symbiotic effectiveness

(Spoerke *et al.*, 1996; Wilkinson and Parker, 1996; Wilkinson *et al.*, 1996; Burdon *et al.*, 1999; Parker, 1999a; Thrall *et al.*, 2000) and other properties such as phage resistance, siderophore production, rhizopine production, and antibiotic (bacteriocin) production (Murphy *et al.*, 1988; Bottomley, 1992; Abd-Alla, 1999; Goel *et al.*, 1999), which suggests that plants may have the opportunity to choose among functionally diverse bacteria.

Opportunity for partner choice: evidence for simultaneous plant interaction with multiple bacterial partners

Individual plants in agricultural settings may be colonized by diverse rhizobial genotypes (Bottomley, 1992; Souza *et al.*, 1992; Silva *et al.*, 1999), suggesting that they have abundant choices. However, there is little information available about the spatial structure of bacterial variation in natural plant populations, where the homogenizing effects of soil tillage are absent (but see Parker, 1995, 1999a; Hagen and Hamrick, 1996a, b; Spoerke *et al.*, 1996; Wilkinson and Parker, 1996; Parker and Spoerke, 1998). Hagen and Hamrick (1996a) found that 80% of the genetic diversity of rhizobia associated with naturalized red clover occurred within individual plants. This study provides the strongest evidence of which we are aware that individual wild plants encounter a diverse bacterial market.

In a preliminary survey of rhizobial diversity in California native lupines, we detected 13 different 16S–23S spacer PCR-RFLP genotypes among 68 *Bradyrhizobium* isolates obtained from wild-collected plants (Fig. 1). Moreover, of 17 plants from which we sampled multiple nodules, 35% hosted more than one genetically distinguishable strain and might therefore exercise partner-choice. This survey, in which we analyzed only a few nodules per plant, is likely to significantly underestimate intra-plant bacterial diversity, since individual lupines usually support tens to hundreds of nodules.

Conflict over commodity valuation

Because legumes and rhizobia disperse independently, the evolutionary trajectories of individual plant and bacterial genotypes are not congruent. Hence, either partner may increase its fitness by selfish actions. Essentially, plants benefit from obtaining as much nitrogen as possible at the lowest possible cost in terms of photosynthate or other resources. A plant might promote its own well being at the expense of compatible bacteria by (1) destroying bacteroids, (2) reducing nodulation under high nitrogen and low phosphorus conditions, (3) regulating nodule number, (4) destroying or restricting the growth of non-differentiated bacterial cells within the nodule, and (5) limiting the timing and forms of carbohydrate rewards supplied to nodule bacteria.

Among bacteria, selection must favor maximum vegetative proliferation and resource acquisition (in-

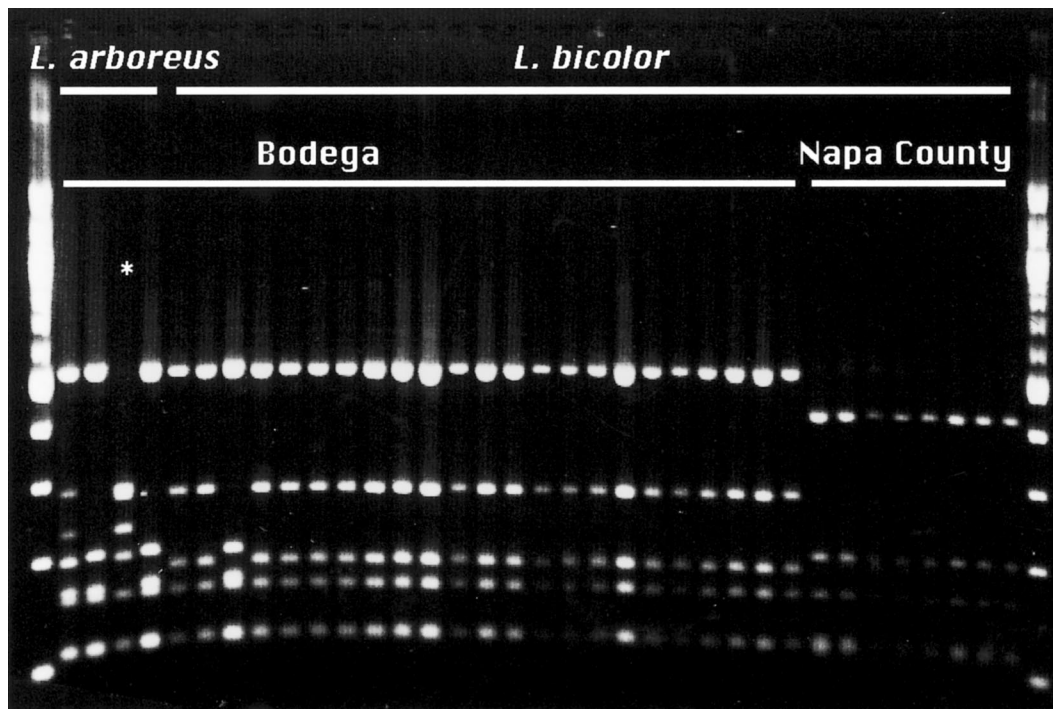


FIG. 1. IGS RFLPs reveal a market of *Bradyrhizobia* encountered by wild CA lupines. The gel above shows Dde I RFLP patterns for the 16S–23S rDNA intergenic spacer region (IGS) seen in *Bradyrhizobia* isolated from *Lupinus arboreus* and *Lupinus bicolor* growing in two Northern California locations, Bodega Marine Reserve (Bodega Head, Sonoma County, CA) and Snell Valley (Napa County, CA). The RFLP pattern of the starred isolate from our collection matches the RFLP pattern (not shown) of the USDA *Bradyrhizobium* sp. (*lupinus*) reference strain 3051 originally isolated in Georgia.

side and/or outside the nodule). Consequently, bacteria might benefit by storing photosynthate rather than spending it on dinitrogen reduction, which is energetically costly. There is clear evidence for bacterial traits that do not serve the interests of their host plants. For example, some *Rhizobium* strains divert nitrogen and carbon into rhizopines, which are released into the rhizosphere where they can be used by reproductive kin of bacteroids (Saint *et al.*, 1993; Murphy *et al.*, 1995; Rossbach *et al.*, 1995; Wexler *et al.*, 1996). Certain *Bradyrhizobium* strains produce rhizobitoxines, compounds that appear to inhibit synthesis of ethylene (Yasuta *et al.*, 1999), a plant hormone involved in defense responses. Rhizobitoxine might improve bacterial fitness by increasing nodule occupancy (Yuhashi *et al.*, 2000) or derailing legume autoregulation. At least one ethylene insensitive legume mutant is hypernodulated (Penmetsa and Cook, 1997). Further, a rhizobitoxine-positive strain of *B. elkanii* produced abundant nodules on *Amphicarpaea edgeworthii*, while rhizobitoxine-negative mutants of the same strain formed few nodules on this host (Parker and Peters, 2001). Rhizobitoxine production has been found in some native strains isolated from this host. Moreover, although rhizobitoxine causes chlorosis in some hosts, and can cause significant yield losses in soybean (Teaney and Fuhrmann, 1993; Vasilas and Fuhrmann, 1993), it does not cause chlorosis in this *Amphicarpaea* species. Additional bacterial behaviors that could cause conflicts of interest include consumption of structural carbo-

hydrates in plant cell walls (Djordjevic *et al.*, 1987), sequestering of energetic compounds in excess of that needed for nitrogen fixation (Denison, 2000), or the subversion of internal plant signaling to increase receipt of photosynthates. Clearly, there exist many potential mechanisms and advantages of cheating by both plants and bacteria.

Partner choice: sanctions and rewards

The conflicts over commodity valuation described above suggest that cheating could be selectively advantageous for rhizobia, unless plants have evolved mechanisms to sanction cheaters (Denison, 2000; West *et al.*, 2002) or reward cooperators. Whether through reward or sanction, the critical element of partner-choice is that it is based on an assessment of partner quality. There are many cues that plants might use to evaluate bacterial quality. Nitrogen flux from a nodule is an obvious possibility. However, plants would be at an advantage if they could monitor the costs associated with a particular nodule (e.g., photosynthate consumption or toxin release) as well as the nitrogen gains. In other words, the full evolutionary implications of the interaction are best understood by considering bacterial quality in terms of plant fitness.

Mechanisms of partner choice: pre-nodulation—specificity as a mechanism of partner choice

As described above, specificity is an important mechanism by which legumes regulate nodulation

(Chanway *et al.*, 1991; Spaink, 1995; Spoerke *et al.*, 1996; Wilkinson and Parker, 1996; Wilkinson *et al.*, 1996; Perret *et al.*, 2000). Specificity in leguminous species prevents infection by inappropriate strains, and may limit the incidence of bacterial cheating. However, the molecular “handshaking” that underlies specificity is not sufficient to prevent all cheating. At issue is the reliability, or honesty (Hill, 1994; Bradbury and Vehrencamp, 2000), of bacterial signals. More than 50 symbiotic genes have been discovered in rhizobia (Spaink, 1995; Long, 1996; Vlassak and Vanderleyden, 1997), and mutations that negatively impact symbiotic quality (*e.g.*, nitrogenase expression) will not necessarily disrupt symbiont recognition by the plant (*e.g.*, nod factor production). That is, plants may be infected by strains that signal reciprocity but have lost the ability to fix nitrogen effectively (*e.g.*, Hahn and Studer, 1986). In fact, among clover rhizobia, effective and ineffective strains can be nearly identical in multilocus genotype, and the two types of strains are completely intermixed on a phenetic tree of genetic distances (Ibekwe *et al.*, 1997). Such rapid evolutionary change in bacterial effectiveness makes it unlikely that specialization in legumes can evolve quickly enough to impose a significant constraint on cheating. Of course, mutations in numerous genes involved in signaling pathways disrupt nodule development (Vlassak and Vanderleyden, 1997; Perret *et al.*, 2000). Our argument is simply that these signaling pathways may not alert the plant to incursion by a cheater strain that differs from an effective strain at loci outside the signaling process. The frequency of nodulation by ineffective strains in numerous field situations provides forceful support for our view (*e.g.*, Quigley *et al.*, 1997; Burdon *et al.*, 1999).

Mechanisms of partner choice: post-nodulation—sanctions and differential rewards

While legumes may be unable to recognize and block infection by poor quality strains, they may still exercise a degree of control following infection (Denison, 2000; West *et al.*, 2002). This type of partner-choice may play an important ecological and evolutionary role in the legume-rhizobium symbiosis.

Selecting bacteria by quality: lateral root responses

Regulation of the development of main lateral roots is the largest scale at which plants may act to constrain cheating. Plants might sanction cheaters by selectively aborting roots that support ineffective nodules, much as certain *Yucca* species selectively abort fruits with a heavy load of seed-eating moth larvae (Pellmyr and Huth, 1994). However, many legumes form nodules just below the soil surface on the central root axis, where selective abortion of the underlying root would almost certainly kill the plant. Therefore, we predict that selective root abortion could occur only in high order lateral roots, and would provide very little selective constraint on cheating by rhizobia.

Plants might also reward particularly effective sym-

bionts through selective resource allocation. Patches of soil nitrogen can induce local root proliferation (Drew *et al.*, 1973), and a nodule or nodules formed by a bacterium that fixes relatively large quantities of nitrogen might similarly induce local root proliferation. If bacterial populations are spatially structured, kin of the nodule occupants might benefit from increased root exudate or from new colonization opportunities. However, if bacterial genotypes are completely mixed in soil (unstructured), then these generalized root responses will provide no advantage to the high-quality bacterial genotype. Instead, mechanisms that operate at the level of single nodules might provide powerful selective constraints on cheating rhizobia.

Selecting bacteria by quality: nodule responses

Responses to bacterial quality at the level of individual nodules might provide the simplest and most elegant solution to the problem of cheating bacteria. Each nodule has a meristem and a unique vascular connection to the rest of the root system. Hence, plants might modulate resource allocation or impose sanctions on individual nodules. Indeed, a recent model by West and colleagues (West *et al.*, 2002) predicts that high rates of N-fixation by rhizobia can be maintained only if plants allocate resources to nodules on the basis of the N-fixation rate of their occupants.

Sanctions could include localized induced defenses, such as hypersensitive responses (Agrios, 1997), or other physiological controls, such as restriction of oxygen diffusion into nodules (Hartwig and Nosberger, 1994; Denison, 1998), which must limit respiration, and hence restrict photosynthate expenditure (Denison, 1998). It remains to be determined if oxygen restriction can be controlled at the level of individual nodules (Denison, 2000).

While many physiological and biochemical mechanisms might be involved in sanctions or differential allocation, these mechanisms should all produce a consistent phenotype in nodules occupied by ineffective bacteria: small size. Limiting the costs of associating with ineffective bacteria would require that plants initiate sanctions or differential allocation at the earliest stages of nodule development at which symbiotic quality can be evaluated.

Bacteroids differentiate and fix nitrogen soon after nodule parenchyma has formed, when nodules are only a fraction of their potential size (whether determinate or indeterminate). Various developmental abnormalities of nodules occupied by ineffective or mutant strains can be observed by this stage (Hirsch *et al.*, 1982; Hirsch and Smith, 1987; Dazzo and Wopereis, 2000). Thus, plants could avoid most of the potential costs of cheating bacteria by restricting nodule development at this early stage. In fact, there are numerous casual references to this phenomenon, as well as some experimental evidence. Gardner *et al.* (1985, p. 140) state, “Nodules smaller than normal are generally an indication of infection by an ineffective strain of *Rhizobium*.” Concrete examples of this sort of observa-

tion include (Jones *et al.*, 1978; Hirsch *et al.*, 1993) among many others.

We have found few quantitative studies of this phenomenon. Mytton and de Felice (1977) studied symbiotic quality and competition for nodulation among rhizobium strains in white clover and found that larger nodules were formed when effective strains were included in the inoculum mixture. However, they did not attempt to identify strains occupying individual nodules. Singleton and Stockinger (Singleton and Stockinger, 1983), in contrast, used fluorescent antibodies to identify soybean nodule occupants after dual inoculation with effective and ineffective strains of *Bradyrhizobium japonicum*. Nodules occupied by the effective strain were 2.5 times larger, on average, than nodules occupied by the ineffective strain. Further compensation was apparent in their observation that the average weight of nodules occupied by effective strains increased as the proportion of nodules formed by the effective strain decreased. These results strongly suggest that legumes can allocate resources differentially to higher quality bacteria, as required by partner-choice models, but require substantiation in other species, especially naturally evolved non-crop species.

For nodule-specific responses to effectively constrain cheating, individual nodules must be occupied by single bacterial genotypes (Denison, 2000; West *et al.*, 2002). Laboratory studies of crop legumes reveal frequencies of mixed-occupancy nodules up to 70%, while field studies have reported lower, but still significant frequencies (Denison, 2000). We are aware of no such studies of wild legumes growing in uncultivated soils.

Selecting bacteria by quality: cellular responses

Punishment or reward of individual bacterial cells is the finest scale at which plants could theoretically constrain cheating. Such mechanisms could be important if nodules are frequently colonized by multiple bacterial genotypes (Denison, 2000). Nodule cortex cells that contain ineffective bacteroids might undergo programmed cell death, although we are unaware of any evidence suggesting that such mechanisms exist.

ACCURATE TARGETING: THE IMPORTANCE OF SPATIAL STRUCTURE

Partner-choice by legumes can explain the stability of the plant/rhizobium mutualism only if it exerts selection favoring effective bacterial symbionts. The review above reveals that the spatial structure of bacterial genotypes is an important determinant of the mechanisms by which plants could potentially target rewards or sanctions. If rhizobium populations are well mixed at all scales, it may be impossible for plants to target rewards or sanctions (West *et al.*, 2002). Notably, mutualism can persist via partner choice only if rhizobial populations occupy some intermediate range of scales of genetic structure. If rhizobium populations are structured at a scale larger than the scope of a single root system, then individual plants will not en-

counter a diverse market, which effectively deprives them of choices. At the other extreme, population structure at too small a scale could prevent plants from accurately rewarding or punishing symbionts for their functional performance. This lower limit will be set by physiological constraints on the scale at which plants can regulate internal resource allocation and growth. Below this threshold, cheaters (free-riders) could benefit by occurring adjacent to beneficial bacteria. Further, under certain mechanisms of plant choice, rhizobial diversity within a single root system may constrain the evolutionary maximum rate of N-fixation that can be maintained in a rhizobium population (West *et al.*, 2002). Clearly, to understand the evolutionary maintenance of mutualism in these systems, further empirical information is needed about the spatial scales of rhizobium population structure and plant responses to bacterial variation. In particular, there is a critical need to document the patterns of spatial organization of rhizobia in the rooting zones of individual plants growing in natural, untillied soil systems.

CAN PARTNER-CHOICE EXPLAIN EVOLUTIONARY PERSISTENCE OF A MUTUALISTIC LEGUME-RHIZOBIUM INTERACTION?

We do not expect that partner-choice by plants is perfect for two simple reasons. First, to detect cheaters functionally, plants must interact with cheating bacteria to a considerable degree, perhaps even provide them some benefit. Second, the ubiquity of ineffective strains in nature suggests that plants are not extremely efficient in detecting and punishing cheaters. Nonetheless, the existence of highly effective strains in the wild and the evolutionary persistence of the mutualism suggest that there must be mechanisms that favor "good" mutualists.

We have reviewed strong circumstantial evidence, primarily from crop plants, for several elements of a bacterial market in legume-rhizobium interactions. However, these studies are far from sufficient to conclude that partner-choice can explain the evolutionary persistence of the legume-rhizobium mutualism. Further, a virtual absence of information from native, co-evolved systems seriously hampers our ability to understand the natural forces selecting traits in nutritional symbioses between plants and microbes.

Major outstanding questions requiring more research include:

1. Do legumes predictably encounter a bacterial market?
2. Can plants evaluate bacterial function, and if so, how?
3. Are bacterial populations spatially structured in a way that facilitates accurate targeting of rewards and sanctions?
4. Do plant rewards and sanctions affect bacterial fitness?
5. What are the fitness costs to bacteria of being beneficial symbionts?

Answers to these questions will reveal which theoretical models best explain the evolutionary maintenance of mutualism between legumes and rhizobia, and in particular reveal whether biological market models are well suited to explain their persistence.

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