Terminal differentiation of symbiotic rhizobia in certain legume species and its implications for legume-rhizobia coevolution

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Dedication

This dissertation is dedicated to my family, friends (especially from Minnesota) and Mark.

Abstract

The symbiotic association between legume plants (Fabaceae) and nitrogen-fixing rhizobia is a classic system of cooperation, but with largely unexplored differences among species in life history traits. Rhizobia transform physiologically and morphologically into nitrogen-fixing bacteroids inside host nodules. The transformation is terminal (bacteroids are swollen and apparently nonreproductive) in some legume host species but not others, regardless of rhizobial genotype. The phylogenetic distribution of this host trait in the Papilionoideae subfamily of legumes suggests that the common ancestor of the papilionoids did not host terminally differentiated bacteroids and there appear to have been at least five independent origins of hosts imposing terminal differentiation on bacteroids. To consider possible advantages of this host trait, I compared the symbiotic efficiency of terminally and non-terminally differentiated bacteroids of a single rhizobial strain with dual-host capabilities. In the two available dual-host cases, I found greater fixation efficiency (N_2 fixation per CO_2 respiration) as well as plant return (host biomass) on investment per nodule mass in the hosts with terminal bacteroid differentiation than in those without. This suggests that host traits leading to terminal bacteroid differentiation may have been derived multiple times because of increased net symbiotic benefits to the host. Lastly, I tested whether legumes hosting terminally differentiated bacteroids impose sanctions, i.e. reduce benefits to the undifferentiated reproductive clonemates of less-mutualistic bacteroids in the same nodule. Host sanctions could maintain the evolutionary stability of the symbiosis despite "cheaters" - less-mutualistic rhizobia that potentially benefit from the fixation by other rhizobia sharing the same individual plant host. Legume roots were split so that half of each nodulated root system was exposed to nitrogen-free atmosphere ($Ar:O_2$) to simulate cheating and the other half was in normal air ($N_2:O_2$). Rhizobial fitness (rhizobia per nodule) was compared between the two halves. A clear host sanctions effect in peas and alfalfa demonstrated that terminal differentiation of bacteroids does not compromise a legume host's ability to sanction. Differences in rhizobial life history suggest various rhizobial symbiotic traits for cooperation and cheating, perhaps leading to different mechanisms in different legume host species that maintain stability of the mutualism.

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CHAPTER 1. Introduction

Significantly modified from Oono et al. (2009) Controlling the reproductive fate of rhizobia: How universal are legume sanctions? New Phytologist. 183: 967-979.

"I use this term [struggle for existence] in a large and metaphorical sense including dependence of one being on another, and including (which is more important) not only the life of the individual, but success in leaving progeny."
—Darwin (1859)

The legumes (Fabaceae) are a diverse family and their symbioses with nitrogen-fixing bacteria (rhizobia) may be equally diverse (Sprent, 2007). In particular, rhizobia are dimorphic (present in two different forms) in nodules of some legume hosts. In those symbioses, the bacteroids (the differentiated rhizobial cells that fix nitrogen) are larger than their undifferentiated clonemates in the same nodule and have typically lost the ability to reproduce. In contrast, bacteroids in other hosts retain the ability to reproduce after fixing nitrogen, perhaps because they are more similar to undifferentiated rhizobia in size and shape. This dissertation focuses on the evolutionary history and implications of rhizobial dimorphism for symbiotic cooperation and conflict. Here, I present the background to my research and discuss some questions and hypotheses that I explored prior to my experiments. Not all of these topics will be explored in the following chapters. Topics directly relevant to a particular chapter of this thesis will be mentioned.

Reproductive potential of bacteroids depends on host species

In pea (*Pisum sativum*) nodules, bacteroids are four to seven times the size of free-living rhizobia (Oke & Long, 1999) and can no longer reproduce (Kijne, 1975, Mergaert *et al.*, 2006). However, these nodules also contain clonally identical undifferentiated rhizobia that do retain the ability to reproduce, but do not fix nitrogen. This is analogous to social insects with worker and reproductive castes. In contrast, in beans (*Phaseolus vulgaris*), bacteroids continue to divide, are similar in size to free-living cells, and are reproductively viable (Fig. 1-1 b, c, d). The size distribution of the cells from a single bean nodule is unimodal, while swollen bacteroids and undifferentiated rhizobia of pea nodules result in a clear bimodal distribution. We refer to bacteroids as swollen if there are distinctly smaller, undifferentiated rhizobia in the same nodule, resulting in rhizobial dimorphism, whether or not there are differences in shape.

The generalization that swollen bacteroids have little or no capacity to divide is based on evidence that varies among species. For example, Zhou et al. (1985) used video microscopy to show that only undifferentiated rhizobia divide in *Trifolium repens* nodules. Using flow cytometry, Ratcliff et al. (2008) showed that colony counts corresponded to the number of smaller-sized (undifferentiated) rhizobia in alfalfa (*Medicago sativa*) nodules rather than total rhizobial per nodule. More generally, swollen bacteroids are also usually found individually within symbiosomes, compartments delimited by plant membranes, whereas a symbiosome may contain several nonswollen bacteroids (Denison, 2000, Izaguirre-Mayoral & Vivas, 1996). Swollen bacteroids tend to have altered cell wall structures that have higher osmotic sensitivity (Sutton & Paterson,

1979). Some studies suggest that swollen bacteroids can revert back to the rod-shaped reproductive forms (Gresshoff *et al.*, 1977, Khetmalas & Bal, 2005). Although this capability to dedifferentiate back to reproductive forms may be possible for some swollen bacteroids, current evidence suggests that the majority of viable rhizobia escaping into the soil are descended from the undifferentiated cell populations in the nodules, not from these swollen forms (Zhou *et al.*, 1985, Ratcliff *et al.*, 2008, Timmers *et al.*, 2000). Another character often attributed to swollen bacteroids is genome endoreduplication, which is thoroughly examined by Maunoury et al. (2008).

Experiments with dual-host rhizobia show that rhizobial dimorphism depends on the legume host. Rhizobia whose bacteroids were swollen in vetch nodules (*Vicia sativa*) were genetically modified to infect *Lotus japonicus* (Bras *et al.*, 2000), where they formed small, rod-shaped bacteroids like those typically found in that host (Mergaert *et al.*, 2006). Vice versa, rhizobia that formed small bacteroids in bean were modified to infect pea (Gotz *et al.*, 1985), where they formed swollen bacteroids typical of that host (Mergaert *et al.*, 2006). Another dual-host rhizobial strain (not genetically modified) makes swollen bacteroids in peanut (*Arachis hypogaea*) nodules and nonswollen ones in cowpea (*Vigna unguiculata*) nodules (Sen & Weaver, 1980).

A recent study published during the dissertation suggests bacteroids lose reproductive viability due to host plant factors known as nodule-specific cysteine-rich (NCR) peptides (Van de velde et al. 2010).

I. Evolutionary history: Is host imposed rhizobial dimorphism ancestral or derived in legumes?

In Chapter 2, I explore the evolutionary history of host-imposed rhizobial dimorphism by testing whether this trait is ancestral or derived in legumes using ancestral character reconstruction analyses for 40 legume genera.

Prior to this thesis research, studies on bacteroid differentiation were limited to a few legumes, mostly species with agricultural benefits (Table 1-1). From the current literature (previous to the publication of Chapter 2), it appeared there might be at least two independent lineages of legumes whose nodules host both undifferentiated rhizobia and relatively larger bacteroids: species within the vicioids, which include alfalfa and peas, and some aeschynomenoid-nodule-forming legumes such as peanuts. However, a comprehensive phylogenetic analysis is required to assess whether host suppression of bacteroid reproduction is derived or ancestral in the legume family.

Elucidating what form of bacteroid differentiation is the derived trait in legumes can help formulate further hypotheses regarding possible benefits to legumes of this trait. If ancestral legumes suppressed bacteroid reproduction, then several lineages, including those with beans and birdsfoot-trefoil (*Lotus corniculatus*), subsequently lost this ability to modify bacteroids. Repeated loss of a trait may suggest that the trait reduced legume fitness. Alternatively, ancestral legume nodules could have hosted nonswollen reproductive bacteroids. This would mean that host-imposed rhizobial dimorphism has arisen at least twice, in vicioids and aeschynomenoids, suggesting that rhizobial dimorphism may somehow be more beneficial to legumes. These inferences are

according to analysis of the currently accepted legume phylogenies (Doyle *et al.*, 1997, Wojciechowski *et al.*, 2004).

No consistent relationship between nodule type and rhizobial dimorphism

Included in Chapter 2 is an analysis for trait correlation between nodule type and rhizobial dimorphism. Nodules can be classified into two general types – indeterminate or determinate. Indeterminate nodules have a persistent meristem and typically elongate during growth, while determinate nodules have a transient meristem and appear spherical. Nodule types can be further classified into subtypes, as discussed in more detail by Sprent (2001, 2008). The most frequently studied legume species (alfalfa, peas, birdsfoot-trefoil, beans, soybeans) show a correlation between nodule type and bacteroid reproduction: among these species, indeterminate nodules have swollen bacteroids and determinate nodules do not (Fig. 1-1 a, Table 1-1). Although a general relationship between bacteroid viability and nodule type has been suggested (Denison, 2000, Lodwig et al., 2005), that apparent pattern does not stand up to closer analysis. There is evidence that peanuts and related legume species, which have aeschynomenoid nodules (a special type of determinate nodule), also have swollen (coccoid) bacteroids (Fleischman & Kramer, 1998) and may have low reproductive viability (Sutton & Paterson, 1980). Also, there are many other legume species not commonly studied, e.g. lupines (Table 1-1), that are known to have indeterminate nodules but appear to have nonswollen rod-shaped bacteroids (Sprent, 2001, Fernández-Pascual et al., 2007) and no rhizobial dimorphism. Therefore, although ancestral legumes are thought to have had indeterminate nodules

(Sprent, 2007), we cannot assume that they hosted dimorphic rhizobia with nonreproductive bacteroids.

II. Are there immediate benefits to individual plants from rhizobial dimorphism?

In Chapter 3, I investigate whether there are immediate benefits to individual plants from rhizobial dimorphism.

Rhizobia descended from the undifferentiated clonemates of nonreproductive swollen bacteroids could evolve differently from those descended directly from reproductive bacteroids. This is because selection for greater or lesser nitrogen fixation by nonreproductive bacteroids must act via effects on the survival and reproduction of their undifferentiated clonemates. This difference in rhizobial evolution is likely to have long-term consequences for the legume species they nodulate and may indirectly benefit future legume hosts. However, natural selection is driven by immediate benefits to individual plants, not future consequences for the species as a whole. Typically, a plant's effects on rhizobial evolution will not preferentially benefit that individual plant, although there may be some exceptions. For instance, legumes with more than one round of nodulation (e.g. perennials) might benefit from the evolution of greater mutualism in rhizobia that are likely to reinfect their own roots. However, any benefits of improving mutualism in soil populations of rhizobia would be shared with nearby competitors. These plants may be kin, due to limited dispersal, but the same limited dispersal makes competition more local, decreasing the effects of kin selection (Griffin & West, 2002). Therefore, in most cases, it is unlikely that host suppression of bacteroid reproduction first evolved (or was lost) because of its effects on future rhizobial evolution. Rather, we

need to consider the immediate fitness effects, for an individual legume plant, of host-imposed rhizobial dimorphism. How might a legume plant benefit from suppressing bacteroid reproduction?

Mergaert et al. (2006) suggest that limiting bacteroid reproduction might prevent them from becoming parasitic and infecting other plant tissues. However, endophytic rhizobia in rice leaves have physiological effects that have been reported to be beneficial under some conditions (Chi *et al.*, 2005).

Alternatively, loss of reproductive viability in bacteroids could be a consequence of bacteroid swelling that benefits hosts. Swollen bacteroids may be advantageous for several reasons. Plants may more easily retrieve nutrients from swollen bacteroids during nodule senescence (Mergaert *et al.*, 2006, Maunoury *et al.*, 2008) because they are easier to lyse. Swollen bacteroids could have better symbiotic performance (Mergaert *et al.*, 2006), e.g. higher nitrogen fixation per g carbon invested in nodule construction or per g carbon respired. Apparent differences in fixation efficiency have been detected using the same rhizobial strain in different hosts. In peanuts, where bacteroids are swollen, nitrogenase activity per mg of bacteroid protein was five times higher than in cowpeas where bacteroids are not swollen (Sen & Weaver, 1984).

If swollen bacteroids prove to be more efficient, what mechanisms are most likely? Sen and Weaver (1984) presented evidence that differences in oxygen supply (perhaps linked to differences in nodule development rather than bacteroid swelling *per se*) may affect nitrogen fixation rate per g nodule, but they did not measure the ratio of nitrogen fixation to respiration. With only one bacteroid per symbiosome, as is typical for dimorphic rhizobia, there may be more direct contact between symbiosome

membranes and bacteroid cell walls, which may improve energy efficiency for resource transfer between host and bacteroid.

Our working hypothesis is that plant traits promoting rhizobial dimorphism (with swollen bacteroids) and the loss of bacteroid reproduction are derived in several legume lineages. Individual plants benefit immediately from bacteroid swelling (e.g., increased nitrogen fixation or easier retrieval of nutrients from senescing bacteroids). Loss of reproductive potential of bacteroids could then be a side effect of swelling.

III. How might sanctions differ when bacteroids are nonreproductive?

Symbiotic partners can have conflicting interests as well as shared interests. In successful mutualistic symbioses, various mechanisms align the interests of the partners or enforce cooperation despite conflicting interests. Three important mechanisms are: i) vertical transmission of symbionts from a host to its own offspring, ii) host sanctions, and iii) minimizing symbiotic opportunities with cheaters (Douglas, 2008). This last mechanism, commonly known as pre-infection partner choice, is discussed more thoroughly in Chapter 4.

Rhizobia spread to new hosts through the soil, not via seeds, and thus mutualism cannot be stabilized by vertical transmission. Even if they were vertically transmitted, the usual presence of multiple strains per individual plant could lead to a "tragedy of the commons" (Denison, 2000). The tragedy is that the individual benefit to a rhizobial strain of diverting resources from nitrogen fixation to its own reproduction may outweigh the

shared cost of reduced photosynthesis in a nitrogen-deficient host plant. Mathematical modeling showed that, with a realistic number of rhizobial strains per individual host plant, strains that invest little or nothing in nitrogen fixation will outcompete those that invest more (West *et al.*, 2002b).

Why do these cheaters, strains that potentially benefit from investing less in nitrogen fixation, not spread through the population? Experiments using nitrogen-free air ($Ar:O_2$) showed that soybean plants impose sanctions on individual nodules that fix less nitrogen (Kiers *et al.*, 2003). Strain-dependent differences in nodule growth consistent with sanctions have also been reported for wild lupines (Simms *et al.*, 2006). Intermediate rates of nitrogen fixation lead to sanctions with intermediate effects on rhizobial fitness, but rhizobia whose nitrogen fixation is between 50 and 100% that of the best strains may pay little fitness penalty for cheating (Kiers *et al.*, 2006). However, strains that fix less than that still persist. Rhizobia isolated from the same soil can vary tenfold in plant benefit (Burdon *et al.*, 1999). A few strains result in less legume growth than an uninoculated control (Nutman, 1954).

If sanctions are so effective, why do we find less-beneficial strains in nature? Possible explanations for the persistence of these strains, despite sanctions, include mixed nodules, conflicting selection regimes, biochemical manipulation of legumes by some strains of rhizobia, or differences in sanctions among host genotypes (Kiers & Denison, 2008). The frequency of mixed nodules has rarely been measured under field conditions. Up to 32% of field soybean nodules contained two strains (Moawad & Schmidt, 1987), which might keep the total nitrogen fixation per nodule high enough to avoid sanctions, even if one strain fixed little nitrogen. There is also evidence that soybean cultivars differ

in their yield response to mixtures of fixing and nonfixing rhizobia under field conditions, perhaps due to differences in sanction strength (Kiers *et al.*, 2007). This could result in the escape of less-beneficial strains. Rhizobia that block plant ethylene signaling are less beneficial to plants, but nonetheless acquire more resources per cell (Ratcliff & Denison, 2009), a possible example of manipulation. All of these experiments used hosts in which rhizobial bacteroids retain the ability to reproduce. Are rhizobial interactions with other legume host species similar?

In Chapter 4, I explore whether hosts with rhizobial dimorphism can impose fitness-reducing sanctions on nonfixing rhizobial strains. In a host where bacteroids irreversibly lose the ability to reproduce, their evolutionary impact on the next generation will depend on how their activities in symbiosis affect the survival and reproduction of their reproductive clonemates, perhaps especially those in the same nodule.

How can nonreproductive bacteroids enhance their inclusive fitness?

Reproductive bacteroids pay a direct fitness opportunity cost when they respire carbon to support nitrogen fixation rather than using it to support their own immediate reproduction, or hoarding carbon resources, like polyhydroxybutyrate (PHB), to support future reproduction (Ratcliff *et al.*, 2008). Neither of these options is available to nonreproductive bacteroids, so we might expect rhizobial cheaters to be rare when bacteroids are nonreproductive. If this is true, do these host species still impose sanctions? Are sanctions still possible, if bacteroids will have no descendants?

In contrast to reproductive bacteroids, nonreproductive bacteroids seem less likely to hoard resources like PHB to support their own long-term survival, because they will have no direct descendants. Instead, there are at least three ways in which nonreproductive bacteroids might have adapted to benefit their reproductive clonemates in the same nodule. First, nonreproductive bacteroids might divert resources (such as rhizopines, discussed below) to be consumed by their reproductive clonemates. This activity may compromise nitrogen fixation. Second, bacteroids might reduce their own rate of resource consumption, thereby freeing more resources for their reproductive clonemates. A simple way to do this would be to reduce respiration in support of nitrogen fixation. Third, bacteroids might elicit host responses that increase net resources to their reproductive clonemates. For example, if the influx of host photosynthate delivered to nodules (and to the reproductive clonemates) were directly proportional to the efflux of fixed nitrogen by bacteroids, then nonreproductive bacteroids might increase their inclusive fitness simply by fixing more nitrogen.

Can nonreproductive bacteroids cheat?

If nonreproductive bacteroids divert resources to their reproductive clonemates, and thereby increase their inclusive fitness at the expense of host fitness, that would be considered a cheating strategy. How to do this? Some nonreproductive bacteroids are known to produce rhizopines, which are compounds synthesized by nonreproductive bacteroids within nodules and catabolized by the undifferentiated rhizobial cells (Murphy *et al.*, 1995).

It has been suggested that rhizopines promote rhizobial mutualism via kin selection by increasing the flux of root exudates to related, reproductively viable rhizobia in the rhizosphere (Olivieri & Frank, 1994, Simms & Bever, 1998). Such a mechanism relies on the assumption that the rhizobia receiving the benefits are closely related to the rhizobia in the root nodules, and that this relatedness arises through limited dispersal (Bever & Simms, 2000). However, this form of kin selection has stringent requirements for spatial genetic structures of the bacterial population outside the nodule (Simms & Bever, 1998). Given that spatial structure also undermines cooperation by making competition more local (West *et al.*, 2001), within-nodule kin selection is likely to be the only form of selection strong enough to consistently promote cooperation.

For rhizopine diversion to be considered cheating, three conditions must be met: (i) rhizopine synthesis must divert energy away from nitrogen fixation, (ii) rhizopines must be consumed by closely related rhizobia, and (iii) catabolism of rhizopines must increase fitness of those rhizobia. The undifferentiated clonemates of bacteroids within the same nodule seem most likely to meet the latter two criteria. Another cheating mechanism that might operate similarly to rhizopines could be excess hydrogen production. Nitrogen fixation always releases some hydrogen gas as a byproduct and some reproductive rhizobia can consume hydrogen (Ruiz-Argueso *et al.*, 1979). Bacteroids that increased hydrogen production from its baseline rate of about 25% of nitrogenase activity (e.g., to 50%) might thereby benefit their reproductive clonemates at the expense of nitrogen fixation.

Even without resource diversion via rhizopines or hydrogen, carbon not consumed by nonreproductive bacteroids may be diverted to the undifferentiated rhizobia

simply by diffusion. This could select for nonreproductive bacteroids that fix less nitrogen and thereby free more carbon for use by their reproductive clonemates. Nodules with ineffective (i.e., nonfixing) nonreproductive bacteroids contained higher levels of starch (Ronson *et al.*, 1981), but whether excess carbon is available to reproductive cells in infection threads is unknown.

Although these cheating mechanisms are plausible, the extent to which nonreproductive bacteroids actually cheat in these ways is unknown. Rhizopine genes have been reported only in rhizobia whose bacteroids are nonreproductive in their usual hosts (Wexler *et al.*, 1995), consistent with resource diversion to reproductive clonemates, but hydrogen consumption has also been reported in reproductive bacteroids, where it may actually increase nitrogen fixation (Albrecht *et al.*, 1979). It also remains uncertain whether carbon that is unused by bacteroids benefits undifferentiated rhizobia enough to select for lower nitrogen fixation.

If these cheating mechanisms are not available, then a nonreproductive bacteroid pays little or no opportunity cost when it consumes carbon to power nitrogen fixation. This contrasts with reproductive bacteroids, where there is a clear tradeoff between nitrogen fixation and hoarding resources to support their own reproduction (Ratcliff *et al.*, 2008, Hahn & Studer, 1986), as discussed in the next section.

Should legumes hosting nonreproductive bacteroids invest in sanctions?

We define sanctions as some action by the host plant that *reduces the relative fitness of less-beneficial rhizobia based on their low rate or low efficiency of nitrogen fixation*. Reduced nodulation, perhaps due to incorrect recognition signals, would be an

example of pre-infection partner choice rather than sanctions (Kiers & Denison, 2008). Also, sanctions are defined by their effects on rhizobial fitness, not host fitness. However, selection among legumes will only maintain sanctions if individual plants that impose sanctions thereby increase their fitness. This legume fitness benefit need not depend on how a given legume response affects rhizobial evolution. Note that rhizobial cheaters may mimic recognition signals of mutualists or retain signals from mutualistic ancestors. We previously showed theoretically that individual plants would benefit from reducing resource consumption in nodules containing less-mutualistic rhizobia (West *et al.*, 2002a). From the plant's point of view, this is simply an efficient use of resources. But, from the point of view of rhizobia, this preferential resource allocation could function as a sanction, reducing the relative fitness of less-mutualistic strains.

If nonreproductive bacteroids have few cheating options, then legumes hosting dimorphic rhizobia may suffer less from less-beneficial rhizobia. For example, <13% of *Sinorhizobium meliloti* and *Rhizobium leguminosarum* strains tested had rhizopine genes (Wexler *et al.*, 1995). However, given the other possible cheating options discussed above, it might be premature to conclude that cheating is rare when bacteroids are nonreproductive.

Furthermore, even if rhizobia with nonreproductive bacteroids are rarely cheaters, i.e., strains that potentially benefit from fixing less nitrogen, legumes may still face considerable variation in symbiont quality. Variation in symbiont quality could occur if rhizobia that are less beneficial to particular hosts arise due to conflicting selection imposed by different host genotypes (Heath & Tiffin, 2007). There could also be significant numbers of mutants that retain the ability to nodulate but are defective in

nitrogen fixation (Denison and Kiers 2004). Given such variation, legumes would benefit from preferential allocation of resources to the most-effective rhizobia (West *et al.*, 2002a), whether or not less-effective bacteroids are actually cheating.

Testing for sanctions by comparing rhizobial strains

When we ask whether a legume host imposes sanctions, we are asking whether they respond to differences among strains in actual symbiotic performance, e.g., N return on C investment, in ways that reduce the fitness of less-beneficial strains. We know that legumes also respond to recognition signals during initial infection and perhaps later. In Chapter 4, I do not consider the extent of host responses to signals but focus on host responses to actual performance.

There are two different types of evidence suggesting that legume species that suppress bacteroid reproduction may also impose sanctions on poorly performing strains. So far, however, each approach has been implemented in ways that limit our ability to draw firm conclusions about these species. One approach is to compare rhizobial strains, to see whether those that provide less benefit to a given host also obtain less benefit from that host, as would be expected if that host imposes sanctions. A second approach is to manipulate a strain's nitrogen fixation rate by adjusting nitrogen gas concentration. One problem with comparing strains is that differences in rhizobial fitness could be due to some host-strain interaction other than performance-based sanctions. For example, a strain that fixes nitrogen very efficiently (i.e. nitrogen fixed per carbon respired) in a given host might also have signal molecules or surface antigens that limit nodulation or nodule growth in that host. A comparison with another strain that fixes nitrogen less

efficiently but forms more nodules or reproduces better in nodules (due to signaling interactions, not performance-based sanctions) could incorrectly be interpreted as evidence against host sanctions. Comparing a larger number of strains would reduce the chance of drawing incorrect conclusions from idiosyncratic associations between signals and symbiotic performance.

Comparing benefits provided and obtained by different strains is also more difficult than generally recognized. It is easy to compare growth of plants, each inoculated with a single strain of rhizobia. However, growth with single-strain inoculation is an imperfect proxy for differences in mutualism among strains under field conditions, where each plant is infected by multiple strains. With single-strain inoculation, a poorly nodulating strain with high nitrogen-fixation efficiency might result in less plant growth than a less-efficient strain that forms more nodules. Plant growth with single-strain inoculation would incorrectly identify the former as a less-beneficial strain. In the field, however, the poorly nodulating strain might only occupy 10% of the nodules on a plant, while providing 20% of the nitrogen. A better method might be to compare the growth of plants with two different strains in their nodules, in different proportions. The more-beneficial strain is the one that increases plant growth as its nodule representation increases. A benefit: cost ratio would also be a better measurement to assess a strain's symbiotic qualities. For example, the ratio of nitrogen fixation rate to respiration rate of nodules or the ratio of host shoot biomass to total nodule mass would be a more accurate evaluation of the strain's symbiotic quality, independent of differences in nodulation efficiency. After all, in the real world, no one strain makes the majority of nodules on a single host.

Measuring differences among strains in rhizobial fitness benefits from symbiosis is also nontrivial. Nodule number from single-strain inoculation is not an appropriate proxy for rhizobial fitness. With single-strain inoculation, the number of nodules formed may depend on whether a strain can suppress the plant's autoregulation of nodule number, e.g. by producing rhizobitoxine, which blocks ethylene signaling (Sugawara *et al.*, 2006). A rhizobitoxine producer may increase total number of nodules but not its own proportional representation (Ratcliff & Denison, 2009). Competitiveness for nodulation in the field depends on survival under field conditions and potentially on negative interactions among rhizobia, such as by producing bacteriocins (Schwinghamer & Brockwell, 1978), neither of which is measured in single-strain inoculation assays. However, the number of nodules per plant in single-strain inoculation can be used to estimate a plant benefit per nodule, somewhat alleviating the problem discussed in the previous paragraph.

Nodule size is widely measured and more promising as a proxy for rhizobial fitness. In soybean, which hosts reproductive bacteroids, much of the difference in rhizobia per nodule with sanctions was explained by nodule weight (Kiers *et al.*, 2003). Similarly, Heath and Tiffin (2007) reported a positive correlation ($r = 0.59$) between reproductive rhizobia (by plate counts) and nodule length in *Medicago truncatula*. However, the relationship between nodule size and the number of reproductive rhizobia inside may not always be comparable among different strains. Therefore, even if nodules containing cheaters have lower average sizes, it does not guarantee that they have lower number of reproductive cells per nodule. Given this uncertainty, reliance on nodule size as a proxy for rhizobial fitness seems unnecessarily risky. We suggest actually counting

viable rhizobia per nodule, which can be done using agar plates with an appropriate growth medium or (with some uncertainty about viability) by flow cytometry. We also recommend measuring PHB/cell, using flow cytometry, because rhizobial cells can accumulate enough PHB to support doubling or tripling of their numbers (Ratcliff *et al.*, 2008). In light of our concerns about single-strain inoculation and nodule size, we hesitate to draw firm conclusions from most of the published data, which are somewhat contradictory.

Miller and Sirois (1982) measured growth of four alfalfa cultivars with each of five rhizobial strains. They found a high correlation (r=0.78) between average nodule weight (perhaps representative of rhizobial benefits) and average yield per plant with single-strain inoculation (perhaps representative of host benefits) (Fig. 1-2). For this dataset, the correlation was similar if plant benefit from rhizobia was corrected for nodules per plant. This is the same sort of correlation seen in soybean (Kiers & Denison, 2008), for which we have direct experimental evidence for sanctions (Kiers *et al.*, 2003). However, we cannot exclude the possibility that healthier alfalfa plants simply support larger nodules (in addition to or instead of more nodules per plant). This could be true even if alfalfa has no ability to selectively support the best nodules within a plant. Preferential host support of the best-performing nodules can be detected using mixed-inoculation experiments. For example, Singleton and Stockinger (1983) found that soybean nodules containing nitrogen-fixing rhizobia were 2.5 times as big as nodules on the same plant that contained an ineffective strain. Heath and Tiffin (2009) also used mixed inoculation, testing three rhizobial strains on four *Medicago truncatula* families. Nodule length varied four-fold, but was not correlated with estimates of genotype-

specific host benefits from each strain. This was interpreted as evidence against sanctions. However, host benefits of each strain were measured in a previous experiment that used single-strain inoculation. With that approach, differences in total nitrogen fixation among strains may be due to differences in nodulation, rather than differences in nitrogen fixation efficiency or rate per nodule.

Another important point is that a given set of strains may not include any whose nitrogen fixation rate is low enough to trigger sanctions, even in hosts that do impose sanctions on nonfixing strains. Therefore, to use comparisons among rhizobia to test whether a given legume genotype can impose sanctions, one should include a nodulating but nonfixing strain. Comparing isogenic strains could reduce the chance of drawing incorrect conclusions about sanctions because of idiosyncratic signaling interactions. Whether rhizobia that are ineffective enough to trigger sanctions are common (or rare, as suggested by Heath and Tiffin for their field locations) is a separate question. Plant alleles for sanctions that are never used would tend to disappear, however, unless they serve some other function.

Given uncertainties about using nodule size as a proxy for rhizobial fitness and plant growth with single-strain inoculation as a proxy for rhizobial mutualism, as discussed above, we consider the results of Miller and Sirois (1982) to be weak evidence for sanctions in alfalfa and the results of Heath and Tiffin (2009) to be weak evidence against sanctions in *Medicago truncatula*. It seems likely that the apparent discrepancy between these two studies is due to differences in methods, rather than a fundamental difference between these closely related legume species.

These same methodological concerns also apply to studies of two other legume species that host dimorphic rhizobia. Nutman (1946) reported that some rhizobia that are ineffective on clover nonetheless produce large nodules. More recently, Laguerre et al. (2007) found rhizobia that produce very large nodules on pea, but result in less plant growth than other strains. Neither report determined whether these large nodules actually contained large numbers of reproductive rhizobia within them. If they did, then either these legumes do not impose sanctions or some rhizobia have means of evading those sanctions.

Manipulating nitrogen fixation to test for sanctions

Given the problems discussed in the previous section, we favor testing for sanctions by manipulating nitrogen fixation rate rather than comparing genotypes that may differ in various ways. We do this by reducing the nitrogen concentration around one or more nodules, thereby limiting nitrogen fixation. Moreover, our approach also allows us to vary nitrogen fixation quantitatively and over time.

In soybean, which hosts reproductive bacteroids, nonfixing nodules (in $Ar:O_2$) grew less and contained fewer viable rhizobia per nodule (Kiers *et al.*, 2003). Intermediate nitrogen concentrations, allowing intermediate rates of nitrogen fixation, led to intermediate sanctions (Kiers *et al.*, 2006). They used both single-nodule chambers and split-root chambers, with similar results.

In Chapter 4, I conduct similar experiments using alfalfa, peas, and peanuts, which impose rhizobial dimorphism. Some indirect evidence prior to this chapter suggests that host sanctions may be widespread. Minchin et al. (1983) found a

physiologically similar response to decreasing nitrogen fixation in a variety of legume species, including some that host nonreproductive bacteroids. Blocking nitrogen fixation in nodulated roots (using either an nitrogen-free Ar:O_2 atmosphere or acetylene, a competitive inhibitor of nitrogenase activity) triggered a decrease in nodule respiration rate, subsequently linked to a decrease in nodule O_2 permeability. Although the fitness effects of decreased respiration for dimorphic rhizobia have not been measured, the decreased nodule O_2 permeability is the same response as Kiers *et al.* (2003) saw in soybean. It therefore seems plausible, although far from certain, that sanctions may operate in a wide variety of legume species, by similar mechanisms.

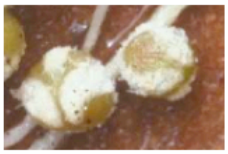
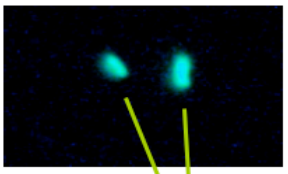
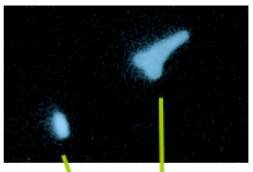
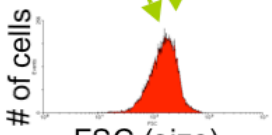
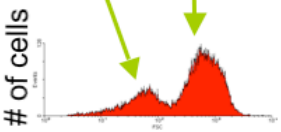
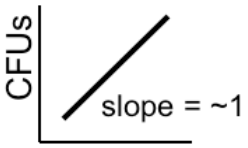
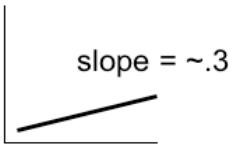
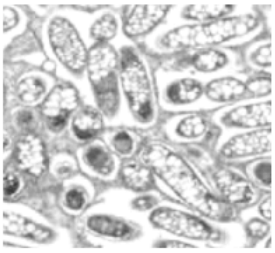
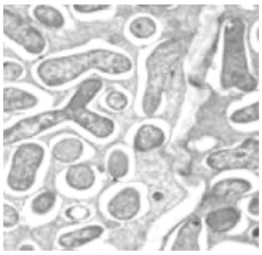
Legume host	Bean (*Phaseolus*)	Pea (*Pisum*)
a) Nodule type	 determinate	 indeterminate
b) Cell types (Microscopy)		
c) Size classes (Flow cytometry)		
d) Bacteroid viability	reproductive 	nonreproductive  ~30% are undiff. rhizobia
e) Next host infected by	descendants of bacteroids	descendants of undifferentiated rhizobia
f) PHB in the bacteroids	Present 	Absent 
g) Cheating mechanism	PHB-hoarding or reproducing	Rhizopine or H_2 synthesis for undiff. clonemates

Figure 1-1. The same *Rhizobium* strain is dimorphic in pea (right) but not in bean (left). Dimorphic rhizobia are found in many vicioid legumes with indeterminate nodules while legumes with determinate nodules, such as beans and *Lotus*, contain a homogenous bacteroid population (**a**). Bacteroid morphologies are evident using microscopy (**b**), including electron micrographs, and flow cytometry (**c**). Bacteroid viability (**d**) is often assessed by comparing total number of cells to number of colony-forming units (CFUs) on plates. A ratio significantly <1 and nonviable cell counts equal to the swollen-cell population suggests that bacteroids are nonreproductive. When bacteroids are swollen, future hosts are infected mainly or exclusively by descendants of undifferentiated rhizobia (**e**). Polyhydroxybutyrate (PHB) is common in nonswollen bacteroids but usually absent from swollen ones (**f**, Lodwig et al. 2005). Differences in PHB and reproductive viability suggest different cheating strategies for rhizobia in different host species.

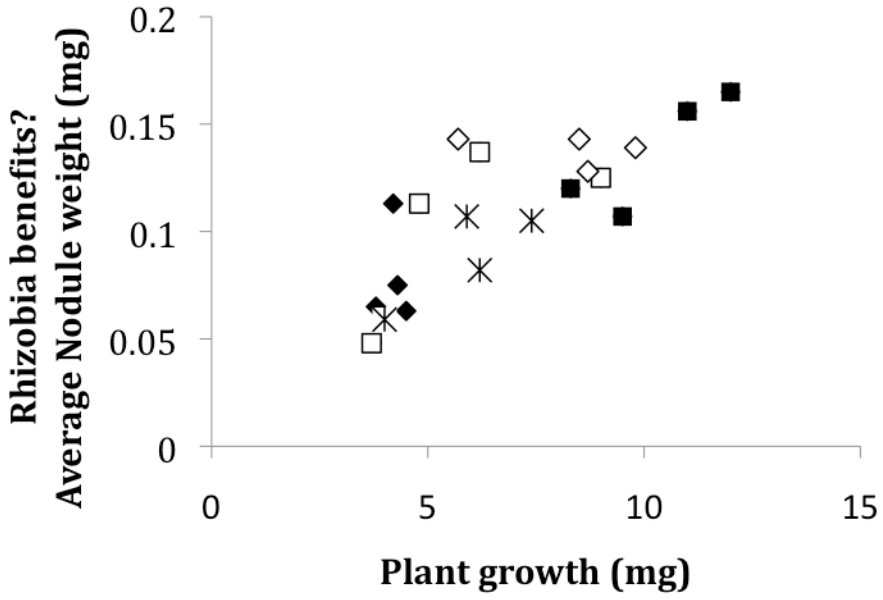


Figure 1-2. Effect of five *Rhizobium meliloti* strains (different symbols) on four *Medicago sativa* cultivars, modified from Miller & Sirois (1982), r = 0.78.

	Alfalfa, pea, vetch	Soybean, bean, cowpea	Birdsfoot-trefoil	Lupine	Peanut
Bacteroid size	5-10 μm long (4, 9, 11)	0.54-1.0 x 2.0-3.1 μm, 3.5 μm^3 (1, 4, 5, 6, 9, 11)	0.23-0.46 x 1.5-2.0 μm (1, 6)	1.2-1.5 μm long (2)	11.4-15.7 μm^3 (5, 6)
Reproductive viability of bacteroid	Not viable (4, 8, 11)	Viable (4, 8, 9, 11)	Viable (4, 8)	Low viability (8)	Low viability (8)
Bacteroid PHB	No accumulation (9, 10)	Up to 50% dry weight (1,6, 10)	Rare (1, 6)	> 500 μg per g of nodule is possible (3)	?
Nodule types (7)	Indeterminate	Determinate	Determinate	Genistoid (indeterminate growth)	Aeschnomenoid (determinate growth)

Table 1-1. Bacteroid properties and nodule type in representative legume species. References: (1) Banba et al. (2001), (2) Fernandez-Pascual et al. (2007), (3) Gerson et al. (1978), (4) Mergaert et al. (2006), (5) Sen & Weaver (1984), (6) Sprent et al. (1987) and references therein, (7) Sprent (2001), (8) Sutton & Paterson, (1980), (9) Ratcliff & Denison (2008), (10) Trainer & Charles (2006) and references therein, (11) Zhou et al. (1985)

CHAPTER 2: Multiple evolutionary origins of legume traits leading to extreme rhizobial differentiation

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SUMMARY

- When rhizobia differentiate inside legume host nodules to become nitrogen-fixing bacteroids, they undergo a physiological as well as a morphological transformation. These transformations are more extreme in some legume species than others, leading to fundamental differences in rhizobial life history and evolution. We analyzed the distribution of different bacteroid morphologies over a legume phylogeny to understand the evolutionary history of this host-influenced differentiation.
- Using existing electron micrographs and new flow cytometric analyses, bacteroid morphologies were categorized as swollen or nonswollen for forty legume species in the subfamily Papilionoideae. Maximum likelihood and Bayesian frameworks were used to reconstruct ancestral states at the bases of all major subclades within the papilionoids.
- Extreme bacteroid differentiation leading to swelling was found in five out of the six major papilionoid subclades. The inferred ancestral state for the Papilionoideae was hosting nonswollen bacteroids, indicating at least five independent origins of host traits leading to swollen bacteroids.

- Repeated evolution of host traits causing bacteroid swelling suggests a possible fitness benefit to the plant. Furthermore, since bacteroid swelling is often correlated with loss of reproductive viability, the evolution of bacteroid cooperation or cheating could differ fundamentally between the two bacteroid morphologies.

INTRODUCTION

Mutualistic symbioses often vary in net benefits depending on partner genotypes and the environment (Bronstein, 1994, Johnson *et al.*, 1997, Denison & Kiers, 2004, Heath & Tiffin, 2007). Some mutualistic species have evolved adaptations that impose selection for more beneficial partners (Kiers *et al.*, 2003, Jander & Herre, 2010). Others have abandoned symbiosis (Hibbett *et al.*, 2000, Sachs & Simms, 2006), which makes maintenance of ancient mutualisms particularly challenging to understand. Their coevolving symbiotic traits and life histories may shed light on mechanisms for evolutionary stability in mutualisms.

In the legume-rhizobia symbiosis, one such ancient system, we find two different rhizobial life histories, which depend on the species of legume host. In nodules of some hosts, including alfalfa (*Medicago sativa*) and peanut (*Arachis hypogaea*), rhizobia that differentiate into nitrogen fixing bacteroids undergo major transformations, including swelling or branching and sometimes amplification of the bacterial genome (Mergaert *et al.*, 2006). As discussed below, this extreme differentiation most likely prevents bacteroids from resuming normal cell division (free-living states), even if they are released from nodules during senescence (Sutton & Paterson, 1983). The next generation of symbiotic rhizobia for these hosts is presumably descended from rhizobia that had

reproduced within the same nodules but not yet differentiated into bacteroids. On the other hand, in hosts like cowpea (*Vigna unguiculata*) and birdsfoot trefoil (*Lotus corniculatus*), the rhizobia still undergo differentiation into bacteroids but this process is not irreversible. Bacteroids in these hosts are less swollen, have no genome amplification, and continue to reproduce after leaving the nodules (Mergaert *et al.*, 2006).

The effect of legume host species on bacteroid differentiation was studied extensively by Sutton & Paterson (1980, 1983), but specific plant mechanisms were unknown and only a handful of closely related species were investigated. Currently, it is widely accepted that the size and shape of nitrogen fixing bacteroids vary widely and are controlled primarily by the legume host rather than the genotype of the rhizobia (Oke & Long, 1999). For example, a single rhizobial strain will differentiate into spherical swollen bacteroids in peanut but remain rod-shaped in cowpea (Sen *et al.*, 1986). Similarly, Mergaert et al. (2006) has shown that recombinant rhizobial strains will transform into different bacteroid morphologies depending on the host species; transgenic rhizobia, which never evolved with one host, nonetheless showed the same level of bacteroid differentiation as the host's wild type rhizobia.

Extreme bacteroid differentiation has recently been shown in *Medicago truncatula* to be imposed by nodule-specific cysteine rich (NCR) plant peptides (Van de Velde *et al.*, 2010). These compounds have similar properties as antimicrobial defensins that block bacterial cell division, often causing genomic endoreduplication and alteration of cell shape (Latch & Margolin, 1997). Differentiation may also involve extreme alteration of the synthesis of peptidoglycan, an elastic polymer of bacterial cell walls known to regulate osmotic pressure and cell shape (Lam *et al.*, 2009). The NCR peptide-

coding sequences are present in EST sequencing databases of closely-related genera of *Medicago* that host swollen bacteroids, but not in the databases of *Lotus japonicus*, *Phaseolus vulgaris* or *Glycine max* (Alunni *et al.*, 2007 and references therein), those species hosting nonswollen bacteroids.

Similarly, the correlation between swelling and loss of bacteroid reproductive viability has been consistent among many tested species, such as *Glycine max* (Gresshoff & Rolfe, 1978, Zhou *et al.*, 1985), *Phaseolus vulgaris* (Ratcliff *et al.*, 2008), *Macroptilium atropurpureum* (Ratcliff *et al.*, 2008), *Lotus japonicus* (Müller *et al.*, 2001), *Trifolium repens* (Zhou *et al.*, 1985) and *Medicago sativa* (McRae *et al.*, 1989, Vasse *et al.*, 1990, Ratcliff *et al.*, 2008). Although Khetmalas et al. (2005) characterized rod-shaped rhizobia inside senescing *Arachis pintoi* nodules as dedifferentiated, formerly spherical bacteroids, the preponderance of evidence suggests that swollen bacteroids rarely dedifferentiate back into free-living forms and that it is the undifferentiated cells that repopulate the soil for future symbiosis. Bacteroid morphology is therefore considered a reasonable proxy for reproductive viability until we find a more effective means of observing bacteroids in their natural states to see how they either dedifferentiate and reproduce or are broken down (by plant, rhizobial, or exogenous enzymes).

Some previous studies also correlated bacteroid type to nodule type, although it has not been shown that this correlation is universal. *Medicago*, *Pisum*, *Vicia* and *Trifolium* (closely related species in the Inverse-Repeat Legume Clade (IRLC)) all have indeterminate nodule types (those with persistent meristems) and swollen bacteroids, whereas *Glycine*, *Phaseolus*, *Macroptilium* and *Lotus*, all have determinate nodule types (transient meristems) and nonswollen bacteroids. It has been observed in many

determinate nodules (within Phaseoloid and Dalbergioid clades) that the rhizobia-infected cells divide and enlarge along with the rhizobia inside them (Chandler *et al.*, 1982, Sprent & Thomas, 1984), whereas host cells of indeterminate nodules (IRLC) are each infected with rhizobia from a branch of the infection thread and do not divide further, and neither do the infecting rhizobia (Sprent & Thomas, 1984).

While we begin to understand the relationship between bacteroid swelling and loss of reproductive viability, we have little understanding of how widely distributed swollen bacteroids are in the legume family. We also know little about the evolutionary effects of swollen bacteroids on the legume-rhizobia interaction. Selection pressures for symbiotic strategies may differ between bacteroid morphologies with implications for rhizobial evolution. For example, when bacteroids themselves are reproductive, hoarding high-energy lipid polymers, like polyhydroxybutyrate (PHB), inside their cells at the expense of nitrogen fixation may directly increase bacteroid fitness. On the other hand, when bacteroids are nonreproductive, it is the undifferentiated rhizobia that reap the benefits of the mutualism in ways that may interact with nitrogen fixation by the bacteroids. Rhizopines, for example, are simple sugar-like compounds produced (possibly by diverting resources from nitrogen fixation) by some swollen bacteroids and catabolized by the undifferentiated rhizobia (Murphy *et al.*, 1995). Only rhizobial strains commonly found in legume species hosting swollen bacteroids are known to possess rhizopine-synthesis genes (Wexler *et al.*, 1995), suggesting rhizobia with evolutionary dead-end bacteroids may have evolved this alternative mechanism for cheating.

Furthermore, if rhizobial cheating strategies or intensities vary, the optimal level of host sanctions (Kiers *et al.*, 2003) may also vary among legume species. Without host-

imposed selection, rhizobia could lose the capacity to fix nitrogen (West *et al.*, 2002). However, host sanctions have not been adequately demonstrated in legume species with nonreproductive bacteroids and other mutualistic systems have shown that levels of sanction can vary among species (Jander & Herre, 2010). In the legume-rhizobia system, the exact mechanisms of host sanctions are still unknown, although limiting oxygen diffusion into nodules may play some role (Kiers *et al.*, 2003) and reduce the fitness of the undifferentiated reproductive rhizobia inside (Oono *et al.*, 2009). However, at present, we have no evidence that sanctions are universal nor that sanctions always work at the whole nodule-level. One could conceive of peribacteroid-level sanctions that only target bacteroids, or that sanctions differ physiologically between species with nonreproductive and reproductive bacteroids.

Given that host-imposed swelling of bacteroids may affect the coevolution of legume-rhizobium symbiosis, we here explore the taxonomic distribution of this trait and its possible evolutionary history in the legume phylogeny. By mapping the life history characteristics of the rhizobia onto the host phylogeny, ecophylogenetic hypotheses can be tested to understand how the interaction evolved (Armbruster, 1992). This has been previously done in studies pertaining to the evolution of nonmutualists among mutualist lineages (Pellmyr *et al.*, 1996, Hibbett *et al.*, 2000) as well as new life-history adaptations among partner lineages, e.g. fragrance-collecting vs. resin-collecting bee pollination (Armbruster, 1992), or dioecy vs. monoecy in figs pollinated by fig-wasps (Weiblen, 2000, Greef & Compton, 2002, Harrison & Yamamura, 2003). In our study, we characterize bacteroid swelling in various legume hosts, to test whether host traits leading to swollen bacteroids were gained or lost among different lineages of legumes. This could

suggest whether there are benefits or costs to legume hosts with this trait. Our specific questions are: Are host traits that cause swollen bacteroids ancestral or derived? How common is bacteroid swelling beyond the well-studied model legume species, and can we assess a broader phylogenetic pattern? We also test whether there is correlated evolution between nodule type and bacteroid type over a wider set of legume species.

MATERIALS & METHODS

1. Determining which legume species host swollen bacteroids

Multiple methods were used to assess bacteroid swelling, as no single method could be used with all the legume species in our phylogeny (Table 2-1). Methods for investigating bacteroid type included flow cytometry (FC), fluorescence microscopy (FM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). All FM and FC data were newly collected for this study. All unpublished SEMs were collected previously by Janet I. Sprent.

We obtained nodules for 19 species either from the field or plants grown in growth chambers. Bacteroid swelling in these hosts was assessed by both FC and FM; bacteroids were considered swollen if rhizobial cells showed a bimodal distribution in flow-cytometric forward scatter (Figure 2-1a, b), which measures size of individual cells. A bimodal distribution suggests a mixture of smaller undifferentiated rhizobia and larger swollen bacteroids within a single nodule. Additionally, the size and shape of the bacteroids were assessed by FM. Size measurements are averages based on more than five of the largest individual bacteroids found in a random view. Bacteroids were confirmed to be swollen if they were either greater than 4 μm long, or wider than 1.5 μm

(for spherical bacteroids) or branched (regardless of size). Bacteroids were assessed as nonswollen if they were smaller than 2.5 x 1.5 μm. Free-living rhizobia are usually less than 2 μm long (Sprent, 2001), and hence would probably not exceed 4 μm long even during cell division. Even bacteroids that are considered nonswollen in our analyses do become slightly larger than free-living rhizobia inside plant nodules, perhaps due to accumulation of carbon resources or osmotic pressure. Legume hosts with bacteroids of intermediate sizes do exist but these species were not included in the analyses (Supporting Information Table S1), due to a coincidental lack of sufficient molecular sequences. When nodules could not be obtained, we examined electron micrographs from unpublished and published studies. We characterized bacteroids as swollen using the same criteria as with the FM method. Some published micrographs had observational statements within the article that suggested either swollen or nonswollen bacteroids as well. Electron micrographs and published data were available for a few of the species for which we already had FM and FC data. Bacteroid data and their individual methods of evaluation are summarized in Table 1 & Table S1.

i. Bacteroid preparation and flow cytometry

Some nodules were collected from the field, surface-sterilized and dried (Somasegaran & Hoben, 1994) for transport to the laboratory where the nodules were rehydrated in water overnight. Other nodules were harvested from plants grown in the growth chamber and were never dried. Rehydrated or fresh nodules were crushed in ascorbic acid buffer (Arrese-Igor *et al.*, 1992) and centrifuged at 100 *g* for 10 minutes to separate rhizobia from plant material. The supernatant, containing bacteroids and

undifferentiated rhizobia, was fixed for 30 minutes in 30% ethanol, pelleted at 5,000 *g* for 5 minutes, and resuspended in phosphate buffer solution (Somasegaran & Hoben, 1994). Nodule rhizobia were diluted (10^7~10^5 cells per ml) for flow cytometric sampling and stained with SYTO 13 (final concentration of 625 nM; Molecular Probes), which binds to nucleic acids, both DNA and RNA. SYTO 13 was measured on FL1 (530 nm BP filter) on a Becton Dickson FACScalibur. Many flow cytometer runs had some background noise, possibly due to staining of RNA or DNA contents from burst cells, but this noise could easily be distinguished from the rhizobial cells. If genome endoreduplication occurred for swollen bacteroids, SYTO 13 can indicate cells that differ in genomic content. We could often detect two distinct clouds of 1C and 2C cells, and occasionally 4C clouds when bacteroids were swollen (Fig. 2-1c, d).

ii. Fluorescence and scanning electron microscopy

For fluorescence microscopy, after surface-sterilization, nodules were crushed directly on top of slides with forceps allowing a high density of bacteroids to be released. Sterile water was added to the slide with 1 μl of SYTO13 (62.5 μM) and cells were observed under an Olympus IX70 Inverted Fluorescence Microscope.

Preparation of the nodules from the unpublished SEMs are previously described (de Faria *et al.*, 1986).

iii. Published electron micrographs and observational data

Bacteroid swelling data from previous studies were all determined from either scanning or transmission electron micrographs and sometimes complemented by

published observations suggesting swelling or lack of swelling. For example, multiple bacteroids per symbiosome suggests bacteroids were able to divide after an initial fragmentation of the symbiosome from the infection thread, consistent with nonswollen bacteroids, whereas a single bacteroid per symbiosome suggests that bacteroids cannot resume division after differentiation. Sutton & Paterson's (1983) compiled dataset suggests there is a correlation between bacteroid viability and number of bacteroids per symbiosome. In their dataset, all species with low bacteroid viability had single bacteroid per symbiosome, whereas those with high bacteroid viability always had multiple bacteroids per symbiosome. However, multiple bacteroids per symbiosome may be found if the symbiosome initially engulfed more than one cell or if the rhizobia divided before differentiating into a bacteroid within the symbiosome.

2. Taxon sampling, phylogenetic analyses

We restricted our analysis to those taxa (40 species in the subfamily Papilionoideae representing 40 different genera) for which molecular sequences (*rbcL* or *matK*) were available and morphological data (bacteroid size/shape) could be generated. *rbcL* and *matK* are two genes previously used to construct phylogenies (Doyle *et al.*, 1997, Kajita *et al.*, 2001, Wojciechowski *et al.*, 2004). No new genes were sequenced for this study. In addition to the *rbcL* and *matK* genes, 5.8S rRNA genes and parts of the *trnL* gene were also used, if available, to increase the number of molecular characters (Table 1). We focused on the papilionoids because it is the only subfamily in which swollen bacteroids are known to exist (Sprent, 2001) and also because the origin of nodulation for the Papilionoideae may be independent from the other two subfamilies –

Caesalpinioideae and Mimosoideae (Doyle, 1994). Reconstruction of ancestral states would be meaningless if analyzed across species with different origins of nodulation.

Six supported nodulating subclades (Wojciechowski *et al.*, 2004) were sampled in proportion to species diversity in each clade (Table 1). Some legume species were not included in the analysis, even though bacteroid characteristics are known (Table S1), because including them would lead to a disproportionate representation of one clade over another. *Indigofera suffructicosa* was included in the analysis despite being outside of the six major subclades because it is a member of the second largest genus (more than 700 species; (Lewis *et al.*, 2005)) within the subfamily, after *Astragalus*. *Sophora secundiflora* was also included because of its inferred basal position in the Papilionoideae (Kajita *et al.*, 2001). Two outgroup species were selected, one from each of the other two subfamilies: *Chamaecrista fasciculata* from the Caesalpinioideae and *Pentaclethra macrophylla* from the Mimosoideae.

Finding species in the Mirbelioids and core Millettioids that had both available DNA sequences and bacteroid data was not possible, but we believed these two lineages were important for phylogenetic diversity and should not be excluded from our analysis. Therefore, bacteroid traits were assessed based on *Gompholobium knightianum* and *Tephrosia virginianum*, whereas the sequence data came from *Gompholobium minus* and *Tephrosia heckmanniana* respectively. Since we recognized the slight possibility of misaligning bacteroid properties to the sequenced species, we modeled the ancestral character reconstruction with the alternative bacteroid trait as well and assessed the robustness of our results.

Sequences were aligned based on the amino acid sequence and reverted back to nucleotides using BioEdit (Hall, 1999). We analyzed 1,391 positions for *rbcL*, 1,598 for *matK*, 164 for 5.8S rRNA, and 347 for *trnL*. Two intron regions (60 and 317 nucleotides long) in *trnL* sequences were excluded from the analyses. We assessed potential conflict between the data portions by checking 75% maximum parsimony bootstrap consensus trees for conflicting topologies (Lutzoni *et al.*, 2004).

MrModelTest (Nylander) was used to determine the best-fitting model for each gene following the more conservative Akaike Information Criterion test: *rbcL* was analyzed by GTR+I+G, *matK* by TVM+G, *trnL* by TIM+G, and 5.8S rRNA by TrNef+I+G.

The B/MCMC analyses were conducted using MrBayes v3.1.1 (Huelsenbeck & Ronquist, 2001). Four simultaneous chains (temperature 0.2) were run three times starting with a random tree, resulting in twelve million generations. Every 100th tree was saved into a file and the first 0.1% of the trees was discarded as burn-in for each run. Log-likelihood scores of sample trees against generations were plotted using TRACER v1.4 (Rambaut & Drummond, 2007) to ensure the Bayesian analysis had reached a stable equilibrium value. For analyses of character evolution we discarded 20,000 trees as burn-in and sampled every 20,000th post burn-in tree for a total of 1000 trees to avoid autocorrelation (Pagel & Meade, 2006).

3. Analyses of character evolution

Evolutions of bacteroid type and of nodule type were traced over 1000 post burn-in trees from the B/MCMC analysis. A combined maximum likelihood and Bayesian

inference approach was implemented using the program package BayesTraits v1.0 (Pagel & Meade, 2006) and a fully Bayesian approach was carried out using SIMMAP v1.0 (Bollback, 2006). In each analysis, we defined constraints for 23 nodes of interest. All of them were supported by a posterior probability of 0.95 or higher except the most basal node of the papilionoids (node 23; Fig. 2-2), which has a posterior probability of 0.79. The consensus tree has a polytomy and cannot resolve the relationship among the Genistoids, Dalbergioids *sensu lato* (*s.l.*). and the other major clades. Although this node has a lower posterior probability than conventionally accepted for constructing ancestral traits, all genera in this clade are monophyletic and the ancestral state of this node is the ancestral state for all the papilionoid species in the analysis.

The trees were rooted with *Chamaecrista fasciculata* as the outgroup for the ML analysis in BayesTraits whereas both *C. fasciculata* and *Pentaclethra macrophylla* were used as outgroups for the Bayesian analysis in SIMMAP. Outgroup taxa were excluded from the analyses in SIMMAP and coded as "2" for trait states in BayesTraits so that the outgroup trait state would not influence the analyses. For bacteroid type ancestral reconstruction, we coded "0" for nonswollen bacteroids and "1" for swollen bacteroids. Scoring was based on the methods available for the particular species as described above. We also reconstructed the ancestral state for nodule type and coded "0" for determinate nodules and "1" for indeterminate nodules.

In BayesMultistate, we reconstructed the ancestral character states using maximum likelihood. The transition parameters were estimated with 10 attempts per tree and an ancestral state probability was calculated for each post burn-in tree based on the estimated parameter values. The ancestral state probabilities for a particular node were

then averaged over all 1000 post burn-in trees. We also constructed ancestral character states using uniform (0, 100) gamma and exponential priors in a Bayesian framework using BayesTraits.

In SIMMAP, we used multiple combinations of gamma prior values to assess the robustness of our results, including flat priors for the bias and rate parameters. The ancestral character posterior probabilities reported here are from a model using the following evolutionary rate parameters: $\alpha = 3.0$, $\beta = 2.0$, $k = 60$. For each tree sampled, 100 draws were carried out from the prior distributions for modeling the rate of evolution.

4. Analyses of correlated traits

Nodule trait (determinate vs. indeterminate) and bacteroid trait (swollen vs. nonswollen) were tested for correlated evolution using the program BayesDiscrete in the BayesTraits package (Pagel & Meade, 2006). Two maximum likelihood models were run; an independent and dependent trait evolution model. The independent evolution model allows the binary nodule trait and the binary bacteroid trait to evolve independent of each other, requiring four transition rate parameters; two for each trait. The dependent evolution model assumes the two traits do not change states independently and the transition rate parameter for each trait is dependent on the other trait's original state. This model requires eight transition rate parameters between the four possible states of trait combinations. We computed under each model the log-likelihood for each of the 1000 post burn-in trees. We then compared the independent and dependent (correlated) evolution models by assessing -2*(likelihood ratio) against a chi-squared distribution

with four degrees of freedom (the difference in number of parameters between the two models; four and eight respectively). The likelihood ratio was measured as the average difference between the log-likelihood of the dependent model and the log-likelihood of the independent model over 1000 trees.

RESULTS

1. Legume species hosting swollen bacteroids

Of 40 papilionoid species used in the phylogeny, fourteen were identified as hosting swollen rhizobial bacteroids in their root nodules. These fourteen species belong to five of the six major subclades: Inverse-repeat loss clade (IRLC), Mirbelioids, Dalbergioids *s.l.*, Millettioids, and Genistoids (Fig. 2-2). Each of these clades contained species hosting both nonswollen and swollen bacteroids but we did not find a single species in the Robinioids hosting swollen bacteroids.

Medicago sativa, *Pisum sativum* (Fig. 2-3a), and *Vicia hirsuta*, all in the IRLC, are well-known for their swollen nonreproductive bacteroids (Mergaert *et al.*, 2006). However, *Cicer arietinum* (1.1 μm ± 0.11 sd) and *Glycyrrhiza lepidota* (2.4 μm ±0.49 sd), also in the IRLC, host nonswollen bacteroids, as does *Biserrula pelecinus* (Nandasena *et al.*, 2004; Table 2-S1).

Mirbelioid species host various sizes of bacteroids, including large swollen (> 4 μm in length), intermediate (between 2.5 to 4 μm in length), and small nonswollen bacteroids (< 2.5 μm in length) (Table 2-S1). Bacteroids in *Gompholobium knightianum* nodules, designated *G. minus* in the phylogeny (as explained above), were 5.6 μm (± 0.88

sd) in length, which is significantly swollen, whereas bacteroids of *Aotus ericoides* averaged 2.1 μm (± 0.72 sd), about double the size of free-living bacteria (Lawrie, 1983).

Arachis hypogaea (Fig. 2-3b), *Aeschynomene indica*, *Stylosanthes hamata*, all in the Dalbergioids *s.l.* have been recognized for hosting unusual spherical bacteroids (Chandler *et al.*, 1982, Sen *et al.*, 1986, Fleischman & Kramer, 1998), but other related legume species within the same clade (*Pterocarpus indicus* and *Discolobium pulchellum*) do not share this trait (Higashi *et al.*, 1987, Loureiro *et al.*, 1994). *Dalea purpurea* (1.5 μm ± 0.56 sd) and *Amorpha fruticosa* also did not host swollen bacteroids (2.3 μm ± 0.31 sd).

None of the four observed Robinioid legume species hosted swollen bacteroids based on our criteria. These included those Robinioid species with indeterminate nodules (*Coronilla varia,* 2.1 μm ± 0.66 sd, and *Robinia pseudoacacia,* 2.3 μm ± 0.31 sd; Fig. 2-3c) as well those with determinate nodules (*Lotus japonicus*; Mergaert *et al.*, 2006, and *Anthyllis vulneraria*, 1.6 μm ± 0.27 sd)

Centrosema virginianum (6.9 μm ± 0.72 sd) and *Tephrosia virginianum* (7.0 μm ± 0.71 sd; Fig. 2-3d), in the Millettioids, have swollen bacteroids based on our criteria. These two appear to be more closely related to each other than to the other Millettioids (Table 2-1, Table 2-S1), but this relationship has weak support in our reconstructed phylogeny as well as in previous studies (Kajita *et al.*, 2001). All other Millettioid members were newly shown or reconfirmed to host nonswollen bacteroids as well as *Indigofera suffructicosa* (Izaguirre-Mayoral & Vivas, 1996), in the close sister clade of the Millettioids.

Three of the Genistoid legume species (*Cytisus scoparius* (Fig. 2-1), *Maackia amurensis*, and *Baptisia australis*) sampled showed bimodal size distribution of nodule rhizobia and two others (*Lupinus angustifolius* and *Genista tinctoria*) have some branching bacteroids (Dart & Mercer, 1966, Kalita & Malek, 2004), both consistent with swollen bacteroids. However, three other *Lupinus* species that were not used in the phylogenetic analysis had nonswollen bacteroids (Table 2-S1). Hence, host effects on bacteroid states apparently changed during the evolution of this very diverse genus. This within-genus variability was not considered in the analysis because our balanced sampling protocol limited us to one species per genus. *Lupinus angustifolius* was the best *Lupinus* species to include for its available *matK* sequence, the longest sequence of the four genes used in the analysis. The implications of ignoring this variability within *Lupinus* are discussed below. *Sophora secundiflora* (1.7 μm ± 0.42 sd), an early branching species within the Genistoids, was categorized as hosting nonswollen bacteroids

2. Phylogenetic analyses

Phylogenetic analyses of the four gene partitions combined included 3500 base pairs, of which 1390 were variable. The tree inferred in the current study does not conflict with previously published topologies that used single molecular loci and larger species numbers (Kajita *et al.*, 2001, Pennington *et al.*, 2001, Hu *et al.*, 2002, Wojciechowski *et al.*, 2004). Six previously reported major lineages are supported by posterior probabilities greater than 0.95. The most recent common ancestor node for all the papilionoids has a lower posterior probability (0.79; Fig. 2-2, node 23). The position

of *Indigofera* as sister group to the Millettioids agrees with previous studies (Wojciechowski *et al.*, 2004). The relationships among the six major clades within the Papilionoideae have never been strongly supported (McMahon & Sanderson, 2006) and were not resolved in this study.

3. Evolution of legume traits affecting bacteroid swelling

Bacteroid states were mapped onto a 50% majority rule consensus tree derived from a Bayesian analysis (Fig. 2-2). Fifteen analyzed nodes had high probabilities for both Bayesian (posterior probability ≥ 0.95) and maximum likelihood analyses (average p > 80%) for either swollen or nonswollen bacteroid states (Fig. 2-2, Table 2-2). Seven of the nodes were particularly robust because they had high posterior probabilities even with uniform gamma and exponential prior distributions using BayesTraits (Table 2-2). The node for the most recent common ancestor of the Papilionoideae subfamily (node 23) was highly supported as hosting nonswollen bacteroids using all methods. According to the two non-uniform prior analyses, host traits leading to swollen rhizobial bacteroids have evolved at least five times (within the IRLC, Mirbelioid, Millettioid, Dalbergioid *s.l.*, Genistoid clades).

Even when *G. minus* and *T. heckmanniana* were coded with the opposite traits, the ancestral state for the papilionoids (node 23) remained hosting nonswollen bacteroids with a posterior probability of greater than 0.95 and an average likelihood of greater than 80%.

4. Correlated evolution of nodule and bacteroid traits

We also analyzed whether a nodule trait (determinate vs. indeterminate growth) and bacteroid trait (swollen vs. nonswollen bacteroids) evolved in a correlated fashion. In order to statistically assess the potential correlation of the two traits, we compared the likelihood fit between two models that allowed the traits to evolve independently or dependently using the program BayesDiscrete. The likelihood for the two models was not significantly different. The average log-likelihood for the dependent model was ln -33.08 versus ln -34.39 for the independent model. The likelihood ratio was 2.62, which is not significant against a chi-squared distribution with four degrees of freedom. This implies that an independent evolution model does not explain nodule and bacteroid trait evolution significantly better than a dependent model or vice versa.

DISCUSSION

Our results suggest that legumes inducing bacteriod swelling evolved independently at least five times from an ancestral papilionoid legume hosting nonswollen bacteroids. Extreme bacteroid differentiation is not significantly correlated with indeterminate nodule types. This finding suggests that generalization from model species, *Medicago truncatula* (hosting swollen bacteroids in indeterminate nodules) or *Lotus japonicus* (hosting nonswollen bacteroids in determinate nodules), is not valid. However, bacteroid morphology is linked to reproductive viability of bacteroids (Mergaert *et al.*, 2006) and can have different implications for rhizobial evolution (Denison, 2000, Oono *et al.*, 2009) depending on their host species.

Legume traits leading to swollen rhizobial bacteroids evolved at least five times

We reconstructed the ancestral state of legume-host effects on bacteroid morphology using maximum likelihood and Bayesian approaches. Our legume phylogeny consists of 40 species, representing only 40 of the 478 genera of papilionoid legumes, but including lineages that have not previously received much attention for their symbiotic properties. Ancestral character reconstruction analysis can depend on the sample size and phylogenetic position of included taxa (Heath *et al.*, 2008). We attempted to sample randomly and diversely in the Papilionoideae, but it is possible that we still missed enough lineages of legumes with swollen bacteroids to overturn our ancestral character state. However, based on the data currently available, the most recent common ancestor of the papilionoids has a very high probability of having hosted nonswollen rhizobial bacteroids.

An ancestral state hosting nonswollen bacteroids suggests five likely independent origins for host-imposed bacteroid swelling. Four of these five lineages had already published cases of swollen bacteroids (IRLC, Dalbergioids *s.l.*, Genistoids, and Mirbelioids) but the cases found in the Millettioids were first discovered by this study. Some of the Dalbergioid legumes are known to have spherical bacteroids (Fig. 2-3b) as opposed to elongated (Fig. 2-3d) or branched ones (Fig. 2-3a) in the other clades. This morphological difference already suggested there were at least two independent origins and two different underlying mechanisms for swelling. However, the possibility of all five lineages having independent origins was uncertain before the present analysis, further strengthening the possibility that inducing swelling in bacteroids could have a host fitness advantage. Furthermore, it would be interesting to investigate NCR or

antimicrobial-like peptides in the other four lineages to see if the host molecular mechanisms imposing bacteroid swelling are similar.

We also confirmed that closely related legume species often host the same type of bacteroids with typically no variation within a genus, suggesting that changes in this trait are relatively rare. However, a closer inspection of *Lupinus*, or the Genistoid clade in general, includes at least some examples of closely related species that differently influence bacteroid differentiation. Species within *Lupinus* may host either swollen or nonswollen bacteroids (Table S1, Dart & Mercer, 1966) and *Cyclopia genistoides* appears to have nonswollen bacteroids, based on TEM (Elliott *et al.*, 2007), yet it is closely related to other Genistoids hosting swollen bacteroids.

Several species not included in the analysis (Table S1), due to lack of adequate DNA sequences, happen to host bacteroids of intermediate lengths. These intermediate morphologies may indicate transitional stages between reproductive and nonreproductive forms indicating a continuous rather than a binary variable.

Why would hosting swollen bacteroids be a derived trait in legume species?

The multiple independent origins we found for host-imposed bacteroid swelling suggest some fitness benefit to hosts. One hypothesis is that swollen bacteroids fix nitrogen more actively than nonswollen ones (Oono *et al.*, 2009), which can explain the greater production of PHB in some nonswollen bacteroids (Lodwig *et al.*, 2005). PHB accumulation and nitrogen fixation compete for the same carbon resources, as confirmed by greater nitrogen fixation in PHB-negative mutants (Cevallos *et al.*, 1996) and greater PHB accumulation in nonfixing mutant bacteroids (Hahn & Studer, 1986). However,

some legume species host nonswollen bacteroids with very little PHB, such as *Lotus* sp. (Banba *et al.*, 2001). More PHB tends to accumulate when respiration and growth are limited by oxygen or other resources (Anderson & Dawes, 1990, references therein), indicating that nodule physiology, which is unrelated to bacteroid morphology, could be important for PHB synthesis. Genomic endoreduplication or differences in surface:volume ratio might also affect the efficiency of swollen bacteroids (Oono *et al.*, 2009).

Although cheating options for swollen nonreproductive bacteroids may be limited (as discussed above), this is unlikely the reason why inducing swelling in bacteroids first evolved among legume hosts. An evolutionary change in rhizobial cheating strategies (e.g. rhizopine synthesis) most likely takes several rhizobial and host generations and would not be an immediate host benefit for inducing swelling and loss of reproductive viability in bacteroids (Oono *et al.*, 2009). However, if inducing swelling has an immediate effect on a bacteroid's ability to cheat (e.g. blocking PHB synthesis and accumulation), this may cause further selection for this host trait.

In hosts where nonswollen bacteroids may have been regained, such as within the genus *Lupinus*, perhaps bacteroid swelling is no longer beneficial to the plant for unknown reasons. On the other hand, some rhizobial strains of *Lupinus* hosts may have evolved traits to overcome host-induced swelling and loss of reproductive viability.

Bacteroid differentiation and its correlation with indeterminate vs. determinate nodule types

To evaluate the effect of nodule type, we grouped legume species into two categories, depending on whether their nodules have determinate or indeterminate growth. There was no consistent relationship between nodule type and host effects on bacteroid swelling, in contrast to some previous generalizations based on fewer species (Denison, 2000). A dependent evolution model for the two traits was not significantly better than an independent one, thus precluding statements about whether the two traits evolve in a correlated fashion.

For simplicity, we used only two categories for nodule type, but we recognize that there are more distinct types of nodules (Sprent, 2007). The determinate nodules of the Dalbergioids are often called aeschynomenoid and have crack entry for infection rather than infection threads. Even determinate nodules with infection threads differ in whether they export amide or ureide to the host. The indeterminate nodules of the Genistoids (including *Lupinus* sp.) lack interstitial cells, which are often found among the infected zone of indeterminate nodules of the IRLC. Some nodules have persisting infection threads where bacteroids reside, as in the indeterminate nodules of *Poecilanthe parviflora.* The bacteroids within persisting infection threads are not highly differentiated and may resemble the initial stages of the ancient symbiosis.

It is easy to understand the perceived correlation between nodule and bacteroid types since these two traits are both conserved in closely related legume species. However, assuming this correlation suggests an ancestral state of swollen bacteroids with

multiple origins of legume hosts releasing bacteroids from inhibition of reproduction, this would drastically change some of our views on the legume-rhizobia symbiosis.

In conclusion, we find multiple origins of swollen bacteroids hosted by different legume species. This suggests swollen bacteroids confer some host fitness benefits, such as optimization for nitrogen fixation efficiency (Oono *et al.*, 2009), which remain to be clarified. Rhizobial strains with a nonreproductive bacteroid life history may also have evolved alternative cheating and cooperation strategies leading to different mechanisms in different legume host species that maintain stability of the mutualism.

	Nodule type		Bacteroid data acquisition method	Bacteroid	Accession numbers from NCBI database			
	I / D	Sub-type	Nodule source or reference	S/N	*rbcL*	*matK*	5.8S rRNA	*trnL*
Genistoids s. l. (7/83)								
Baptisia australis	I	I	FC, FM[Ur]; SEM[JS]	S		AY386900	AY091572	AF309831
Cyclopia genistoides	I	I	TEM (1)	N	Z70124		AJ409895	
Cytissus scoparius	I	I -i	FC, FM[CH]	S	Z70086	AY386902	AF351120	
Genista tinctoria	I	I -i	TEM (2)	S	Z70099		AF007471	DQ417001
Lupinus angustifolius	I	L -i	TEM (3)	S	Z70064		AF007477	DQ417006
Maackia amurensis	I	I	FC, FM[Du]	S	Z70137	AY386944		
Poecilanthe parviflora	I	I	TEM (4)	N		AF142687	AF187089	AF208897
Dalbergioids s. l. (7/53)								
Amorpheae (2/8)								
Amorpha fruticosa	I	I	FC, FM[PMN]	N	U74212	AY391785	AY426774	AF208899
Dalea purpurea	I	I	FC, FM[WNS]	N		AY391798	AY426794	
Dalbergioids (5/45)								
Aeschynomene indica	D	A -i	TEM (5)	S	AF308701	AF272083S2	AF068141	AF208927
Arachis hypogaea	D	A -i	FC, FM[TA]; SEM, TEM (6)	S	U74247	EU307349	AF156675	DQ131546
Discolobium pulchellum	D	A -i	TEM (7)	N		AF270873	AF189059	AF208963
Pterocarpus indicus	D	A -i	SEM, TEM (8)	N		AF142691	AF269177	AF208953
Stylosanthes hamata	D	A -i	TEM (9)	S		AF203594	AF203550	AJ131247
Mirbelioids (2/32)								
Aotus ericoides	I	I	TEM (10)	N		AY386884		
Gompholobium minus/knightianum	I	I	SEM (11)	S		AY386891	AY233086	
Millettioids (13/168)								
"core Milletieae" (1/56)								
Tephrosia heckmanniana/virginiana	I	I	FC, FM[SH]	S	U74211	AF142712	AF467497	
Phaseoloids (12/112)								
Amphicarpaea bracteata	D	DesU	FC, FM[CC]	N	AF181930	AY582971	AF417015	EF543424
Cajanus cajan	D	DesU	SEM[JS]	N	AB045790	EU307315	EU288918	EF200131

Calopogonium mucunoides	D	DesU	TEM (12)	N	AB045792		AY293845	
Centrosema virginianum	D	DesU	FC, FM[SH]	S	AF308706			
Erythrina crista-galli	D	DesU	SEM[JS]	N	Z70170	AY386869		
Glycine max	D	DesU	TEM (13)	N	Z95552	AF142700	AF144654	DQ131547
Kummerowia stipulaceae	D	DesU	FC, FM[SH]	N	U74229			
Lespedeza cuneata	D	DesU	FC, FM[SH]	N	U74215			
Macroptilium atropurpureum	D	DesU	TEM (1)	N		AY509938	AF115138	
Oxyrhynchus volubilis	D	DesU	SEM[JS]	N	AF308717	AY509935	AF069114	
Phaseolus vulgaris	D	DesU	FC, FM[HF]; TEM (14, 16)	N	EU196765	DQ445990	AF069128	EF543430
Vigna unguiculata	D	DesU	FC, FM[HF]; SEM, TEM (6)	N	Z95543	AY589510	AY748433	AB304074
Robinioids (4/34)								
Coronilleae, Robinieae (2/11)								
Coronilla varia	I	I	FC, FM[GM], SEM[JS]	N	U74222	AF543846	AF218537	
Robinia pseudoacacia	I	I	FC, FM[CH]	N	U74220	AF142728	EF494737	AF529391
Loteae (2/23)								
Anthyllis vulneraria	D	DesA	SEM[JS]	N		AF543845	AF218499	
Lotus japonicus	D	DesA	FM (15), TEM (16)	N	NC_002694	NC_002694	DQ311975	DQ311703
IRLC (5/54)								
Cicer arietinum	I	I	TEM (17)	N	AF308707	AY386897	AJ237698	DQ315487
Glycyrrhiza lepidota	I	I	FC, FM[PMN]	N	AB126685	AF142730		AF124238
Medicago sativa	I	I	FC, FM[UM] (18)	S	Z70173	AY386881	AF053142	DQ131554
Pisum sativum	I	I	FC, FM[HF] (15)	S	X03853	AY386961	AY143486	DQ311717
Vicia hirsuta	I	I	FC, FM[GM] (15)	S		AF522157	DQ351827	
Species in other major lineages								
Indigofera suffructicosa	I	I	TEM (12)	N		AF142697	AF467051	
Sophora secundiflora	I	I	SEM[JS]	N	Z70141	AF142693	U59885	
Outgroups								
Caesalpinioid, *Chamaecrista fasciculata*	I	I			U74187	AY386955	EF590760	
Mimosoid, *Pentacletra macrophylla*	I	I			AM234250	AF521853		AF365051

Table 2-1. Bacteroid morphology assessment, nodule type and sequence data sources for legume species in the ancestral character reconstruction analysis. Legume species are categorized within their subclades with fractions indicating the proportion of genera represented in the analysis, out of the total recognized genera in the clade (Lewis *et al.*, 2005). Methods for investigating bacteroid type were flow cytometry (FC), fluorescence microscopy (FM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). All nodules prepared for FM and FC were collected for this study. Seed sources or plant location are indicated by superscripts; CC = Cedar Creek, MN; CH = Chapel Hill, NC; Du = Durham, NC; GM = Garden Makers, Rowley, MA 01969; HF = Henry Field's Seed and Nursery Co., Aurora, IN 47001; JS = previously collected by Janet I. Sprent; PMN = Prairie Moon Nursery, Winona, MN 55987; SH = Sandhills, NC; Ur = Urbana, IL; TA = Texas AgriLife Research and Extension Center, Lubbock, TX 79403 (Mark Burow); UM = University of Minnesota, Department of Agronomy and Plant Genetics, St. Paul, MN 55108 (Keith Henjum); WNS = Western Native Seeds, Coaldale, CO 81222. Nodule types: indeterminate (I), determinate (D), aeschynomenoid (A), lupinoid (L), lacking interstitial cells (-i), desmodoid exporting ureide (Des-U), desmodoid exporting amide (Des-A), persistent infection thread (P). References for nodule types are indicated for each species respectively or based on Sprent 2001 (2001) or Pueppke & Broughton (1999). Bacteroid state "S" stands for swollen and "N" stands for nonswollen. (1) Elliott et al. 2007, (2) Kalita et al. 2004, (3) Dart & Mercer 1966, (4) Sprent et al. 1987, (5) Fleischman & Kramer 1998, (6) Sen & Weaver 1986, (7) Loureiro et al. 1994, (8) Higashi et al. 1987, (9) Chandler et al. 1982, (10) Lawrie 1983, (11) Sprent 2001, (12) Izaguirre-Mayoral & Vivas 1996,

(13) Hahn & Studer 1986, (14) Cevallos et al. 1996, (15) Mergaert et al. 2006, (16) Banba et al. 2001, (17) Lee & Copeland 1994, (18) Ratcliff et al. 2008.

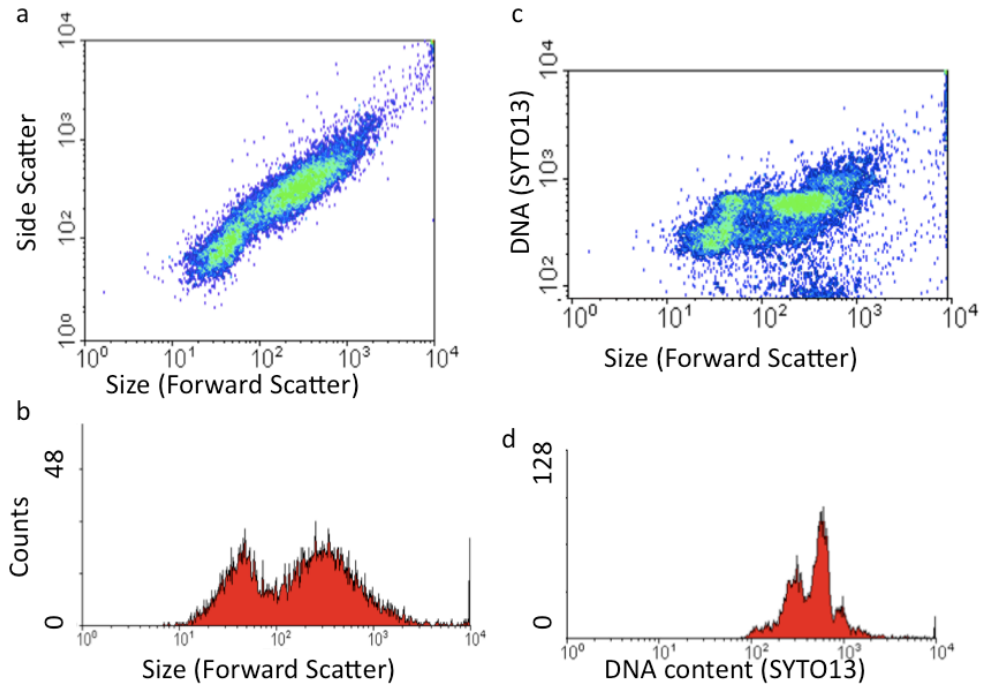

Figure 2-1. *Cytisus scoparius* nodule run through flow cytometer. a) Two distinct populations of rhizobia were detected, based on forward- (cell volume) and side- (inner complexity) scatter. b) Bimodal distribution of size indicates small undifferentiated rhizobia and larger swollen bacteroids inside a single nodule. c) DNA fluorescence due to SYTO13 staining reveals several distinct populations within a single nodule. d) The three peaks for SYTO13 detection signifies approximate doubling of fluorescence expected from genomic endoreduplication; geometric mean FL1 of 275, 558, and 1033.

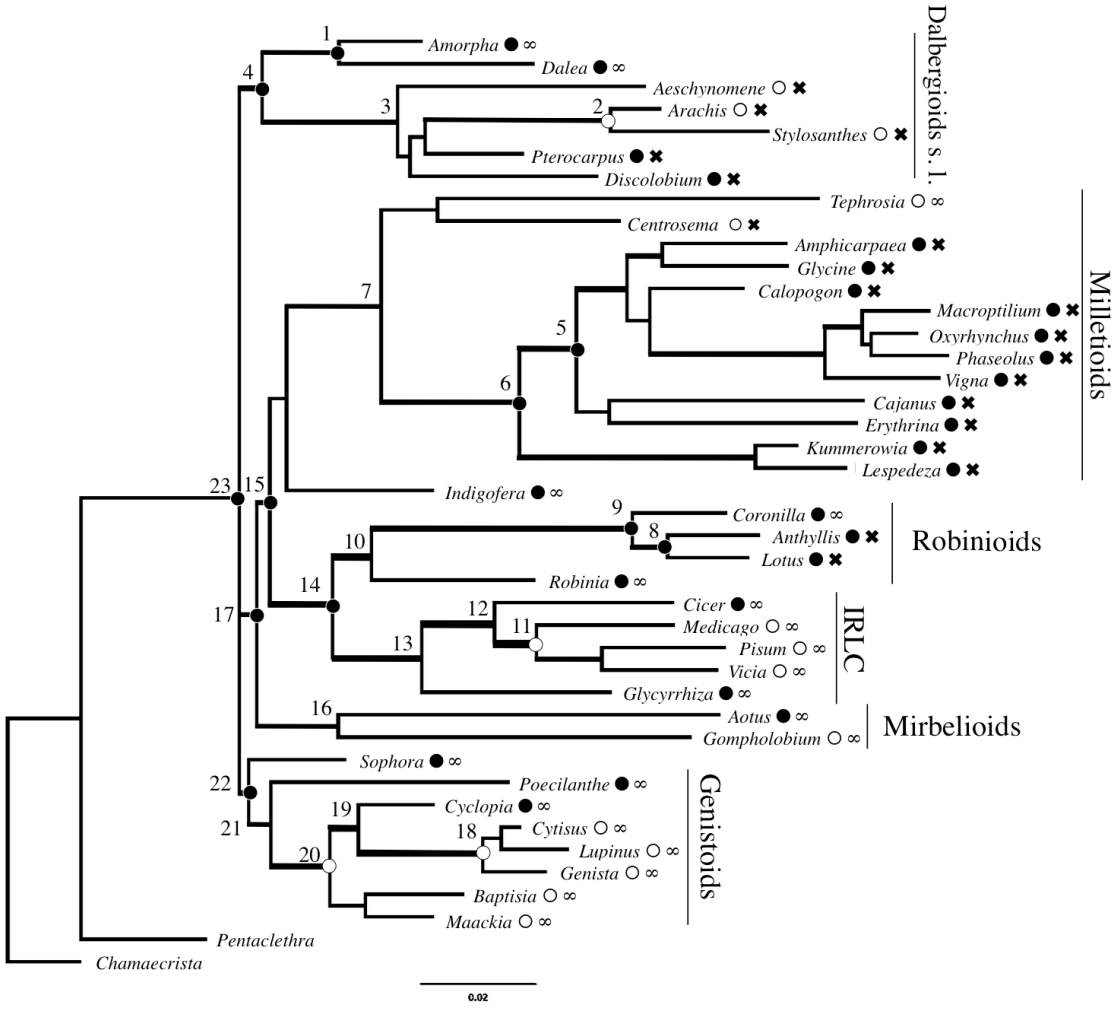

Figure 2-2. 50% majority rule consensus tree of 40 Papilionoid species based on 108,000 post burn-in trees from a Bayesian analysis. This phylogeny is based on combined *matK*, *rbcL*, partial *trnL*, and 5.8S rRNA sequences. Thickened branches indicate support of posterior probability ≥ 0.95. There were 23 nodes analyzed for ancestral character states of swollen (○) or nonswollen bacteroids (●). Trait states are indicated on the respective nodes if reconstructed with significant statistical support (probability ≥ 0.95 as well as ML bootstrap of ≥ 80%). Numbered nodes with no symbol indicate insignificant

reconstructions. Extant character states for swollen and nonswollen bacteroids as well as indeterminate (∞) and determinate nodules (✖) are indicated next to species names.

Node #	MCMC	ML
1*	1	0.932
2*	0	0.004
3	0.934	0.638
4	1	0.821
5	1	0.874
6*	1	0.962
7	0.863	0.536
8*	1	0.97
9*	1	0.938
10	1	0.747
11	0	0.034
12	0.803	0.426
13	0.9993	0.751
14	1	0.831
15	1	0.893
16	0.965	0.49
17	1	0.9
18*	0	0.001
19	0.125	0.727
20	0.043	0.188
21	0.974	0.583
22	1	0.887
23*	1	0.976

Table 2-2. Ancestral character state probabilities for nonswollen bacteroids at 23 nodes in the phylogeny in Fig. 2-2. Analyses were integrated over 1000 trees from a Bayesian MCMC analysis. Maximum likelihood was carried out using BayesTraits (BayesMultistate), and trait probabilities were averaged over all trees. Bayesian framework was executed by SIMMAP with gamma prior for overall evolutionary rate with $\alpha = 3.0$, $\beta = 2.0$, $k = 60$. Highlighted boxes indicate probability >95% for MCMC and 80% for maximum likelihood. Asterisks (*) by node indicate >95% for Bayesian framework using uniform gamma and exponential priors in BayesTraits, which indicate extreme prior values. For all nodes, state "2" (coded for the outgroups) had negligible

posterior probabilities and likelihoods and therefore, nodes with <5% posterior probabilities or <20% likelihood for hosting-nonswollen-bacteroid states can be considered as strongly supported for hosting-swollen-bacteroid states.

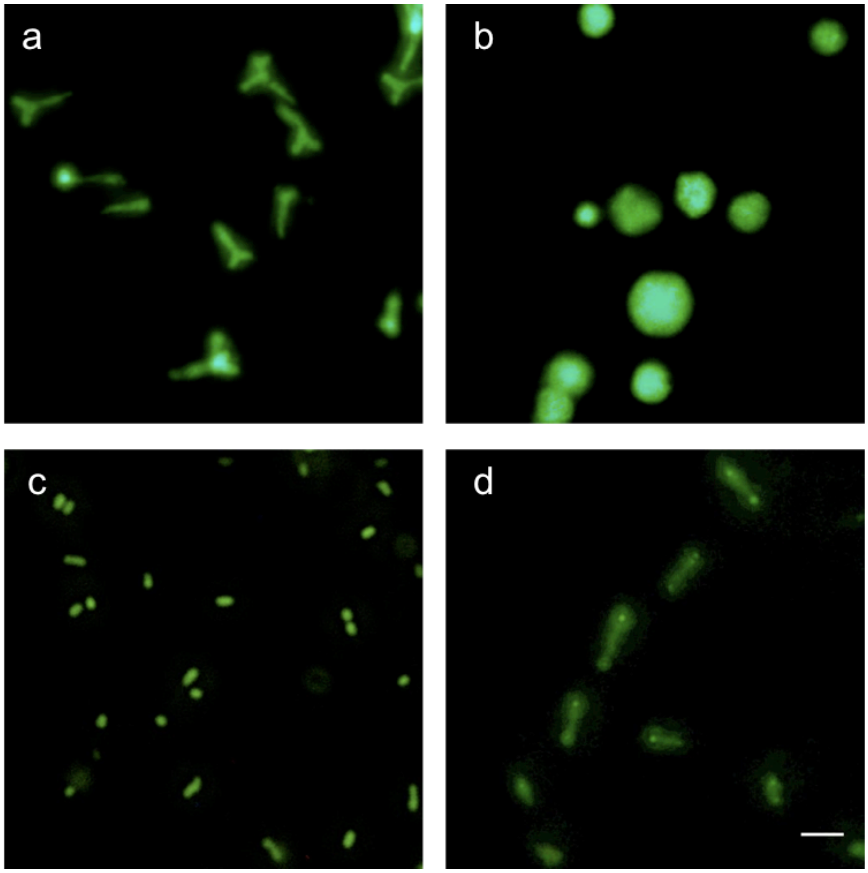

Figure 2-3. SYTO13 stained rhizobia (bacteroids and undifferentiated cells) harvested from nodule of a) *Pisum sativum* nodulated by *Rhizobium leguminosarum* A34, b) *Arachis hypogaea* nodulated by *Rhizobium* sp. 32H1, c) *Robinia pseudoacacia* wild nodule, d) *Tephrosia virginianum* wild nodule. Bar = 5 µm.

SUPPORTING INFORMATION

	Data method; reference or new data	Bacteroid State
Milletioid		
Pseudarthria hookeri	SEM (2.18 ± 0.31 x 0.62 ± 0.07)	Nonswollen
Vigna sinensis	TEM; Dart & Mercer 1966	Nonswollen
Ptycholobium biflorum	SEM (3.08 ± 0.74 x 0.65 ± 0.11)	?
Leptoderris sp.	SEM (1.65 ± 0.32 x 0.72 ± 0.05)	Nonswollen
Mirbelioid		
Mirbelia spinosa	SEM (4.85 ± 0.67 x 0.50 ± 0.06)	Swollen
Daviesia horrida	SEM (3.65 ± 0.86 x 0.64 ± 0.10)	?
Jacksonia sp.	SEM (2.86 ± 0.45 x 0.65 ± 0.12)	?
Dillwynia uncinata	SEM (3.56 ± 0.76 x 0.53 ± 0.09)	?
Viminaria juncea	TEM; Dart & Mercer 1966	Nonswollen
IRLC		
Galega orientalis	SEM (4.32 ± 0.69 x 0.53 ± 0.08)	Swollen
Astragalus canadensis	FC, FM^{PMN}branched	Swollen
Biserrula pelecinus	TEM; Nandasena et al. 2004	NonSwollen
Robinioid		
Anthyllis montana	SEM (2.19 ± 0.20 x 0.51 ± 0.06)	Not
Genistoid		
Lupinus diffusus	FC^{SH}, unimodal rhizobial size	Nonswollen
Lupinus bicolor	FC^{PMN}, unimodal rhizobial size	Nonswollen
Lupinus albus	TEM; Fernandez-Pascual et al. 2007	Nonswollen
Crotalaria anagyroides	TEM; Izaguirre-Mayoral & Vivas 1996	Swollen
Lamprolobium sp.	SEM; (2.57 ± 0.70 x 0.54 ± 0.04)	?

Supporting Information Table 2-S1. Papilionoid species for which bacteroid dimensions are known but were not included in the phylogenetic analysis; all abbreviations are the same as in Table 1.

Chapter 3: Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids

Symbiotic rhizobia differentiate physiologically and morphologically into nitrogen-fixing bacteroids inside legume host nodules. The differentiation is apparently terminal in some legume species, such as peas and peanuts, likely due to extreme cell swelling induced by the host. In other legume species, such as beans and cowpeas, differentiation into bacteroids, which are similar in size and shape to free-living rhizobia, is reversible. Bacteroid modification by plants may affect the effectiveness of the symbiosis. Here, we compare symbiotic efficiency of rhizobia in two different hosts where the rhizobia differentiate into swollen nonreproductive bacteroids in one host and remain nonswollen and reproductive in the other. Two such dual-host strains were tested; *Rhizobium leguminosarum* A34 in peas and beans and *Bradyrhizobium* sp. 32H1 in peanuts and cowpeas. We found that both of these strains confer more host benefit per cost in terms of nodule construction (plant mass per nodule mass) and nitrogen fixation efficiency (H_2 production by nitrogenase per CO_2 respired) in the host that imposed terminal differentiation (swollen bacteroids) than in the host with less extreme bacteroid differentiation. Terminal bacteroid differentiation among legume species has evolved independently multiple times, perhaps due to the increased host fitness benefits observed in this study.

INTRODUCTION

Legume-rhizobia interactions vary widely across a diverse paraphyletic group of nitrogen-fixing soil bacteria and over 18,000 species of legumes throughout the world (Lewis *et al.*, 2005). At least five independent lineages of legume species within the Inverted Repeat-Lacking Clade, Genistoids, Dalbergioids, Mirbelioids, and Milletioids, show evidence of convergent evolution for a trait that induces swelling of rhizobial cells during the process of differentiation into nitrogen-fixing bacteroids (Oono *et al.*, 2010). Swelling apparently leads to terminal differentiation; swollen bacteroids no longer divide normally. In other legume host species, bacteroid differentiation is less extreme, leading to nonswollen bacteroids. Nonswollen bacteroids are similar in shape and size to free-living rhizobia and divide normally once outside of their nodules. The evidence for multiple independent origins of hosts causing swollen bacteroids led us to believe that there may be a correlated evolutionary advantage for the legume hosts. Could the swelling of bacteroids improve nitrogen fixation efficiency or some other aspect of the symbiosis? In this study, we compare symbiotic efficiencies of rhizobia in legume hosts that are evolutionarily diverged but share a common effective rhizobial strain with and without terminal bacteroid differentiation.

Variations in benefits and costs of cooperation with rhizobia among different host species or genera are not commonly explored (Thrall *et al.*, 2000) because legume species typically nodulate with only one group of rhizobia (i.e. *Sinorhizobium* sp. in *Medicago*), although some legumes and some rhizobia are more promiscuous. We studied two strains, a transgenic strain that nodulates beans (*Phaseolus vulgaris*) and peas

(*Pisum sativum*) and a second wild strain harvested from cowpeas (*Vigna unguiculata*) that also nodulates peanuts (*Arachis hypogaea*). Beans and cowpeas are both within the Phaseolid group and do not induce terminal differentiation of rhizobial bacteroids. Peas and peanuts both host terminally-differentiated bacteroids but are in distant clades and likely have different genetic origins for traits that induce terminal differentiation (Oono *et al.*, 2010). Also, bacteroids in peas are branched while those in peanuts are spherical.

Differences in symbiotic qualities between swollen and nonswollen bacteroids have been previously explored in peanuts and cowpeas by Sen and Weaver (1980, 1981, 1984), who also hypothesized that swollen bacteroids are more beneficial to the host plant than nonswollen ones. They found 1.5 – 3x higher acetylene reduction by nitrogenase (as well as plant nitrogen) per nodule mass in peanuts than in cowpeas at multiple nodule ages (Sen & Weaver, 1980). Acetylene reduction per bacteroid was also greater in peanuts than in cowpeas when measuring whole nodules, but this difference disappeared when isolated bacteroids were measured directly (Sen & Weaver, 1984). They concluded that the terminal differentiation of peanut bacteroids *per se* was not responsible for the higher nitrogenase activity. They suggested that in cowpea nodules, with greater numbers of smaller bacteroids per nodule volume, availability of oxygen to each bacteroid might be restricted such that the rate of oxidative phosphorylation, necessary for nitrogen fixation, is reduced. Fixation rates may be different between hosts due to nodule permeability, interior oxygen concentrations, or bacteroid concentrations within nodules, but fixation efficiency (nitrogen fixed per carbon dioxide respired) would not necessarily be affected by these and may be more important for the host than the relative rates of fixation.

Rhizobial performances are often compared by measuring the symbiotic benefits, *e.g.* rates of acetylene reduction or plant growth (Sen & Weaver, 1984, Hashem *et al.*, 1997, Lodwig *et al.*, 2005), but rarely by measuring the symbiotic costs, *e.g.* carbon consumed or respired. Up to 25% of a legume's net photosynthate may be required for nitrogen fixation by rhizobia (Minchin *et al.*, 1981). Faster fixation rates (mol N per sec) can be beneficial for hosts if carbon costs are equal, but rhizobia that fix more nitrogen per carbon respired could free more carbon for other functions, including the option of supporting more nodules with the same amount of photosynthate. Cabrerizo et al. (2001) found CO_2 enrichment increased plant/nodule mass ratios but not specific nitrogen fixation in pea plants, suggesting that legume growth is carbon-limited. CO_2 enrichment can also improve plant water status, however, as higher external CO_2 concentrations allow photosynthetic rate to be maintained with partially closed stomata. If plants are carbon-limited, then at least sometimes, improved carbon-use efficiency, rather than increased rate of fixation could enhance plant fitness. Measuring both benefits and costs is key to an accurate understanding of the symbiotic efficiency of a rhizobial strain.

While we recognize the many physiological differences between peas and beans or peanuts and cowpeas, the fact that terminal differentiation induced by host legumes evolved multiple times independently (Oono *et al.*, 2010) suggests there may be some consistent host symbiotic benefit, such as improved fixation efficiency. Here, we measure efficiency by comparing host biomass to total nodule mass (return on nodule construction cost) as well as hydrogen (H_2) production by nitrogenase relative to carbon dioxide (CO_2) respiration (return on nodule operation cost) of the two rhizobial strains in different hosts.

MATERIALS AND METHODS

Plant/Rhizobia Culture and Experimental Conditions

Rhizobium sp. NGR234 has the largest known host-range but does not nodulate any legume species currently recognized to induce terminal differentiation of rhizobial bacteroids (Pueppke & Broughton, 1999). Some *Sinorhizobium fredii* strains apparently nodulate certain cultivars of soybean (*Glycine max*, hosting nonswollen bacteroids) and alfalfa (*Medicago sativa*, hosting swollen bacteroids) (Hashem *et al.*, 1997), but our efforts to replicate these results in our lab did not lead to successful nodulation. Therefore, we compared two strains – 1) *Rhizobium leguminosarum* A34, a transgenic strain, previously studied by Lodwig et al. (2005) and Mergaert et al. (2006) among others, that nodulates beans and peas and 2) a wild strain, *Bradyrhizobium* sp. 32H1 (=USDA3384), that nodulates cowpeas and peanuts.

Seeds of peas (*Pisum sativum* 'Maestro') and beans (*Phaseolus vulgaris* 'Royal Burgundy') were surface-sterilized with 0.09% hypochlorite (3% commercial bleach) for 5 min, rinsed in deionized water, and inoculated with 1 ml (approximately 10^9 cells) of stationary phase *Rhizobium leguminosarum* 4292 (Johnston *et al.*, 1982), A34 (Gotz *et al.*, 1985), or 3841 (Johnston & Beringer, 1975), which were grown in TY media (Somasegaran & Hoben, 1994). A34 and 4292 are both derived from *Rhizobium phaseoli* 8002 (Lamb *et al.*, 1982) but contain different plasmids allowing nodulation in peas (PRL1J1) or beans (PRL2J1), respectively. A34 retained ability to nodulate beans (albeit with delayed nodulation compared to 4292) and was used as the common strain to compare pea and bean host effects. 4292 only nodulates beans and 3841 only nodulates

peas. Hence, these strains were used to measure natural (control) host effects on symbiotic efficiency to compare with that of the common (A34) strain. Peas and beans grew in plastic growth pouches with nitrogen-free Fahreus nutrient media (Fahreus, 1957) using growth conditions previously described (Ratcliff *et al.*, 2008). Plants were not randomized within the growth chamber space, but random plants were chosen for harvest at each time point.

Seeds of peanuts (*Arachis hypogaea* 'Starr') and cowpeas (*Vigna unguiculata* 'California Blackeye') were surface-sterilized and planted in 15-in deep cones with sterile vermiculite:perlite (1:1) mixture. Growth chambers were set at 29° C light 16 h and 22° C dark 8 h. Peanuts and cowpeas were inoculated with *Bradyrhizobium* sp. 32H1, grown in MAG media (Somasegaran & Hoben, 1994). Since peanuts and cowpeas commonly share many strains, we did not include control strains to assess natural host effects on symbiotic efficiency. These plants were also watered with N-free Fahreus media (Fahreus, 1957) and mixed throughout the growth chamber (nonrandomly). Plant individuals were randomly chosen for harvest.

Harvesting Nodules

Peas and beans were grown for 74 days, and four to six plants each were harvested at five different time intervals. Cowpeas and peanuts were harvested intermittently between day 50 and 100. All nodules were harvested from each plant and their total fresh weights were recorded. The host shoots were dried in an oven overnight and weighed. Pea and bean shoots were further processed for nitrogen content using elemental combustion analysis. Pea and bean root weights were estimated from typical

shoot:root ratios estimated from a separate set of peas and beans nodulated with A34 (pea root = 0.25*shoot, $r^2 = 0.47$, $n = 7$, bean root = 0.64*shoot, $r^2 = 0.77$, $n = 11$). For peanuts and cowpeas, actual root weights were used. Nodule fresh weight was measured and dry weights were estimated by regression based on a separate experiment (pea dry nodule weight = 0.17*wet nodule weight, $r^2 = 0.99$, bean dry nodule = 0.18*wet, $r^2 = 0.99$, peanut dry nodule = 0.21*wet, $r^2 = 0.99$, cowpea dry nodule = 0.25, $r^2 = 0.99$).

Comparing Polyhydroxybutyrate (PHB) in bacteroids

We also measured the amount of a carbon storage polymer, polyhydroxybutyrate (PHB), per bacteroid in each host since PHB is known to compete with nitrogen fixation for photosynthates (Trainer & Charles, 2006) references therein). We tested whether there is less PHB in the swollen bacteroids than in the unswollen bacteroids, which might help explain any differences in symbiotic efficiency between hosts, if they are present.

Five to eight random nodules from each host plant were weighed individually and saved for PHB analysis via flow cytometry. Procedures for PHB analysis are described by Ratcliff et al. (2008).

Nodules for H_2:CO_2 Efficiency Measurements

Healthy, mature, pink nodules were harvested and used immediately for measuring H_2 production and CO_2 respiration in nitrogen-free air, using a method adapted from Witty et al. (1998)'s open-flow through system. Witty et al. (1998) showed respiratory cost (mol CO_2/mol ethylene from acetylene reduction by nitrogenase) remained relatively constant with plant age for detached nodules, so nodules of various

ages were pooled as long as they looked healthy. These nodules were detached from plants but still connected to some root fragments in order to minimize wounding or introduction of ambient oxygen into nodule interior. Detached nodules have lower fixation and respiration rates but the relationship between them apparently does not change (Witty & Minchin, 1998). Detached nodules were pooled from two or more individual host plants to obtain detectable levels of H_2 production. Peanut and cowpea plants were acclimated to a cooler growth chamber of 20° C for 24 hours before harvesting at room temperature (20° C) in order to reduce the temperature shock for the nodules.

Productions of H_2 and of CO_2 by nodules (total fresh weight between 0.5 *g* and 1 *g*) were assayed in a flow-through chamber (Fig 3-3a). Argon:O_2 flowed through the nodules from below at a controlled rate (100ml/min). Due to the absence of N_2, 100% of nitrogenase activity went to H_2 production. A subsample of gas from above the nodules was pulled through an H_2 analyzer (Witty & Minchin, 1998) and infrared gas analyzer for CO_2 (Qubit Systems) at a lower flow rate than the supply rate, with excess gas vented (Fig. 3-3a). After equilibrating under 21% O_2 in Argon (Ar), oxygen percentage was increased in steps to 33%. Increments of oxygen increases varied from 1% (v:v, i.e., 1 kPa) every 4 min to 3% every 10 min depending on nodule sensitivity to O_2 (Fig. 3-3b). When external O_2 partial pressure is increased gradually, internal O_2 partial pressure will rise enough to reduce O_2-limitation of nodule interior respiration (increasing nitrogenase activity as well as respiration) but not enough to damage nitrogenase irreversibly (Denison *et al.*, 1992, Witty & Minchin, 1998). Efficiency was calculated from linear regression of nitrogenase activity (H_2 production) on respiration (CO_2 production) (Witty

et al., 1983). If nitrogenase was damaged at high external oxygen concentrations, then when external oxygen was returned back to 21% at the end of the experiment from 33%, there would be a definitive drop in H_2 evolution from the initial 21% reading (Fig. 3-3c). In that case, CO_2 and H_2 values at some high oxygen percentages were not included in the regression (Witty & Minchin, 1998) unless they remained linear. The H_2 sensor was calibrated at 0.5 ppm (ambient air) and at 50 ppm (H_2 standard).

Statistics

In order to test if the effect of nodule weight on plant weight was significantly different between the two strains on the same host species or between two host species with the same strain, controlling for a constant effect of plant age, we compared linear models using a *t*-test in R. Formula: plant weight = $b + m$*(nodule weight) +I(strain:host species combination) + plant age + I(strain or host species)*m*(nodule weight), where I designate indicators for the model, b is the intercept of the model and m is the coefficient on the nodule weight variable or the slope of the linear regression that was estimated. Slopes indicate plant growth per nodule growth, and allow discounting differences in initial seed weights. Fixation efficiencies were compared with unpaired equal variance two-tailed t-test.

RESULTS

Plant return on nodule construction cost

Plant dry weights were always greater per nodule dry weight for the host species with swollen bacteroids (peas and peanuts) than for those with nonswollen ones (beans and cowpeas) when nodulated with the common rhizobial strain. Pea hosts had similar shoot dry weights per nodule wet weight when nodulated by A34 and the control strain, 3841 (Fig. 3-1, Table 3-1). Bean hosts also had similar shoot growth per nodule fresh weight when nodulated with the common A34 strain and the control strain, 4292 (Fig. 3-2, Table 2). Peas grew about five times more than bean plants (with common A34 strain) per nodule weight (10.68 vs. 2.09, Fig. 3-4a, Table 3). Nitrogen percent per aerial mass did not differ significantly between peas and beans (2.7% and 2.5% respectively, $p = 0.18$, $n = 50$). The pea and bean biomasses are lower than expected under field conditions since they were grown in plastic pouches in the growth chamber.

Peanuts grew about three times more than cowpeas (with common 32H1 strain) per nodule dry weight (39.16 vs. 12.62, Fig. 3-4b, Table 2). This three-fold difference in growth regression between peanuts and cowpeas is consistent with results reported for multiple rhizobial strains in previous experiments conducted by Sen & Weaver (5.15 vs 1.5, Fig. 3-4b inset, (Sen & Weaver, 1981), $t = 5.83$, $d.f. = 7$, $p < 0.001$, using t-test on a linear model of plant weight on nodule weight with host species as a factor).

Nitrogen Fixation Efficiency

Nitrogen fixation efficiency, measured by the ratio of H_2 evolution by nitrogenase per CO_2 production by respiration, in nitrogen-free air ($Ar:O_2$), was also higher in peas than with the same rhizobial strain in beans. Pea nodules with strain A34 averaged an efficiency of 0.50 H_2/CO_2 (s.d. ± 0.04) while beans averaged 0.34 H_2/CO_2 (s.d. ± 0.01) over three independent measurements each (Fig. 3-4c). Nitrogen fixation efficiency of peanuts averaged 0.62 H_2/CO_2 (s.d. ± 0.18) while efficiency in cowpeas averaged 0.28 H_2/CO_2 (s.d. ± 0.04) when they were both nodulated with 32H1 (Fig. 3-4d).

H_2 evolution is a by-product of the nitrogenase reaction, with at least 25% of nitrogenase activity going to H_2 production in ambient air. For *R. leguminosarum* A34, there were detectable levels of H_2 produced in air ($N_2:O_2$), which increased three-fold when switched to $Ar:O_2$, indicating that, in air, approximately 33% of nitrogenase activity was used in H_2 production instead of nitrogen fixation. However, when sampling *Bradyrhizobium* sp. 32H1 in either cowpeas or peanuts, we could barely detect any signs of H_2 production when nodules were in $N_2:O_2$, although we could easily detect H_2 once we switched to $Ar:O_2$. Hence, 32H1 may contain hydrogen uptake (hup) enzymes, commonly found among cowpea rhizobia (Martins *et al.*, 1997), which oxidize hydrogen and can recycle it for further nitrogenase activity. If 32H1 expresses hup genes, our measurements of hydrogen production may not be equally proportional to nitrogen fixation in cowpeas and peanuts because different fractions of hydrogen may be recycled depending on host species, e.g. differences in nodule permeability. However, if H_2 uptake is equally saturated in both species by the greater H_2 production in $Ar:O_2$, then presence

of hydrogenase would not affect the slope of the H_2 vs. CO_2 line, used to calculate efficiency.

PHB Accumulation Per Bacteroid

R. leguminosarum A34 bacteroids lacked PHB inside pea nodules (Fig. 3-5), which is a similar result seen in natural pea rhizobia, such as 3184. However, A34 accumulated high levels of PHB in beans (Fig. 3-5), as did 4292 and many other natural bean rhizobia (Lodwig *et al.*, 2005). PHB in swollen bacteroids was analyzed separately from PHB in the undifferentiated cells by distinguishing large and small cells by forward scatter in flow cytometry.

Bradyrhizobium 32H1 bacteroids had low levels of PHB in both cowpeas (0.045 pg ± 0.03 s.d., n = 20 plants) and peanuts (0.03 pg ± 0.02 s.d., n = 14 plants) compared to beans (0.25 pg ± 0.13 s.d., n = 20). PHB in cowpea bacteroids was significantly lower than normally found in closely related legume species with nonswollen bacteroids, *e.g.* bean bacteroids can accumulate up to 0.72 pg/cell, *Macroptilium atropurpureum* (siratro) bacteroids accumulate on average 0.35 pg/cell (Ratcliff et al. *in prep*).

DISCUSSION

Peas grew about five times more per nodule mass than beans with *R. leguminosarum* A34. Peanuts also grew about three times more per nodule mass than cowpeas with *Bradyrhizobium* sp. 32H1. The greater efficiency in peanuts compared to cowpeas can be generalized to other strains that nodulate both species as seen by Sen and

Weaver (1981) (Fig. 3-4b inset), who compared four different effective strains' host to nodule mass ratio, on peanuts, cowpeas and siratro (not shown). The study of Sen & Weaver (1981) shows that the superiority of peanut symbiosis over cowpeas was not restricted to the 32H1 strain. The nitrogen fixation efficiency with the same strains between cowpeas and siratro plants, which both host nonswollen bacteroids, were comparable.

Peas and peanuts also had higher nitrogen fixation efficiency (H_2 production per CO_2 respiration) than beans and cowpeas respectively. It should be noted that the possible presence of hup genes in *Bradyrhizobium* sp. 32H1 may impede accurate measurement of nitrogen fixation rate via H_2 production in peanuts and cowpeas, but a consistent loss to uptake would not affect the slope used to calculate efficiency.

Similar methods have not shown consistent effects of bacteroid differentiation in the past, however. Witty et al. (1983) measured fixation efficiency in twelve different legume genera but there was no discernible difference between those species with terminal differentiation of bacteroids and those without. For example, peas ranged from 2.25-4.52 CO_2 / C_2H_4 whereas beans ranged from 2.65-3.29 CO_2 / C_2H_4 depending on host cultivar and rhizobial strain. They also found no clear difference between cowpeas and peanuts (1.97 CO_2 / C_2H_4 moles per min per nodule weight in cowpeas and 2.08 in peanuts) that were nodulated by the same strain of rhizobia RCR 3824. These comparisons by Witty et al. (1983), among others (Hunt et al. 1989), gave similar values to our results; found 2-3 CO_2 / H_2 assuming a 1 mol of H_2 for 1 mol of C_2H_4 conversion. More strains and cultivars need to be tested in order to determine if the level of bacteroid differentiation is the significant factor influencing symbiotic efficiency. *R.*

leguminosarum A34, however, did not effectively nodulate other bean cultivars, such as 'Early Contender', and the experiment could not be extended. A34 effectively nodulated another pea cultivar, 'Green Arrow', albeit with delayed nodulation. Fixation efficiency of A34 in cv. 'Green Arrow' was even higher than in cv. 'Maestro,' 0.70 H_2/CO_2 (± 0.18 s.d., $n = 3$).

Higher fixation efficiency often correlates with the production of more plant mass relative to nodule mass. Legumes will typically continue to form nodules until they have attained an adequate nitrogen source, i.e. legumes will form many more, but usually smaller, nodules with less-effective rhizobial strains than with more-effective ones. Hence, the construction cost of nodules is much greater for legumes when they do not find effective strains. Peas had fewer nodules per plant (157 nodules per g of plant mass) than beans (414 nodules per g of plant mass) while the average nodule for the two hosts weighed about the same (1.6 mg). However, peanuts had on average a greater number (68 nodules per g of plant mass) of smaller nodules (0.3 mg) per plant than cowpeas (53 nodules per g of plant mass, 1.4mg), which contradicts the normal nodule number and efficiency relationship. This further highlights the difference in peanut nodule development from the other plant hosts.

If terminally-differentiated bacteroids indeed have higher symbiotic efficiency, to what could this be attributed? We hypothesized that if host plants could control PHB accumulation in swollen bacteroids but not in nonswollen ones, this could lead to the differences we saw in fixation efficiency. PHB tends to be absent from bacteroids of peas, and other vicioid legumes with swollen bacteroids, but abundant in nonswollen bean bacteroids. But we did not see significantly less PHB in swollen peanut bacteroids

compared to nonswollen cowpea bacteroids. Plant manipulation of internal biochemical pathways of bacteroid PHB synthesis to increase nitrogen fixation efficiency seems unlikely in peanuts.

Could the swollen morphology of the bacteroids allow increased nitrogen fixation efficiency? When bacteroids are reproductive, there are multiple bacteroids per peribacteroid membrane, which may lead to some bacteroids not having any contact with host cells, essentially creating a metabolically wasteful void in the middle of a symbiosome. Single swollen bacteroids per symbiosome might also increase energy efficiency for nutrient transport across rhizobia-plant membranes. Y-shaped bacteroids may have polar-localization allowing partitioning of metabolic functions in different areas of the cytoplasm (Young 2006), which might increase fixation efficiency Terminally-differentiated bacteroids are also known to have genomic endoreduplication and their genomic size can be commonly observed as 4C (Oono *et al.*, 2010). Genomically-enhanced rhizobia may have more efficient metabolism or higher levels of nitrogenase activity.

The terminal differentiation of bacteroids has been shown to be mediated by plant factors known as nodule-specific cysteine-rich peptides (Van de Velde *et al.*, 2010). These peptides interfere with normal rhizobial cell division (cytokinensis) once inside the symbiosome, which leads to a single bacteroid per host symbiosome. This also can lead to increased rhizobial genome copies per cell if DNA synthesis was already occurring. The cells typically become larger because daughter cells cannot split off from each other during cytokinesis. This tightly links the three characteristics of 1) genomic

endoreduplication, 2) single bacteroid per host cell and 3) swelling, making it difficult to assess which characteristic might be most beneficial for the host.

Further investigation of the biochemistry and genetics of legume host species imposing terminal bacteroid differentiation may allow us to modify other host species to have higher nitrogen fixation efficiency. Terminally-differentiated bacteroids are currently not the dominant trait among legumes (Oono *et al.*, 2010). This may be because of certain trade-offs depending on its environmental conditions, much like C4 photosynthesis has a greater carbon fixation efficiency than the dominant C3 systems, but only under certain environmental conditions.

As many more non-model legume species are explored, other unique symbiotic interactions are expected to be discovered, e.g. rhizobial strains that produce plant hormones that manipulate resource allocation to nodules (Ratcliff & Denison, 2009). As the underlying genetic mechanism for terminal differentiation unfolds (Van de Velde *et al.*, 2010), it will become possible to conduct comparative genetics on independently arising lineages of hosts with terminal differentiation and understand selection pressures and evolution of these underlying genes. Transcription profiles of swollen and nonswollen bacteroids may also reveal crucial differences between the two phenotypes that ultimately determine how terminally differentiated bacteroids could be more efficient.

FIGURES

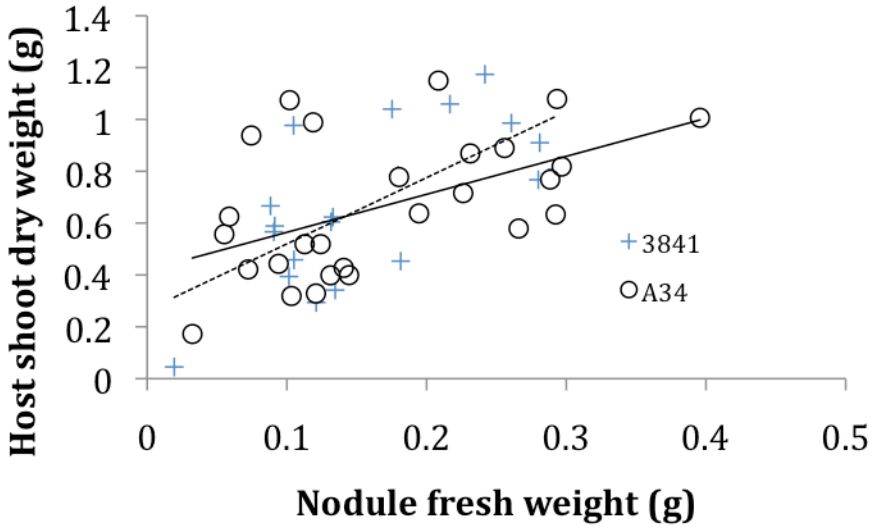

Figure 3-1. Pea plants nodulated by *R. leguminosarum* A34 and 3841 across four harvest dates (four plants each for 3841 and six plants each for A34). Dashed line indicates regression for strain 3841. Solid line indicates regression for strain A34. Nodule weight effect on plant weights was not signficantly different between rhizobial strains, controlling for constant linear effect of plant age (Table 1).

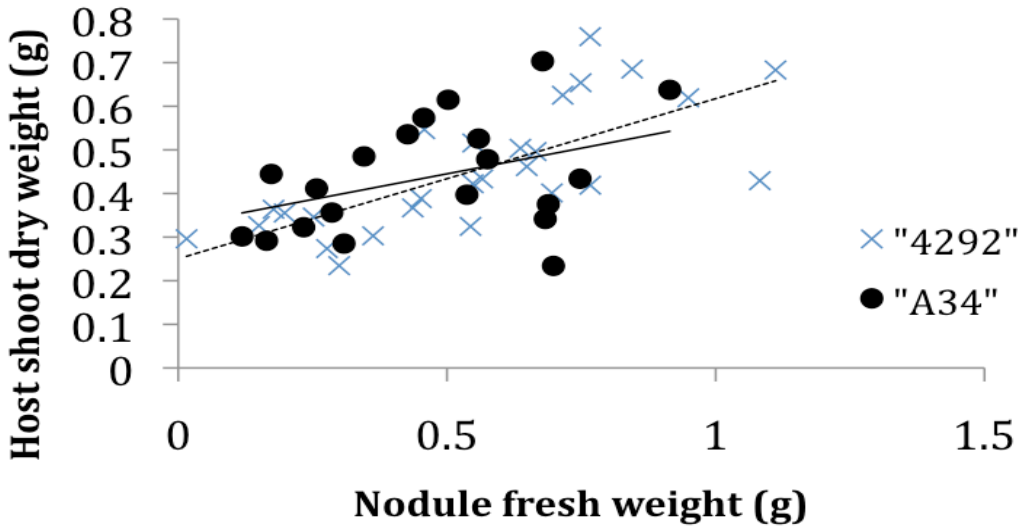

Figure 3-2. Bean plants nodulated by *R. leguminosarum* A34 and 4292 across five harvest dates (four plants each). Dashed line indicates regression for strain 4292. Solid line indicates regression for strain A34. Nodule weight effect on plant weights was not signficantly different between rhizobial strains, controlling for constant linear effect of plant age (Table 1).

Bean/Pea	Estimate	Std. Error	t-value	Pr (>|t|)
Nodule weight	1.54	0.33	4.62	1.3 e^{-5} ***
Plant age	0.006	0.001	5.03	2.7 e^{-6} ***
Nodule weight *A34:bean	-1.43	0.37	4.36	0.0002***
Nodule weight * 4292:bean	-1.21	0.35	7.27	0.0009***
Nodule weight * 3841:pea	0.83	0.58	1.44	0.15

Table 3-1. T-test on linear model controlling for a constant effect of plant age with all strain:host combinations as factors, using pea plants nodulated by *R. leguminosarum* A34 as baseline comparison. Nodule weights of pea plants nodulated by *R. leguminosarum* A34 have a significant effect on plant weight (first line). Plant age also has an effect on plant weight (second line). The effect of nodule weight on plant weight does not significantly differ between the two strains (3841 and A34) on pea plants, controlling for a constant effect of harvest date (fifth line). However, the effect of nodule weight on plant weight is significantly different between pea plants nodulated by A34 and bean plants nodulated by A34 (third line), as well as bean plants nodulated by 4292 (fourth line). This same test was rerun using bean plants nodulated by A34 as baseline, which showed that nodule weight effect on plant weight did not significantly differ between bean plants nodulated by A34 and 4292.

Peanut/Cowpea	Estimate	Std. Error	t-value	Pr (>|t|)
Nodule weight	11.12	3.71	3.00	0.007 **
Plant age	0.01	0.01	1.09	0.29
Nodule weight * peanut	25.14	5.34	4.71	0.0001***

Table 3-2. T-test on linear model controlling for a constant effect of plant age, using cowpea plants nodulated by *Bradyrhizobium* sp. 32H1 as baseline comparison. Nodule weights of cowpea plants nodulated by *Bradyrhizobium* sp. 32H1 have a significant effect on plant weight. The effect of nodule weight on plant weight was significantly different between the two host species, controlling for a constant linear effect of plant age. Nodule weights of peanut plants nodulated by *Bradyrhizobium* sp. 32H1 also have a significant effect on plant weight when rerunning the model with peanuts as baseline comparison.

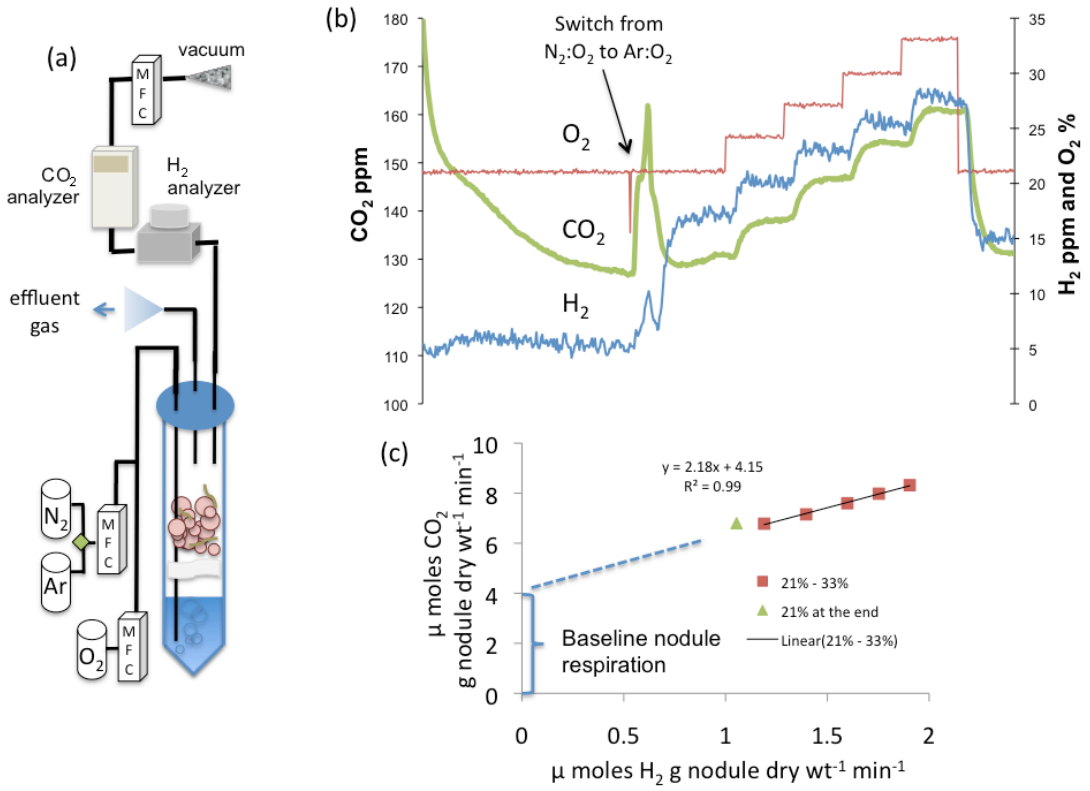

Figure 3-3. Method for measuring nitrogen fixation efficiency. (a) Pure compressed N_2 and Ar were directed to a two-way valve into one mass flow controller (MFC). Pure compressed O_2 was connected to another MFC. Tubes from the two MFC mixed O_2 with either N_2 or Ar before entering the nodule-containing tube. The bottom of the tube contained water to humidify the gas mixture before affecting the nodules, which were suspended in the middle of the tube with tissues. Gas samples were taken from the top of the nodules and directed to an H_2 analyzer and an IRGA monitor. (b) Increasing external oxygen step-wise from 21% in a nitrogen-free atmosphere (argon: oxygen mixture) raises respiration and nitrogenase activity. Nitrogen fixation is measured by hydrogen production. (c) Slope of H_2:CO_2 regression line defines efficiency for nitrogen fixation while CO_2 intercept defines baseline respiration for nodules and rhizobia in the tube.

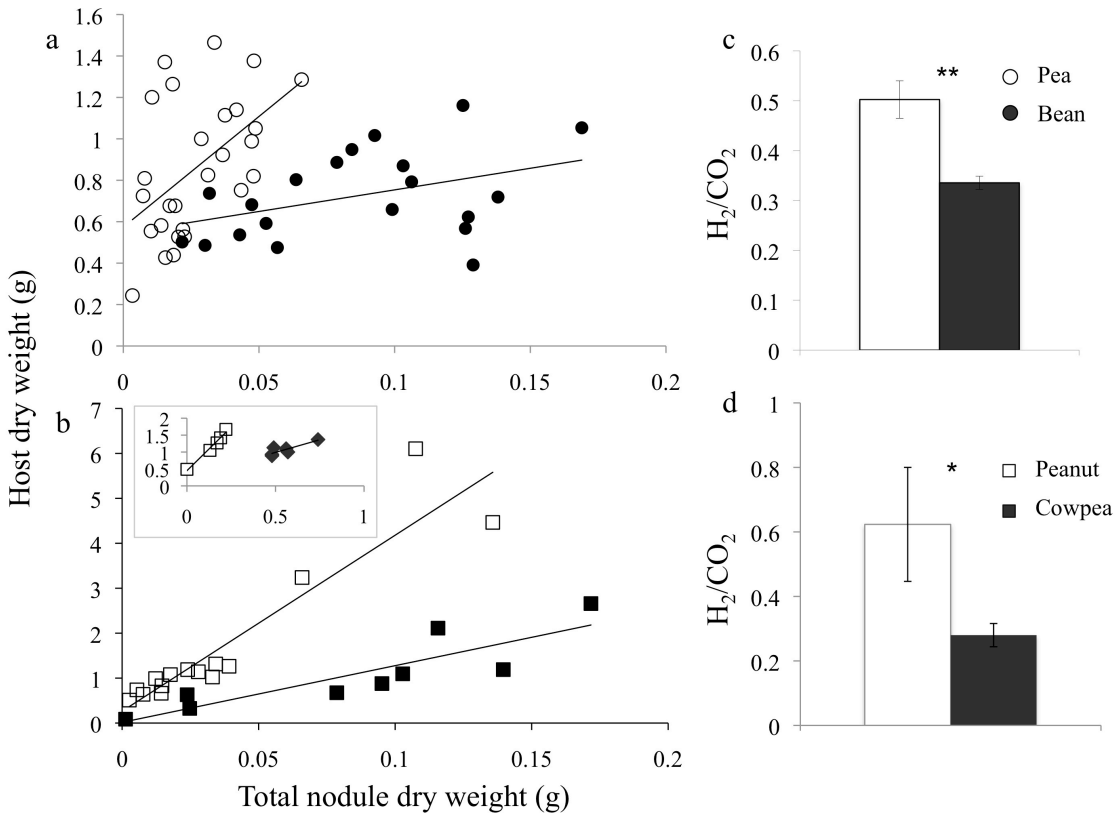

Figure 3-4. Peas (open circles) and peanuts (open squares) grow more per nodule mass as well as have greater fixation efficiency than beans (closed circles) and cowpeas (closed squares) respectively when inoculated by common rhizobial strain. (a) Pea biomass (including shoots and roots) increased greater per nodule biomass than beans ($p < 0.001$). (b) Peanut biomass greater than for cowpeas ($p < 0.001$). (b inset) Peanut plant mass grows about three times greater than cowpeas with other rhizobia strains ($p < 0.001$). (c) Nitrogen fixation efficiency of rhizobia inside peas (0.50 H_2/CO_2) are greater than in beans (0.33 H_2/CO_2) and (d) peanuts (0.62 H_2/CO_2) are greater than cowpeas (0.28 H_2/CO_2). Pea vs bean ($p < 0.01$, $n = 3$), peanut vs cowpea ($p < 0.05$, $n = 3$), error bars are one standard deviation.

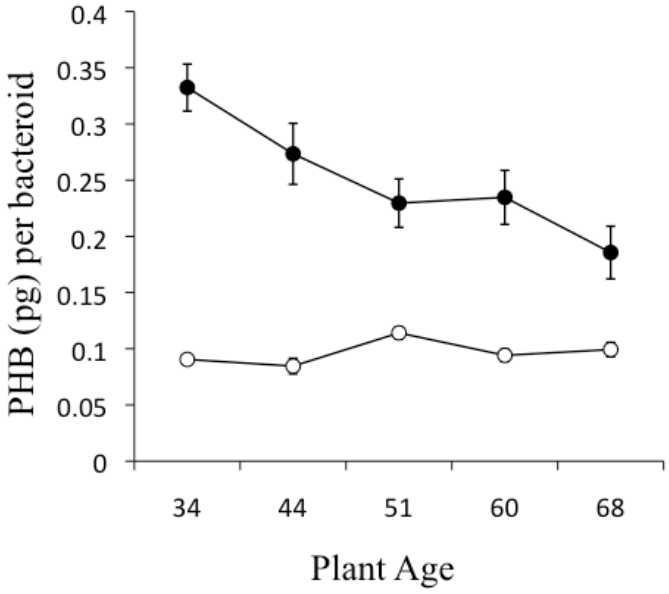

Figure 3-5. PHB per bacteroid in peas (open circles) and beans (closed circles) of *R.leguminosarum* A34 over plant age. Eight nodules were sampled from four to five plants at each time period. Error bars indicate one standard deviation.

Chapter 4: Failure to fix nitrogen (N_2) by nonreproductive symbiotic rhizobia triggers fitness-reducing sanctions against their reproductive clonemates in two legume host species

The legume-rhizobia symbiosis is a classical mutualism where fixed carbon and nitrogen are exchanged between the species. However, within root nodules of certain legume species, some rhizobia differentiate into nonreproductive nitrogen-fixing bacteroids. Rhizobial cells that have yet to differentiate into bacteroids inside such nodules can still go back into the soil and produce the next generation of symbiotic rhizobia. The limited cheating options available to nonreproductive bacteroids might dispel the conflict of interest that reproductive bacteroids otherwise have with their host plants. Host sanctions were therefore tested in three legume species that host nonreproductive bacteroids to see if sanctions were less stringent than in hosts with reproductive bacteroids. We demonstrate that even legume species that host nonreproductive bacteroids can severely sanction the undifferentiated rhizobia within the same nodule. Hence, host sanctions play a role in maintaining nitrogen fixation by a diverse set of legumes, but other mechanisms of selection, such as pre-infection partner choice, may still play a role in stabilizing the mutualism.

1. INTRODUCTION

Mutualism is a cooperative relationship between different species that is ubiquitous and can also be ancient (Herre *et al.*, 1999). "Cheaters", which have been

defined as individuals or genotypes that cooperate less while benefiting from the cooperation of others (West *et al.*, 2007, Kiers & Denison, 2008), have been reported in many mutualisms (Douglas, 2008). One of the major questions in evolutionary biology is why ancient mutualisms have not been broken down by cheating (Sachs *et al.*, 2004, Sachs & Simms, 2006). Theoretically, cheaters gain the benefits of mutualism without paying the costs and potentially have a competitive advantage over mutualists. Mutualistic interactions could be stabilized if each partner imposes fitness-reducing sanctions on cheaters (Kiers *et al.*, 2003, Simms *et al.*, 2006, Jander & Herre, 2010) or if each partner could choose not to form any relationships with cheaters (Mueller *et al.*, 2004, Heath & Tiffin, 2009, Gubry-Rangin *et al.*, 2010). In the legume-rhizobia symbiosis, a classic mutualism model where fixed carbon and nitrogen are exchanged between the species, both types of mechanisms of stabilization have been demonstrated (Kiers *et al.*, 2003, Simms *et al.*, 2006, Heath & Tiffin, 2009) but it is still debated if these mechanisms are universal, i.e. found in all legume host species (Oono *et al.*, 2009, Gubry-Rangin *et al.*, 2010).

Rhizobia are facultative mutualists that fix nitrogen (N_2) and reproduce inside legume nodules, both of which are metabolically costly processes that can compete with each other for energy resources. A legume plant associates with multiple horizontally-transmitted rhizobial genotypes (Bailly *et al.*, 2006) with high variability in N_2-fixation among strains (Burdon *et al.*, 1999). Since the different rhizobial strains on a given host are potential competitors once back in the soil, this could lead to a tragedy of the commons. The potential tragedy is that every symbiotic rhizobia could increase their benefits if they all fixed N_2 and contributed to a healthier host, but a single cheating strain

could obtain even greater benefits in the short-term if it selfishly reaped the benefits without paying the cost of N_2-fixation. But what if there is no obvious way for rhizobia to benefit from fixing less nitrogen? Does the tragedy of the commons still exist?

Inside nodules of legume species like peas (*Pisum sativum*), alfalfa (*Medicago sativa*), and peanuts (*Arachis hypogaea*), there are plant compounds that transform rhizobia into swollen cells. These larger cells are the bacteroids, which fix nitrogen for the plants but cannot reproduce anymore even once back in the soil (Van de Velde *et al.*, 2010). Some rhizobial cells remain undifferentiated within the nodule, acting as a germline for additional bacteroids, as well as for soil populations that may nodulate the next generation of plants. These undifferentiated rhizobial cells, however, cannot fix N_2. Inside nodules of other legume host species, like soybeans (*Glycine max*) or some wild lupines (*Lupinus arboreus*), bacteroid differentiation is not as morphologically extreme and the bacteroids are not swollen and remain reproductive. These reproductive bacteroids pay an opportunity cost when fixing N_2 because the carbon received from plants could otherwise be used for bacteroid reproduction or stored up for future reproduction once in the soil. However, it is unclear whether nonreproductive bacteroids can increase their inclusive fitness by fixing less N_2 (Oono *et al.*, 2009), i.e., whether there is any opportunity cost for fixation.

Host sanctions, defined as the ability of a host plant to discriminate among rhizobia based on their fixation benefits and to impose selection for more-beneficial rhizobia, have been demonstrated in legume species that do not induce terminal differentiation of their bacteroids: soybeans (*Glycine max*) by Kiers et al. (2003) and wild lupines (*Lupinus arboreus*) by Simms et al. (2006). Kiers et al. (2003) simulated cheaters

by reducing atmospheric N_2 around nodules to near zero, thereby preventing genotypically identical rhizobia from fixing N_2. They found about 50% less rhizobia per nonfixing nodule compared to controls. Simms et al. (2006) inoculated hosts with multiple rhizobial strains with varying levels of N_2-fixation and found that nodules occupied by the poor-fixing strain were smaller. On the other hand, a study using *Medicago truncatula,* a host species that induces terminal differentiation of bacteroids, found no effects of host sanctions, based on a lack of significant difference in nodule size among strains differing in benefits to the host (Heath & Tiffin, 2009). However, pre-infection partner choice appeared to play a role in stabilizing mutualism for *M. truncatula*, based on plants forming more nodules with the more-beneficial strains, which was also found in another study by Gubry-Rangin et al. (2010). This latter study also concluded that there were no host sanctions in *M. truncatula*, based on no difference in number of viable (undifferentiated) rhizobia per nodule between fixing and nonfixing strains, rather than based on nodule size or weight data. In contrast to Heath and Tiffin (2009), however, they did find greater growth of nodule biomass with the fixing strain, and concluded that the host exercises post-infection partner choice. Both studies found evidence for pre-infection partner choice and none for the reduced rhizobial fitness expected with host sanctions (albeit the use of different methods).

Pre-infection partner choice, the ability for host legumes to preferentially nodulate with rhizobia having greater cooperative benefits, seems a theoretically unlikely mechanism for stabilizing rhizobial mutualism because rhizobia have a much shorter generation time and can evolve faster than their hosts. The opportunity to reproduce inside nodules would impose strong selection for signals (nod factors) that allow them to

nodulate plants, regardless of their ability to fix N_2. On the other hand, if lower N_2-fixation does not increase the inclusive fitness of nonreproductive bacteroids, then these hosts may rarely face problems with cheaters that break down the mutualism. Even if nonreproductive bacteroids have no way to increase their inclusive fitness at the expense of N_2-fixation, higher fixation would still be selected because it would increase the total benefits allocated (perhaps equally in the absence of host sanctions) among all rhizobia. The interests of rhizobia and host plants would then be aligned, diminishing the tragedy of the commons and the need for host sanctions. This could lead to weakened selection for mechanisms of host sanctions. There would still be some small selection for hosts, however, to have mechanisms for aborting or inducing early senescence of nodules containing less-beneficial rhizobia, which can be present in nature even if there is no greater benefit from lower fixation for these rhizobia. We assume that legume evolution has been shaped by the fitness benefits of reducing losses to less-mutualistic rhizobia, with rhizobial fitness changes being mere side effects.

Furthermore, even nonreproductive bacteroids may have some cheating options. A few rhizobial strains that associate with hosts imposing terminal differentiation of bacteroids have the ability to synthesize rhizopines, nutritional inositol compounds synthesized by bacteroids and catabolized by undifferentiated rhizobia (Wexler *et al.*, 1995). Rhizopine synthesis might divert carbon use from N_2-fixation and may be an adaptive cheating strategy to terminal differentiation if it allows nonreproductive bacteroids to increase their inclusive fitness.

In this study, we measure rhizobial fitness directly in order to assess the evolution of cooperative and cheating behavior when bacteroids are nonreproductive. Since

genotypically distinct strains (even isogenic ones) can have pleiotropic differences that may interfere with nodulation (Gordon *et al.*, 1996) or bacteroid development (Yarosh *et al.*, 1989), we adapt the methods of Kiers et al. (2003) in eliminating N_2 gas from one half of a plant's nodulated roots and comparing the rhizobial fitness of a single genotype between the two sides. We also compared the amount of energy-rich polyhydroxybutyrate (PHB) in the reproductive clonemates of fixing and nonfixing bacteroids, a resource that could be crucial for rhizobial fitness (Ratcliff *et al.*, 2008).

2. MATERIALS AND METHODS

(a) Plant growth conditions and rhizobial inoculum

Seeds of peas (*Pisum sativum* cv. 'Green Arrow', Henry Field's Seed & Nursery Co., Aurora, IN 47001), peanuts (*Arachis hypogaea* cv. 'Starr', Texas AgriLife Research and Extension Center, Lubbock, TX 79403 (Mark Burow)), alfalfa (*Medicago sativa* 'ARC', University of Minnesota, Department of Agronomy and Plant Genetics, St. Paul, MN 55108 (Keith Henjum)), were surface-sterilized with 0.09% hydrogen peroxide for 3 minutes, rinsed with sterile deionized water and planted in plastic pouches containing nitrogen-free Fahreus nutrient media (Fahraeus, 1957).

Between four and seven days after germination, the main seedling roots were cut one to two centimeters below the cotyledons to allow even formation of lateral roots between the two halves of the pouches. After development of split-roots, legumes were inoculated with 1 ml (approximately 10^9 cells) of stationary phase rhizobial inoculum on each half of their roots. Peas were inoculated with *Rhizobium leguminosarum* A34,

peanuts with *Bradyrhizobium* sp. 32H1 (=USDA3384), and alfalfa with *Sinorhizobium meliloti* MP6. *R. leguminosarum* and *S. meliloti* were grown in tryptone yeast media with antibiotics (500 μg/ml streptomycin for A34 and 400 μg/ml streptomycin and 6 μg/ml tetracycline for MP6). *Bradyrhizobium* sp. was grown in modified arabinose glutamate media (Somasegaran & Hoben, 1994).

(b) Nitrogen (N_2)-free gas treatment

Split-root plants inoculated in pouches were transferred into glass chambers with silicone-dividers between root halves (Kiers *et al.*, 2003). A mass-flow control system mixed pure argon (Ar) and oxygen (O_2) gases, approximately 79:21 ratio. This N_2-free Ar:O_2 mixture were introduced into one half of each chamber (randomly chosen) while pure air was introduced into the other half at a slightly lower flow rate, to prevent leakage of N_2 into the N_2-free side. Gases were introduced near the bottom of each side (approximately 75 ml in volume) and flowed out around the plant stem. Nodules of comparable sizes were identified from each side before treatments were assigned. Initial pea nodule length range was 1.0 – 2.3 mm for younger nodules and 2.0 – 3.9 mm for older nodules, initial alfalfa nodules ranged from 1.0 – 2.0 mm, and initial peanut nodule diameters ranged from 1.2 – 1.8 mm. These nodules were harvested after ten days of gas treatments. Flow rates on the argon side ranged from 60ml/min for peas and peanuts to conserve gas, but increased to 90ml/min for alfalfa to prevent incursion of atmospheric N_2.

(c) Rhizobia fitness assessment and flow cytometry

For peas and alfalfa, nodules were harvested and rinsed with sterile deionized water three times before being crushed in ascorbic acid buffer (Arrese-Igor *et al.*, 1992). There was no surface-sterilization performed on the nodules since in the past, this led to low colony counts on antibiotic media compared to counts from flow cytometry. *S. meliloti* MP6 and *R. leguminosarum* A34 could be accurately counted on media containing strain-specific antibiotics with serial dilution. For peanuts, the minority of fast-growing nodule-surface contaminants (mostly fungal) swamped internal rhizobia in many plate counts since there were no antibiotics in the media. Hence, the viable rhizobia were counted by estimating number of small (i.e. undifferentiated) rhizobia via flow cytometry. Flow cytometric counts may include non-rhizobial cells, but are typically correlated to rhizobial cells (Ratcliff *et al.*, 2008).

Nodule extracts (both bacteroids and undifferentiated rhizobia) were stained with Nile red and analyzed for mean PHB (pg) per undifferentiated rhizobial cell in the flow cytometer Smaller undifferentitaed rhizobia could be distinguished from larger swollen bacteroids because they have lower forward and side scatter with the flow cytometer.

(d) Statistics

Number of undifferentiated rhizobia per nodule, PHB (pg) per undifferentiated rhizobial cell, and nodule fresh weights were compared between argon and control treatments using a two-tailed paired t-test. Regressions of nodule weight to viable

rhizobia per nodule were compared between treatments using Student's *t* in R. We tested to see if these regressions had significant positive slopes using an *F*-test in R.

3. RESULTS

Sanctions in peanuts

Results for peanuts were similar to those for pea and alfalfa, but most apparent differences were not statistically significant. On average, peanut nodules on the N_2-free side decreased in size (-.15mm) while nodules on the air side only increased by 0.05 mm ($p = 0.13$, $n = 6$). Nodule weights were not statistically different (2.9 mg in air vs. 2.1 mg in Ar:O_2, $p = 0.16$). Apparent difference in number of undifferentiated rhizobia per nodule was also not statistically significant (6.5×10^5 cells per nodule in air vs 1.8×10^5 cells per nodule in Ar:O_2, $p = 0.30$) due to high variability. PHB per undifferentiated rhizobial cell were not different (both had 0.07 pg of PHB, $p = 0.66$). Nodule weights (both argon and control treatment put together) were weakly correlated with number of undifferentiated rhizobia per nodule ($r^2 = 0.08$) with a positive slope (7.1×10^7 cells (g nodule)$^{-1}$, $p < 0.05$).

Sanctions in peas

After ten days in N_2-free atmosphere (Ar:O_2), pea nodules were visibly senescing compared to control treatment nodules (Fig. 4-1a, b). Plate counting revealed young nonfixing nodules contained only 25% as many undifferentiated rhizobial cells as fixing

nodules while old nonfixing nodules contained only 10% (Fig. 4-2a). Final nodule weights were significantly different between the treatments in younger nodules but not in older ones (Fig. 4-2c). There was a significant positive relationship between number of viable rhizobia and nodule weight among fixing nodules ($r^2 = 0.56$, 6.6 x 10^8 cells g^{-1}, $p < 0.0001$) and a much weaker positive relationship for the nonfixing nodules ($r^2 = 0.04$, 7.1 x 10^7 cells g^{-1}, $p < 0.05$). The slopes were significantly different between the two treatments (Fig. 4-3, *d.f.* = 187, $t = 2.18$, $p < 0.05$).

PHB per undifferentiated rhizobial cell was analyzed by flow cytometry. There was no significant difference in PHB between treatments for younger nodules at the end of the experiment, but in older nodules, PHB per undifferentiated rhizobial cell was 70% greater in the N_2-fixing control than in nodules in the N_2-free Ar:O_2 treatment.

Sanctions in alfalfa

Alfalfa nodules in N_2-free air also contained fewer viable rhizobia per nodule (27% of controls) and had lower nodule fresh weights than control nodules (Fig. 4-4a, c) after ten days. Nodule weights were weakly correlated with number of viable rhizobia per nodule ($r^2 = 0.17$ for argon and $r^2 = 0.38$ for control) and had significantly positive slopes for both treatments (1.0 x 10^9 cells (g of nodule)$^{-1}$ control, $p < 0.0001$, and 9.3 x 10^8 cells (g of nodule)$^{-1}$ for argon, $p < 0.0001$). Unlike pea nodules, these regressions of viable rhizobia to nodule weights were not different between treatments for alfalfa (*d.f.* = 134, $t = 0.34$, $p = 0.73$). PHB per undifferentiated rhizobial cell per nodule was greater in the nodules in N_2-free Ar:O_2 than in N_2-fixing nodules in air (Fig. 4-4b), contrary to results from peas.

4. DISCUSSION

Rhizobia in nodules of some legume species, including peas, alfalfa, and peanuts, apparently lose the ability to reproduce when they become N_2-fixing bacteroids. A fraction of the rhizobia (clonally identical to the bacteroids) within the same nodule remains undifferentiated and reproductive. Previous work suggests hosts cannot sanction or limit increase of undifferentiated reproductive rhizobia per nodule in nodules fixing less nitrogen (Heath & Tiffin, 2009, Gubry-Rangin *et al.*, 2010). This is in contrast to results for soybean or lupines whose nodules only contain reproductive bacteroids. Our results provide clear evidence that even host species with terminally-differentiated bacteroids, like peas and alfalfa, can impose such sanctions, at least when rhizobia fix almost no N_2.

As discussed above, Heath and Tiffin (2009) found little evidence for fitness-reducing host sanctions in *Medicago truncatula*, based on comparisons of nodule size among strains with relatively small differences in performance. Another experiment with *M. truncatula*, comparing strains with larger differences in performance, found that nodules containing the higher-fixing strain were significantly larger, more than twice the weight of nodules with an inferior strain (Gubry-Rangin *et al.*, 2010). Curiously, this difference, which the authors referred to as "post-infection partner choice", was not reflected in differences in plate counts of viable rhizobia per nodule. In this current study, we imposed large differences in N_2-fixation using an $Ar:O_2$ mixture to essentially

eliminate N_2-fixation. We found strong evidence for host sanctions in both peas and alfalfa by measuring reproductive rhizobia per nodule using plate counts. We also found weak evidence for sanctions in peanuts by measuring reproductive rhizobia per nodule indirectly with flow cytometry. Hence, mutualism in nonreproductive bacteroids can be facilitated by kin selection, via the exposure of their undifferentiated clonemates to host plant sanctions.

A lack of evidence for host plants to impose fitness-reducing sanctions when there are only small differences in N_2 fixation among treatments or strains (Heath & Tiffin, 2009) is consistent with previous results for soybean (Kiers *et al.*, 2006). The results of Gubry-Rangin et al. (2010) are harder to understand, particularly the effect on nodule weight without an apparent effect on the number of reproductive rhizobia inside. We found that nodule weight and the number of undifferentiated rhizobia per nodule had a positive relationship for all host species. Positive correlations between nodule weight and number of viable rhizobia per nodule have been found in other studies, including indeterminate nodules of *Medicago truncatula* (Heath & Tiffin, 2007), and *Lupinus arboreus* (Simms *et al.*, 2006).

While nodule weight is typically positively correlated with viable rhizobia (of any strain) per nodule, its regression slope could be different between fixing and nonfixing rhizobia (Fig. 4-3), suggesting nodule size or weight does not fully reflect rhizobial fitness of different rhizobial phenotypes. In peas, we found fewer reproductive rhizobia per nodule mass in nonfixing than fixing nodules, but there are also examples where there were more rhizobia per nodule mass in nodules that fixed less N_2 (Sachs *et al.*, 2010) or greater nodule mass for rhizobial strains that fix relatively little N_2 (Laguerre *et al.*,

2007). When older pea nodules were forced to stop fixing N_2, we did not see a significant difference in nodule weight compared to nodules that continued to fix N_2 for the next ten days (Fig. 4-2c). Nodules may have stopped growing at that stage, but the number of undifferentiated rhizobia within the nodules clearly changed (Fig. 4-2a). Some rhizobia may stop fixing N_2 earlier than others (Cevallos *et al.*, 1996) in order to reproduce or hoard PHB while others continue to fix nitrogen, a possible cheating phenotype. If total duration of nitrogen fixation varies among rhizobia, final nodule weights may not allow detection of sanctions since nodule weights only marginally change at later stages but rhizobial numbers can change significantly.

Similarly, PHB per rhizobial cell cannot be detected from nodule weight (Ratcliff et al. *in prep*), but could significantly contribute to fitness differences among rhizobia (Ratcliff *et al.*, 2008). PHB amounts per undifferentiated rhizobial cell were not significantly different between fixing and nonfixing nodules in most cases. In older pea nodules, fixing nodules had slightly more PHB per undifferentiated cell than nonfixing nodules. This relationship was the opposite for alfalfa, an interesting contrast that warrants further investigation. In both peas and alfalfa, we detected less than 0.1 pg of PHB per cell, which may not have significant consequences for survival. At least 0.1 pg of PHB per cell is necessary to fuel any reproduction (Ratcliff *et al.*, 2008), and therefore, we doubt that the differences we detected in PHB would overturn the fitness disadvantage we measured in terms of cell numbers.

We detected differences in the regressions for viable rhizobia per nodule vs nodule weight between fixing and nonfixing strains in peas but not in alfalfa. This could be a result of differences in the architecture of infection threads between the two species

as well as differences in meristematic growth. Pea nodules tend to grow longer and fewer of them make branches (Fig. 4-1a). Alfalfa nodules tend to grow more branches and become thicker on the meristematic ends as they become older (Fig. 4-1c), while pea nodules tend to become fat around the middle of the nodule. Hence, nonfixing pea nodules may have grown slightly more in the bacteroid region (increasing nodule weight) compared to the meristematic region (where the undifferentiated rhizobia reside). Nonfixing alfalfa nodules may have grown slightly only in meristematic regions (increasing viable rhizobial cells as well as nodule weight). It is also possible that rhizobia in nonfixing pea nodules died during the treatment, a possible host sanction strategy not necessarily linked to simply decreasing resources or oxygen permeability to that nodule (Kiers *et al.*, 2003). It is unlikely that rhizobia inside a nodule would starve to death during a ten day treatment, but perhaps some plant factors may have acted as an antibiotic within the nodule. Killing rhizobia, rather than just cutting off resources, could be a valuable legume adaptation if it gives the plant access to amino acids etc. in the rhizobia.

While it is now clear that host sanctions are possible even in legume species that impose terminal differentiation of bacteroids, it is not understood whether they play an equal or greater role than pre-infection partner choice in maintaining the mutualism. In alfalfa, we found significant sanction effects on the viable rhizobia population per nodule, but this was only true when we used an $Ar:O_2$ flow rate (90ml/min) great enough to thoroughly flush N_2 from that side of the chamber. In previous alfalfa experiments where the $Ar:O_2$ flow rate was equal to that which was sufficient to trigger sanctions in pea (60ml/min), we did not find strong host sanction effects. Note, our experimental flow

rates are lower than previously used to test sanctions in soybeans (130ml/min, Kiers et al. 2003). Our experience suggests that alfalfa imposes sanctions only on strains that fix almost no N_2. This would explain results by Heath et al. (2009) who did not find any evidence for sanctions against naturally occurring rhizobial strains, which all fixed N_2 to some extent. While Gubry-Rangin et al. (2010) found no evidence for sanctions as measured by rhizobial plate counts in one fix$^-$ strain (STM 5472), they did find differences in nodule growth, which was the criterion Heath and Tiffin (2009) used to infer a lack of sanctions against the strains they tested. There have also been studies showing sanctions (smaller nodules containing few viable rhizobia) against a fix$^-$ strain due to a single insertion-mutation in the nifD gene (Maren Friesen, *pers. comm.*).

Pre-infection partner choice favoring more-beneficial rhizobia may play an essential role in maintaining the quality of rhizobia if legumes can evolve fast enough to counter the evolution of good-strain recognition signals in less-beneficial rhizobia. There should be further studies that investigate the biochemical mechanisms for pre-infection partner choice.

Decreased nodule O_2 permeability appears to be involved in sanctions in soybean (Kiers *et al.*, 2003), but we did not investigate this for our nonfixing nodules. Blocking N_2-fixation, however, causes a decrease in nodule gas permeability in many legume species, including peas (Witty *et al.*, 1984). Since undifferentiated rhizobia are spatially segregated from bacteroids, whole-nodule sanction appears to be operating. This is easier to understand than a host cutting off resources specifically to reproductive rhizobia in response to the behavior of nonreproductive bacteroids.

By inducing terminal differentiation of bacteroids, plants may reduce conflict with symbionts, but it appears that peas and alfalfa can nonetheless sanction their rhizobia more severely than soybeans in terms of relative viable cell numbers per nodule. It remains to be seen whether intermediate levels of fixation are sanctioned in these hosts and whether there are ways in which nonreproductive bacteroids can effectively cheat their hosts.

FIGURES

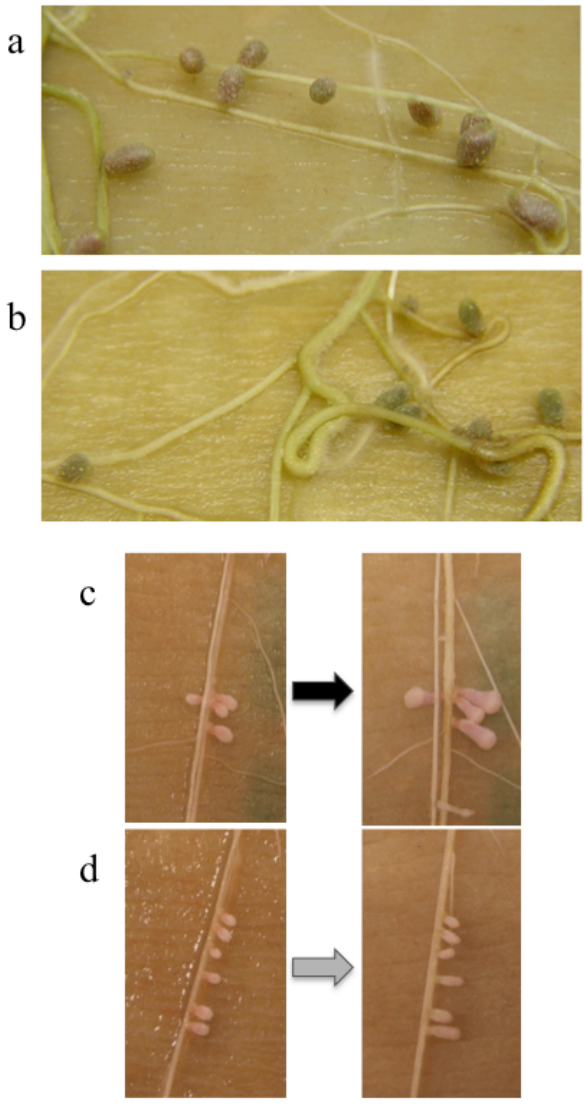

Figure 4-1. Split-root experiments of peas and alfalfa, half of nodulated roots were exposed to a Ar:O_2 (79:21) mixture for ten days while the other half received purified air. (a) N_2-fixing pea nodules after the treatment were pinkish-red while (b) non-fixing nodules on argon side were green and senescing. (c) Alfalfa nodules before and after air treatment. (d) Alfalfa nodules before and after argon treatment from the same host plant as (c).

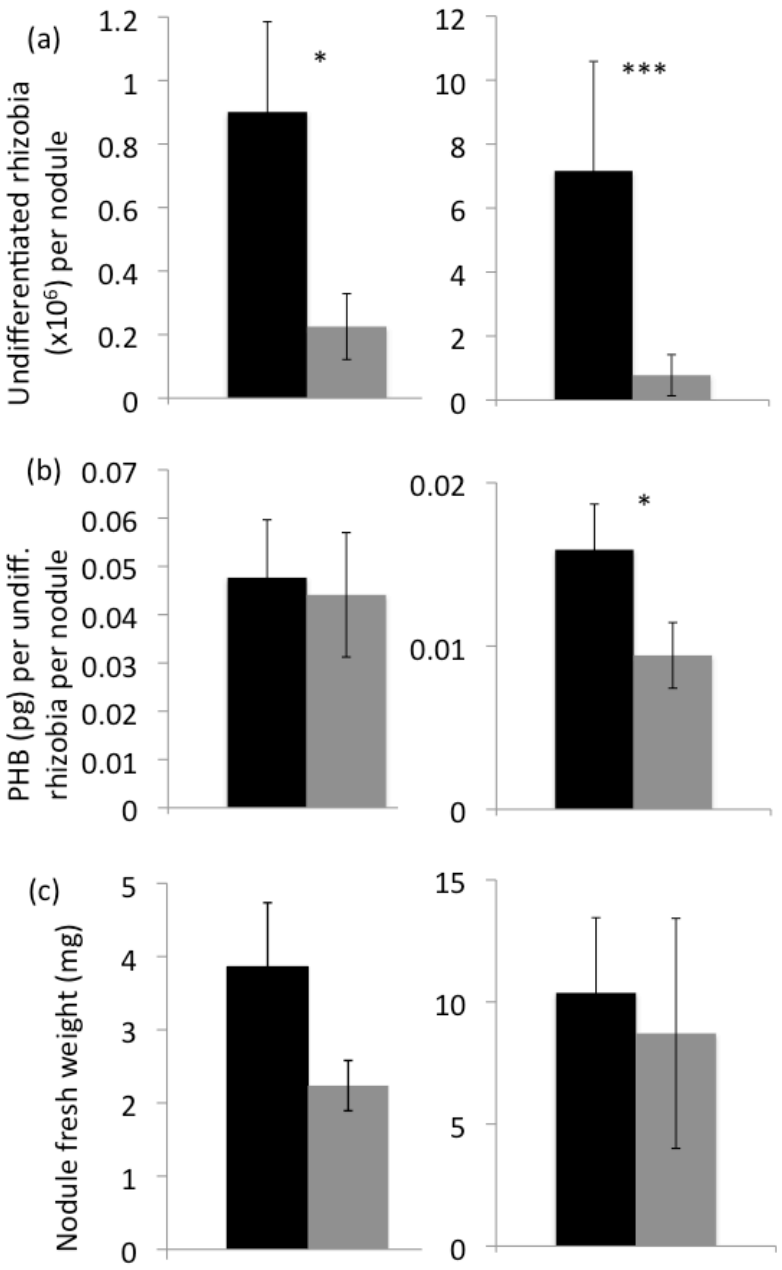

Figure 4-2. Comparison of a) reproductive rhizobia per nodule, b) PHB per cell in small reproductive rhizobia, and c) nodule fresh weight after a ten day argon ($Ar:O_2$, gray bars) versus control ($N_2:O_2$, black bars) treatments, in young (left column) versus old (right

column) pea nodules. Error bars indicate one standard deviation. Paired two-tailed equal variance t-tests were performed * $p < 0.05$, *** $p < 0.001$.

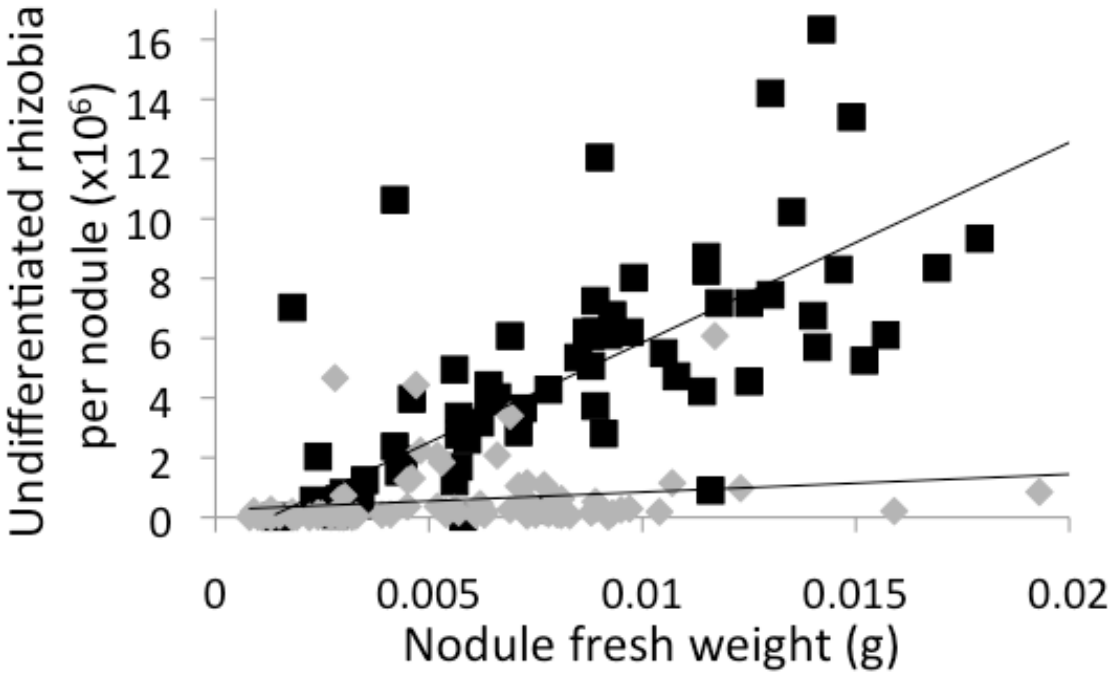

Figure 4-3. Regressions of nodule fresh weight to number of colony-forming rhizobia per nodule of the two treatments (N_2:O_2 -black squares vs Ar:O_2 –gray diamonds) for peas (d.f. 187, t = 2.18, p < 0.05).

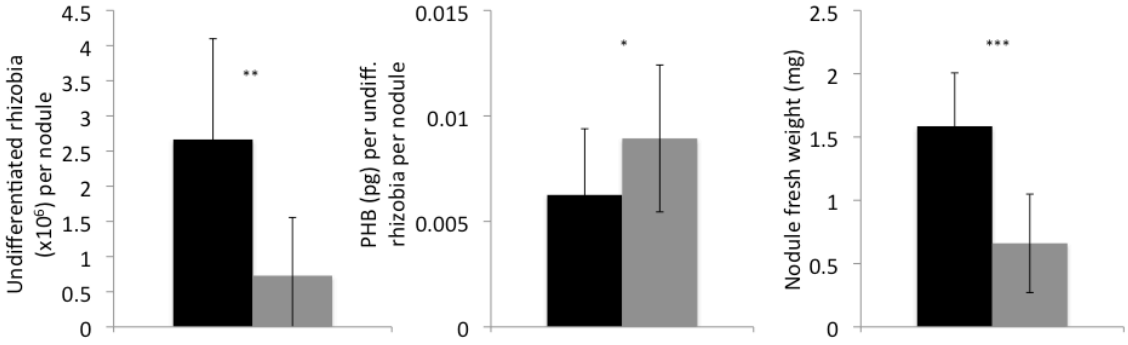

Figure 4-4. Comparing reproductive rhizobia per nodule, PHB per reproductive (nonswollen) rhizobial cell, and nodule fresh weights after ten days of argon ($Ar:O_2$) treatment (gray bars) on young alfalfa nodules and controls ($N_2:O_2$, black bars). Error bars indicate one standard deviation. Paired two-tailed equal variance t-tests were performed * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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