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Review

Why are most rhizobia beneficial to their plant hosts, rather than parasitic?

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Abstract

Multiple strains per plant and root-to-root (not seed-borne) transmission should favor rhizobia that invest in their own reproduction, rather than symbiotic N_2 fixation, as analogous factors may favor pathogen virulence. But legumes can select for greater mutualism, controlling nodule O_2 supply and reducing reproduction of rhizobia that fix less N_2.

Keywords: Symbiosis; Rhizobium; Nitrogen fixation; Fabaceae; Oxygen; Evolution; Virulence

1. Introduction

Rhizobia (bacteria classified as *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, etc.) can survive and reproduce in the soil, but they are best known for fixing N_2 inside the nodules that develop on legume plants whose roots they have infected. A single founding rhizobium may produce a billion or more descendants inside a nodule [1]. But once established inside a nodule, what benefits do rhizobia obtain by expending much of their energy fixing atmospheric N_2 and supplying it to their host plants? Given the extensive literature on this symbiosis, it is remarkable how little attention has been paid to this central question.

Respiration in support of N_2 fixation consumes reduced carbon compounds that rhizobia could otherwise have used to support their own current or future reproduction, thereby enhancing their Darwinian fitness. For example, instead of fixing N_2, rhizobia could use carbon received from the plant to synthesize energy-rich storage molecules like polyhydroxybutyrate (PHB), which could enhance rhizobium survival and reproduction after they return to the soil [2].

By supplying its host with nitrogen, an individual rhizobium can enhance host photosynthesis [3], presumably increasing the rhizobium's own access to photosynthate. But does this also benefit other rhizobia—future competitors—infecting the same individual plant? If so, then why have not rhizobia that fix less N_2 in symbiosis (and thereby hoard more resources) outcompeted and displaced those that fix more N_2, over the course of evolution?

Rhizobia range from mutualists, which supply their hosts with nitrogen at a reasonable carbon cost, to parasites, which infect legume plants but then fix little or no N_2 inside their nodules. Even at a single location, different strains of rhizobia can vary 10-fold in the net benefits they provide their hosts, as measured by growth of test plants [4].

This variation in the degree of mutualism is reminiscent of variation in virulence among pathogens. Are the factors favoring the evolutionary stability of mutualism also similar to those that limit the evolution of virulence?

This question would be especially important if mutualists could readily evolve into pathogens, or vice versa. Some mutualists do have close relatives that are pathogens. *Sinorhizobium meliloti*, which fixes N_2 in nodules of *Medicago* species, may be more closely related to the plant pathogen *Agrobacterium* than it is to some fellow rhizobia, at least if phylogeny is based on nonsymbiotic, chromosomal genes [5]. Relatedness is a tricky concept for rhizobia, however, as the phylogeny of symbiotic genes often differs from that of the remainder of their genome [6]. Horizontal transfer of symbiotic genes among distantly related bacteria appears to be an important factor in the past and ongoing evolution of rhizobia [7,8].

It has been suggested that irreversible specialization may limit evolutionary change between the extremes of mutualism and pathogenicity [9]. Even if this turns out to be true, comparing evolution of mutualism and of virulence may still be informative.

Two factors that can affect the evolution of both mutualism and virulence are (1) within-host competition, especially among unrelated microbial strains, and (2) the mode of transmission among hosts. Host responses to symbiont be-

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havior may also be important, as we have recently shown for symbiosis between *Bradyrhizobium japonicum* and soybean *(Glycine max)* [10]. These factors will be discussed in turn.

2. Effects of multiple infection

When a host is infected by a single strain of a pathogen, premature death of the host due to overexploitation by a highly virulent strain could decrease transmission to new hosts. Highly virulent strains might therefore become less common over time.

But if mixed infection is typical, within-host competition will tend to select for increased virulence. Restraint by any one strain will not delay host death, so self-restraint (limiting reproduction within the host) is not an evolutionarily stable strategy. Therefore, it has been suggested that multiple infection will often favor the evolution of aggressive microbial exploitation of host resources, typically resulting in greater virulence [11].

Similarly, qualitative [2,12] and quantitative [1] arguments have suggested that competition among several strains of a microbial mutualist infecting the same host individual should favor the evolution of decreased mutualism. Maynard Smith [13] noted that it "may be better to keep alive the goose that lays the golden eggs than to kill it. But this argument depends on the assumption that, if you do not kill the golden goose, no one else will either: that is, it assumes that the host is infected by a single clone of symbionts."

In the case of rhizobia, each individual plant often hosts 10 or more rhizobium strains at the same time [14]. Theoretical considerations therefore seem to predict that rhizobia should evolve to be less mutualistic in each generation [1,2]. This apparently has not happened, for reasons discussed below.

The effects of multiple infection may be more complicated than previously recognized, however. Sometimes the ability of a pathogen to exploit its host depends on some individually costly collective activity, such as production of a toxin that suppresses host defenses. In that case, mixed infections may actually decrease virulence, by favoring strains that avoid the cost of collective action against the host [15].

Collective production of rhizobotoxine by rhizobia in soil near the root can trick some plants into allowing more rhizobia to found nodules [16]. Once inside a nodule, however, the most obvious form of collective action by rhizobia is fixing N_2, an activity which benefits rather than harms the host.

3. Effects of horizontal transmission

Another major generalization from pathology is that parasites transmitted only vertically (i.e. from parent to offspring) should evolve lower virulence than those that are freely transmitted horizontally, among unrelated individuals [11]. Virulent pathogens that kill the host before it is able to reproduce would disappear from the population if they are only transmitted in eggs or seeds. Similarly, mutualistic symbionts that increase the reproductive success of their host should be favored by natural selection if they are only transmitted to their host's offspring, but not if they spread readily between hosts [12].

This theory, too, predicts that rhizobia should evolve to be less mutualistic. Although Bergersen et al. [17] have suggested that rhizobia accumulate PHB to support "the continuation of N_2 fixation at high rates until the last stages of seed development", seed production by an individual plant is not essential to the rhizobia infecting its roots. Rhizobia spread between host plant roots through the soil. Although rhizobia may be found in dust on the surface of legume seeds [18], those rhizobia do not necessarily come from the plant that produced the seeds.

Of course, if none of the legumes in a region produced seeds, that would adversely affect future opportunities for rhizobia. But natural selection is blind both to future consequences and to the greater good of a species as a whole [19]. A rhizobium mutant that sacrificed its own reproduction (even partially) to ensure the reproduction of its plant host would be outcompeted by less altruistic strains infecting the same host, even if that eliminated symbiosis as a future option for any of them.

So, multiply infected hosts and horizontal transmission should select for less mutualistic rhizobia, just as they may select for more-virulent pathogens. What about factors specific to mutualisms in general, or specific to rhizobia in particular?

4. Spatial structure

A popular idea is that "spatial structure of populations leading to repeated interactions" might enhance the evolutionary stability of mutualism [12,20]. For example, limited physical mixing of untilled soils could increase genetic relatedness among rhizobia infecting a given individual plant. This would increase the chances that a hypothetical altruistic rhizobium (assumed to increase photosynthate supply to other rhizobia infecting the same plant, indirectly, by investing in high rates of N_2 fixation) would mainly benefit other rhizobia that carry the same altruist gene. Similarly, rhizobial cheaters would be more likely to share a plant with other cheaters, so all would suffer reduced fitness.

Reduced mixing of soils also has an opposite effect, however, which undermines cooperation rather than favoring it. Consider two (related or unrelated) rhizobium strains infecting the same host, which may indirectly benefit one another by providing their common host with nitrogen. How do benefits to a second strain affect the fitness of the first strain? If soils are thoroughly mixed, then the two clones are unlikely to encounter each other in the future, so fitness of the second strain has no effect on the first. But if soils are not mixed, then rhizobia that fix more N_2 are indirectly helping a likely future competitor. In other words, reduced soil mixing makes competition more local, rather than global, so strains that fix more N_2 would suffer more future competition. The

contrasting effects of spatial structure in (1) increased within-plant relatedness, and (2) making competition more local, tend to balance each other [1]. Therefore, spatial structuring of soil populations will not necessarily favor greater mutualism.

Repeated interactions should favor coevolution of legumes and rhizobia, but the resulting adaptations could include more sophisticated forms of exploitation, not necessarily greater cooperation.

5. Recognition signals

Legumes and rhizobia exchange elaborate recognition signals prior to infection. If the legumes could recognize and exclude all but the best mutualists, they would enhance their own growth and also selectively enrich the soil with the best rhizobium strains. Recognition signals may provide some protection against some of the worst rhizobia. For example, some soybean cultivars have an allele that excludes some rhizobia that produce toxins harmful to the plant. But there is no intrinsic link between toxin production and the recognition signals, so other toxin-producers are admitted [21]. Similarly, mutant rhizobia with nonfunctional nitrogenase can retain all of the normal recognition signals, so they compete strongly for nodulation opportunities, even though they will not provide the host with any nitrogen [22,23]. Recognition signals therefore offer only a weak and inconsistent defense against parasitic rhizobia.

6. Host sanctions: mutualism's last chance?

So, why have not rhizobia that use plant resources for their own reproduction, rather than for N_2 fixation, outcompeted and displaced N_2-fixing strains? We have proposed [1,2] and subsequently shown experimentally [10] that legumes monitor actual behavior—not just identity—of rhizobia in individual nodules, and then impose sanctions on rhizobia that fail to fix enough N_2.

For host sanctions to explain the evolutionary persistence of mutualism in rhizobia, they would have to reduce the reproductive success of strains that fix less N_2 in symbiosis. In *B. japonicum* and some other species, the rhizobia that eventually escape from senescing nodules back into the soil are apparently descended from bacteroids, the differentiated form of rhizobia that typically fixes N_2 in nodules. Host sanctions against less mutualistic bacteroids would therefore reduce the frequency of these strains in the soil [2].

S. meliloti, on the other hand, typically loses the ability to reproduce when the free-living form differentiates into bacteroids. This is also true of other rhizobium species that infect legumes whose nodules have indeterminate growth. However, indeterminate nodules also contain significant numbers of undifferentiated, reproductive rhizobia. Only host sanctions directed against these reproductives would have any direct effect on rhizobium evolution [2].

Although legumes have a collective interest in selectively favoring the most beneficial strains of rhizobia, natural selection within a legume species will not maintain imposing sanctions unless an individual plant also benefits from doing so. Fortunately, saving resources that would otherwise be wasted could provide such an individual benefit [24].

7. Testing the sanctions hypothesis

The legume–rhizobium mutualism is unusually suitable for experimental manipulation. Unlike some microbial mutualists, rhizobia can be grown and cultured on selective antibiotic media. Furthermore, their symbiotic behavior inside nodules can be manipulated and monitored noninvasively.

In our experiments, we used an N_2-free (Ar:O_2) atmosphere to prevent N_2 fixation in a rhizobium strain that normally fixes N_2 at high rates. We could have tested the sanctions hypothesis by looking at plant responses to "ineffective" strains of rhizobia, which fix little or no N_2. It has been shown previously that nodules containing some ineffective rhizobia grow less [25], although effects on rhizobium numbers were not determined. However, such responses could have been based on strain identity (surface antigens, recognition signals, etc.) or some metabolic defect, rather than N_2 fixation per se. Our argon treatments eliminated N_2 fixation without such confounding factors.

When we used Ar:O_2 to prevent N_2 fixation by all the rhizobia infecting a plant, rhizobium population growth inside nodules and in the rhizosphere was less, relative to controls in air. But this result could have occurred even without specific host sanctions, because overall plant growth was less in the Ar:O_2 treatment than in air. We therefore conducted two additional experiments using Ar:O_2 to prevent N_2 fixation in either (1) roughly half of the nodules of a root system (a split-root experiment) or (2) in a single-nodule.

In the latter experiment, the small chambers that controlled gas composition around single-nodules (one each for Ar:O_2 and air, on each of six replicate plants) also held optical fibers that allowed us to measure nodule interior O_2 concentration by noninvasive spectrophotometry (Fig. 1). Reversible binding of O_2 to leghemoglobin in the nodule interior changes absorbance at 660 nm (and other wavelengths not used in these experiments [26]). The dynamics of these changes in response to brief (<30 s) exposure to low or high O_2 concentrations was used to calculate nodule O_2 permeability and O_2-saturated respiration rate [27]. The latter is assumed to respond to carbon substrate availability, among other factors.

The split-root and single-nodule experiments confirmed the results of the whole-plant experiment. Rhizobia prevented from fixing N_2 grew to about half the population of those in control nodules in air. In the split-root experiment, rhizobium numbers in the rhizosphere were also less on the nonfixing side, presumably because fewer rhizobia were released from the nodules (we plan to test an alternative hypothesis, that N_2-fixing nodules support greater reproduction

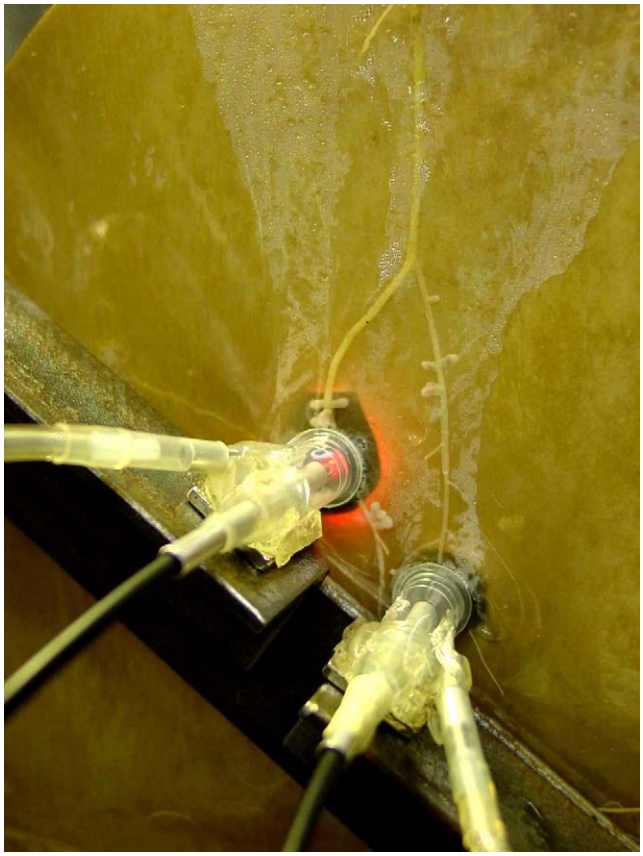

Fig. 1. Single-nodule chambers, applied here to alfalfa (*Medicago sativa*) roots, allow experimental control of N_2 fixation by varying N_2 concentration. An optical fiber touching the nodule surface monitors transmission of red light through the nodule, which depends on the oxygenation status of the leghemoglobin inside.

of rhizobia already in the rhizosphere, in future experiments). Nodule growth was also less in the Ar:O_2 treatment.

Monitoring nodule O_2 relations revealed plant responses to the cessation of N_2 fixation in individual nodules. Within 48 h, nodule interior O_2 concentration in the Ar:O_2 treatment was 50% that of control nodules in air. This change resulted from a decrease in the gas permeability of the nodule. Our spectrophotometric method measures O_2 permeability, but permeability to other gases, such as H_2, has previously been shown to change in parallel with O_2 permeability [28]. On the other hand, O_2-saturated respiration did not differ between treatments, so limitation by carbon substrate supply was apparently no more severe in the Ar:O_2 treatment. The decreased respiratory demand resulting from lower O_2 supply may, however, have allowed the plant to reduce carbon allocation to nodules without increasing the extent to which respiration was carbon-limited.

Cessation of N_2 fixation led to decreased O_2 supply and reduced rhizobium reproduction inside nodules, but did the decreased O_2 supply cause the decreased reproduction? To test the hypothesis that O_2 supply was the main factor controlling rhizobium reproduction, we will repeat the single-nodule experiment using air, Ar:O_2, and a third treatment: Ar:O_2 plus supplemental O_2. Nodule interior O_2 concentration in this third treatment will be adjusted to match the level in the control nodule, by dynamic adjustment of external O_2 concentration. After a week or two, we will count rhizobia in each nodule. If cutting off O_2 supply was the main plant sanction reducing rhizobium reproduction in the Ar:O_2 treatment, then supplemental O_2 should increase rhizobium numbers in the Ar:O_2 treatment to control values. Otherwise, we will conclude that plants impose some other fitness-limiting sanction on rhizobia that stop fixing N_2.

8. Escaping legume sanctions

In the absence of host sanctions, we would expect rhizobia that do not fix N_2 to outcompete those that do, since they would use a larger fraction of resources they get from plants to support their own reproduction [1,2]. Sanctions turn this argument on its head. Given sanctions, the reproductive success of rhizobia that did fix N_2 was about double that of rhizobia prevented from fixing N_2, at least in our experiments with soybean. Why, then, have not rhizobia that fix more N_2 in nodules outcompeted and completely displaced "ineffective" strains that fix little or no N_2? We consider several hypotheses, which are not necessarily mutually exclusive.

One rather boring hypothesis is that the coexistence of fixing and nonfixing strains simply reflects a balance between natural selection and mutation. Under this hypothesis, selection imposed by host sanctions consistently increases the frequency of N_2-fixing strains in rhizobium populations. But there must be many different mutations that would reduce N_2 fixation rate. These would include mutations in nitrogenase and related genes, as well as mutations that would increase the rate of processes, like PHB accumulation, that compete with N_2 fixation for ATP or other resources. If this hypothesis is true, then we would expect strains that fix little or no N_2 to exhibit a wide variety of different mutations that reduce N_2 fixation.

Results consistent with a hypothesis do not prove that hypothesis, however [29]. In particular, diversity in nonfixing mutants would not prove that high rates of mutation are responsible for the presence of nonfixers in significant numbers. Selection could also favor nonfixers under some conditions. Although rhizobial cheating might seem to be a complex trait requiring an entire suite of related genes, it appears that any mutation that knocks out nitrogenase activity may automatically result in redirection of resources to PHB accumulation [22]. If PHB enhances rhizobium survival after nodule senescence, then any mutation that reduces N_2 fixation would tend to increase rhizobium fitness, assuming that nonfixing rhizobia escape fitness-reducing sanctions.

One way nonfixing rhizobia might escape sanctions would be to share a nodule with a fixing strain. Hahn and Studer [22] showed that a nitrogenase-knockout mutant accumulated more PHB when it shared a nodule with its N_2-fixing parent. Presumably, the nonfixing strain would have lower fitness than the fixing strain when each was the sole occupant of a nodule, due to host sanctions imposed on nonfixing

nodules. In that case, the balance between fixing and nonfixing strains would depend on the frequency of mixed nodules. There have been few studies on the frequency of mixed nodules under field conditions, especially using methods that distinguish closely related strains, which might have similar antibody reactions.

It is also possible that some rhizobium strains that fix little or no N_2 nevertheless manage to avoid host sanctions by interfering with a host's signaling pathways that lead to sanctions. This would be analogous to the toxins some rhizobia produce that can interfere with the mechanisms their hosts use to limit nodule number [16].

9. Host sanctions in a broader context

Are host sanctions important to the evolutionary stability of other mutualistic symbioses? The mycorrhizal symbiosis is another case where several different microbial strains (or even species) are associated with each individual plant. Theoretical arguments analogous to those we have made for rhizobia therefore suggest that host sanctions might also be needed to maintain mutualism in mycorrhizal fungi [30]. If the benefits they provide their hosts are costly to the fungi, strains that avoid those costs would otherwise increase in frequency over generations, at the expense of more cooperative strains.

Does anything analogous to host sanctions limit the evolution of virulence in pathogens? Host defenses like the immune system in vertebrates or the hypersensitive reaction in plants certainly respond to parasites in ways that can limit parasite reproduction. The question is whether these responses selectively favor less-virulent strains or selectively punish more-virulent strains. To the extent that defenses exhibit this sort of selectivity, they would be functionally equivalent to the sanctions we have seen in the legume–rhizobium symbiosis.

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