

Cooperation and conflict in the legume-rhizobium mutualism

Background

Rhizobia are soil bacteria capable of growing inside the roots of legume plants, where they convert nitrogen gas from the air into plant fertilizer; in return, the rhizobia are supplied with food and a safe home. This mutualism is critical to agriculture: legumes account for 27% of the world's primary crop production, and 33% of our dietary protein comes from grain legumes (such as beans, peas and lentils) alone.¹ Nitrogen fertilizer supplied by rhizobia is essential for much of this productivity. Yields, however, are reduced significantly by 'cheating' rhizobia, strains that provide little fertilizer despite consuming plant resources.² Why do rhizobia cheat?

Cooperative rhizobia use most of the energy provided by the plant to make fertilizer, using only a small amount for reproduction, while cheaters use most of this energy to reproduce instead. Cooperation is a good strategy when the benefits of increased *future* reproduction outweigh the costs of reduced *immediate* reproduction. This occurs when legumes are only infected with a single rhizobium strain--cooperators help their plant grow well, allowing the rhizobia inside the roots of large healthy plants to leave many more offspring than cheating rhizobia in small, sickly plants. Unfortunately, legumes are usually infected by both cooperators and cheaters at the same time.³ Under these conditions cooperators forgo immediate reproduction to make fertilizer, increasing the future growth of *both cheating and cooperating strains in the same plant equally*, but as a result leave fewer offspring than cheaters. Yet cheaters have not displaced cooperators entirely because plants punish rhizobia that cheat, limiting the reproduction of cheaters inside plant roots.⁴ This raises a central question of my thesis: *Why are cheaters globally persistent in the face of effective legume punishment?*

Goals and Objectives

I propose the following answers to the above question:

- 1- Some cheaters have evolved counter-measures to evade plant punishment.
- 2- Cheaters hoard more resources that help them survive in the soil once they leave the plant, allowing them to succeed despite plant punishment.
- 3- Cooperation among free-living rhizobia favors strains that cheat legumes.

Design and Methodology

Answer #1- Some cheaters have evolved counter-measures to evade plant punishment.

Punishment of cheating rhizobia by legumes should, over thousands of years, eliminate cheaters. The global distribution of cheaters, however, suggests that punishment is not effective. Why? In the arms race between rhizobia and legumes, any mechanism that allows rhizobia to grow more while evading punishment would likely evolve. Some rhizobia synthesize rhizobitoxine, a chemical that prevents legumes from making the hormone ethylene. Ethylene in turn plays a critical role in this interaction by limiting the infection of roots by rhizobia.⁵ Thus, I hypothesized that a rhizobitoxine-producing strain would reproduce more in the root than a non-producing strain. To test this, I infected legumes with both a rhizobitoxine-producing strain and non-producing (but otherwise genetically identical) mutant. I found that the rhizobitoxine-producer didn't grow more, but instead accumulated 47% more resources per cell as a bacterial fat (poly-3-hydroxybutyrate, abbreviated PHB). The effect of the rhizobitoxine on the plant was negative, however, reducing growth by 37% when it was infected with only rhizobitoxine-producing rhizobia. Because PHB is a high-energy resource, rhizobia may be able to use it for reproduction in the soil after the plant has died. If so, rhizobitoxine would be an example of a

mechanism used by cheaters to manipulate the legume. So does accumulating more PHB actually help rhizobia? I tested this in my next experiment.

Answer #2- Cheaters hoard more resources that help them survive in the soil once they leave the plant, allowing them to succeed despite plant punishment. Even without rhizobitoxine production, most rhizobia still make PHB. Previous work has shown that PHB is energy-intensive to synthesize, and that strains that make more of it produce less fertilizer for the plant.⁶ While plants punish rhizobia that make little fertilizer by restricting reproduction, might the benefits to rhizobia of storing more PHB exceed this cost? When the plant dies, rhizobia return to the soil and seek out new roots to colonize. Food in the soil can be scarce, so I examined the ability of rhizobia to use PHB to aid survival and reproduction during starvation. I first developed a high-throughput method for measuring rhizobium PHB content. Then, I generated populations of genetically-uniform rhizobia that varied in PHB per cell, using both a culture technique and centrifugation. Rhizobia were starved and sampled for population size and average PHB/cell weekly. I found that PHB was an excellent source of energy for both survival and reproduction, allowing rhizobia to fuel several cell doublings and survive months of starvation. Plant punishment reduces the reproduction of cheaters by up to 66%⁴, but I found that cheaters could store enough energy in PHB to reproduce up to 5 times after leaving the plant, compensating for their lower numbers. Storage of energy in PHB for later reproduction and survival in the soil may limit the efficacy of plant punishment in eliminating cheaters, and may help explain the global persistence of cheating rhizobia. Further, we now know the role of rhizobitoxine: it has evolved at least in part because it allows rhizobia to make more PHB without being punished by the legume.

Answer #3- Cooperation among free-living rhizobia favors strains that cheat legumes. I found that rhizobia exposed to starvation form both high and low-PHB cells. By following individual rhizobia during starvation with a microscope, I found that this difference in PHB is caused by unequal division: the daughter cell gets very little PHB, while the mother cell retains the majority of the remaining PHB (Fig. 1). This mode of division only occurs during starvation: rhizobia grown in the presence of food split PHB evenly between both mother and daughter cells. Does this uneven split benefit rhizobia? To find out, I measured rhizobium susceptibility to long-term starvation. Early in starvation (two weeks), 86% of both high and low-PHB rhizobia were viable. However, after 16 months of starvation, more than 90% of low-PHB rhizobia died, while only about 40% of the high-PHB rhizobia died. In addition, high-PHB rhizobia were resistant to antibiotics, while the low-PHB cells were not. The high-PHB cells, it turns out, were going dormant, while the low-PHB cells remained metabolically active.

This unequal division allows a single genotype of rhizobia (rhizobia with the same genetic composition, a.k.a. clonemates) escaping from plants to 'hedge their bets' against environmental catastrophe, because *some* cells are ready to take advantage of the arrival of unexpected food, while other cells are not growing (thus paying an opportunity cost if food is available) but are capable of surviving rough times. Interestingly, cooperation between clonemates can reduce the opportunity costs of going dormant. Any food that is not used by high-PHB dormant cells to reproduce can instead be used by low-PHB clonemates for

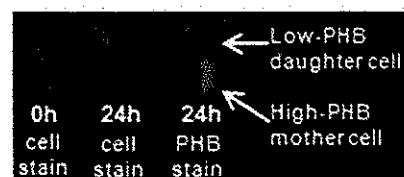


Fig. 1- Rhizobia viewed at 1000-fold magnification. Initially, all cells had large amounts of PHB. Daughter cells produced after 24 hours of starvation contained little PHB; most PHB was retained by mother cells. The green cell stain used, GFP, is nontoxic.

reproduction. Evolutionarily speaking, reproduction by either high- or low-PHB clonemates is identical, since they have identical genomes. Thus, this cooperation can remove the opportunity cost of dormancy. A mathematical model I made shows that cooperation between rhizobia in the soil favors strains that form many dormant cells, potentially making high-PHB accumulating rhizobium strains difficult to eradicate from soils. I was recently awarded a NSF Doctoral Dissertation Improvement Grant (\$10,515) to use rhizobia as a model system for understanding the role of cooperation and conflict in the evolution of dormant, 'persister' bacteria, whose antibiotic resistance makes them medically problematic as well.

Potential significance of the research

Science called the 'problem' of cooperation - including how mutualism evolved and avoids collapse caused by cheating - one of the 25 most important questions facing scientists in the next quarter century.⁷ *This research helps solve the problem by determining the mechanisms that facilitate and restrict cheating in the legume/rhizobium mutualism.* My results have also contributed to work on the evolutionary history of rhizobia/legume co-evolution by another student in our lab. Substantial agricultural benefits are also feasible. Understanding the evolutionary persistence of cheaters may facilitate the development of new legume varieties that are less susceptible to cheaters and allow development of management strategies to reduce the frequency of cheaters in agricultural fields. For example, if cheating rhizobia benefit mainly by storing extra energy in PHB, farmers may be able to remove this advantage (and thus the incentive to cheat) by providing rhizobia exiting dying legumes with external food. This could be done by adding organic material such as compost or straw to the field. This should a) reduce bet-hedging and cooperation among free-living rhizobia, and b) remove the growth advantage high-PHB rhizobia possess. Increasing the amount of nitrogen fertilizer made by rhizobia will decrease demands for inorganic nitrogen fertilizer in developed countries, and improve crop yields in developing countries where cheating rhizobia substantially depress yields² and nitrogen fertilizer is unavailable or cost-prohibitive.

Progress to date and schedule for completion

The hypotheses above constitute the first three chapters of my thesis. The experimental work for these chapters has been completed, the first chapter is in press, and the second chapter published in 2008. I am currently working on further experiments on the evolution of dormant 'persister' bacteria, which will constitute the 4th chapter of my thesis. Finally, the 5th and 6th chapters are focused on the evolution of aging (not described here), the 5th chapter is theoretical (submitted for publication) and the 6th an experimental test of this theory. This fellowship will allow me to concentrate full-time on research in my final year, completing the experiments for the 4th and 6th chapters of my thesis, and writing and submitting chapters 3, 4, and 6 for publication. I expect to defend my dissertation and graduate by the spring of 2010.

Key References

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