Applying Reversible Mutations of Nodulation and Nitrogen-Fixation Genes to Study Social Cheating in *Rhizobium etli*-Legume Interaction

Jun Ling¹, Huiming Zheng¹*, David S. Katzianer²*, Hui Wang¹, Zengtao Zhong¹*, Jun Zhu¹,²*

¹ Department of Microbiology, Nanjing Agricultural University, Nanjing, China, ² Department of Microbiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, United States of America

Abstract

Mutualisms are common in nature, though these symbioses can be quite permeable to cheaters in situations where one individual parasitizes the other by discontinuing cooperation yet still exploits the benefits of the partnership. In the *Rhizobium*-legume system, there are two separate contexts, namely nodulation and nitrogen fixation processes, by which resident *Rhizobium* individuals can benefit by cheating. Here, we constructed reversible and irreversible mutations in key nodulation and nitrogen-fixation pathways of *Rhizobium etli* and compared their interaction with plant hosts *Phaseolus vulgaris* to that of wild type. We show that *R. etli* reversible mutants deficient in nodulation factor production are capable of *intra*-specific cheating, wherein mutants exploit other *Rhizobium* individuals capable of producing these factors. Similarly, we show that *R. etli* mutants are also capable of cheating *inter*-specifically, colonizing the host legume yet contributing nothing to the partnership in terms of nitrogen fixation. Our findings indicate that cheating is possible in both of these frameworks, seemingly without damaging the stability of the mutualism itself. These results may potentially help explain observations suggesting that legume plants are commonly infected by multiple bacterial lineages during the nodulation process.

Introduction

Rhizobia can form symbiosis nodules with legumes in which the bacteria fix atmospheric nitrogen into ammonia that can be utilized by the host plant. Two-way chemical signaling is required for this process. For example, *Rhizobium etli* is able to identify flavonoid substances released by its host legume *Phaseolus vulgaris* signaling it to produce nodulation factors (Nod), which in turn induce cortical cell division and form symbiosis nodules, where nitrogen is fixed [1]. Nodulation and nitrogen fixation is achieved by *nod* and *nif*/*fix* gene clusters, which play pivotal regulatory roles in symbiosis [2]. All Rhizobia strains contain the common nodulation genes *nodDABC*, which encode a transcription factor, as well as enzymes that are required to produce Nod factors [3]. In bacteroids that rhizobia differentiate in the infection thread, *nif* and *fix* genes are expressed through the FixL/FixJ two-component regulatory system and the transcriptional activator NiFA [4] which leads to the production of nitrogenase and ultimately nitrogen fixation.

Due both to the ability of Rhizobia to form symbiotic nodules on host legumes and their ability to fix inert atmospheric nitrogen (N₂) to plant-useable ammonia, mutualistic interactions are commonly studied within the Rhizobia-host system [5]. In this context, *Rhizobium* individuals cooperate with the plant host by fixing nitrogen into usable ammonia while benefiting from receiving host-derived resources, such as carbon [5]. Nitrogen-fixation, however, is an energetically costly process, and can be predicted to benefit Rhizobia only if the plant supplies efficient resources to outweigh the cost of ammonia production on the bacteria [6]. Given this cost, opportunity exists for non-cooperating partners to parasitize these social dynamics by reaping benefits (carbon or other resources) while failing to supply nitrogen-fixation in return [7]. It has been demonstrated that one way of mitigating this potential burden is through the differential allocation of host resources in attempts to punish these non-fixing “cheaters” within a community, though the mechanism of these sanctions is unknown [8]. Though these studies show that plants can reduce rhizobial nodule occupancy in scenarios where whole nodule cheating is forced, they do not address how plants respond to increased parasitism in the event of nodules containing non-fixing *cheaters* within a community, though the mechanism of these sanctions is unknown [9].

Similar dynamics can be studied within the colonization process itself. For effective colonization, Rhizobia secrete nodulation factors (Nod factors) which induce cortical cell division in their host legume [1]. For instance, previous studies have shown that it is possible for mutants deficient in nodulation to form mixed
nODULES WITH NOD FACTOR-PRODUCING STRAINS [10], SUGGESTING THAT RHIZobia CAN hitch-hike WITHIN THEIR OWN SPECIES DURING THE NODULE-FORMATION PROCESS. THIS SYSTEM, IN TURN, CAN BE PERMEABLE TO CHEETERS (NODULE DEFICIENT MUTANTS) THAT BENEFIT BY COLONIZING WITH OTHER INDIVIDUALS, ALLOWING FOR HOST COLONIZATION AND RESISTANCE SEQUESTRATION WITHOUT THE BURDEN OF PRODUCING NOD FACTORS THEMSELVES. IF A STRONG METABOLIC COST TO PRODUCE THESE NOD FACTORS EXISTS, MUTANTS DEFICIENT IN NODULEATION CAN BE EXPECTED TO ARISE AT HIGH FREQUENCIES, AS WHAT TYPICALLY HAPPENS WITH ANY COMMON GOOD [11]. AS WITH NITROGEN FIXATION, THE SELECTIVE ADVANTAGE GAINED BY MUTANTS DEFICIENT IN NODULEATION FACTOR PRODUCTION WOULD DEPEND UPON THE METABOLIC COST OF PRODUCTION. IN THIS STUDY, WE CONSTRUCTED REVERSIBLE AND IRREVERSIBLE MUTATIONS IN THE KEY nod AND nif GENES OF R. etli AND INVESTIGATED THEIR NODULEATION AND N2-FIXATION BEHAVIORS WHEN THEY WERE INOCULATED INDIVIDUALLY OR CO-INOCULATED WITH WILD TYPE STRAINS. WE SHOW THAT R. etli IS CAPABLE OF BOTH INTRA-SPECIFIC CHEATING THROUGH THE MUTATION OF A KEY GENE IN THE NODULE-FORMATION PATHWAYS AS WELL AS INTER-SPECIFICALLY CHEATING THEIR HOST THROUGH THE MUTATION OF A GENE ESSENTIAL TO NITROGEN FIXATION, SUGGESTING A MECHANISM FOR THE EXISTENCE OF MULTIPLE BACTERIAL STRAIN POPULATIONS IN NATURE AS WELL AS A REASON FOR THEIR PERSISTENCE.

MATERIALS AND METHODS

BACTERIAL STRAINS, VECTORS AND CULTURE CONDITIONS

R. etli CE3, a streptomycin-resistant derivative of R. etli CFN42 [12], was used in this study. Escherichia coli strains were grown on LB at 37°C [13]. R. etli strains were grown on peptone–yeast extract (PY) medium [12] or tryptone-yeast extract (TY) [14] at 28°C. All solid media contained 1.5% agar, and antibiotics were added at the following concentrations for R. etli: streptomycin (Sm), 100 μg/ml; kanamycin (Km), 100 μg/ml; tetracycline (Tc), 10 μg/ml; rifamycin (Rif), 5 μg/ml; chloramphenicol (Cm), 20 μg/ml. Sucrose was added to a final concentration of 10% in media for counter-selection of in-frame deletion strains and their derivatives [15]. Double-crossover deletions (irreversible) of nodB and nifA were constructed by cloning the regions flanking sequences of nodB and nifA with antibiotic resistant cassettes (chloramphenicol-resistant and tetracycline-resistant genes, respectively) into the suicide vector pEX18Gm containing a sacB counter-selectable marker [16]. The resulting plasmids were introduced into R. etli by conjugation and deletion mutants were selected for double homologous recombination events. Single-crossover mutations (reversible) of nodB and nifA were constructed by cloning internal fragments of nodB and nifA into pVTK112 [17]. The resulting plasmids were then introduced into R. etli and insertionional mutants were selected for single homologous recombination events. Under certain selective pressure, revertants through a second homologous recombination event of insertionional mutants may persist.

NODULEATION ASSAYS

Phascolus vulgaris seeds were treated with 75% ethanol and 0.1% HgCl2 and were surface-sterilized. The treated seeds were then placed in Petri dishes and germinated in the dark at 28°C for 2–3 days. Seedlings were then immersed in approximately 105 R. etli cells (either single inoculation or mixed inoculation) cultured in PY medium containing appropriate antibiotics and resuspended in sterilized water. After 20 mins, seedlings were transferred to pots containing sterile vermiculites and were grown in a plant growth chamber at 28°C with a 12 h/12 h day/night cycle. It has been shown that inocula containing high cell numbers may increase the frequency of mixed nodules [18]. The plants were watered as necessary with sterile nitrogen-free plant nutrient solution [19] for 30 days. The plants were then pulled out to count the number of nodules from both main roots and lateral roots.

To determine the nodule occupancy, nodules formed by mixed inoculation of wild type and mutants were surface-sterilized, crushed, and sequentially stabbed on PY agar containing selective antibiotics. After incubation at 28°C for 72 hrs, plates were inspected for the identity of strains. Nodule occupancy is based on at least 250 nodules isolated from 15–30 plants. To determine the competitive index of different mutant strains, colony forming units (CFUs) of bacterial cells in nodules containing mixed strains were determined by serial dilution and plating onto PY medium containing selective antibiotics. Competitive index (output ratio of two strains in a mixed nodule) was calculated as the output ratio of mutation rate gained by mutants deficient in nodulation factor production would depend upon the metabolic cost of production. INDIVIDUAL Nodulation Assays

Acetylene reduction assay was used to estimate nitrogenase activity following protocols previously described [20,21]. Briefly, all nodules were collected from each plant, placed in 20 ml headspace bottles with 2 ml acetylene (10%), and incubated upside down at 28°C for 1 hr. Gas chromatography was conducted to measure peak height of ethylene and acetylene with 100 μl gas by an HP 6890 Series Gas Chromatograph System. The approximate nitrogenase activity is calculated as % of acetylene production per gram of nodule dry weight.

Data Deposition

All raw data used in this study are available in Data S1.

RESULTS

CONSTRUCTION OF IRREVERSIBLE AND REVERSIBLE MUTANTS IN KEY NODULEATION AND N2 FIXATION GENES

Rhizobium-legume symbiosis interaction has been studied extensively in recent years. However, how rhizobia interact with each other during the symbiosis and how the host affects this interaction are not fully understood. To study the R. etli cell-cell interaction in the context of the R. etli-Phascolus vulgaris symbiosis, we constructed R. etli mutants in nodB, a common noduleation gene
involved in the synthesis of Nod factors in rhizobia [22]; and in nifA, encoding a conserved transcriptional activator which is required for expressing N2-fixation related genes [23,24]. We constructed two kinds of mutations: in addition to double-crossover replacement of nodB and nifA with antibiotic cassettes (annotated as ΔnodB, ΔnifA, respectively) (irreversible), we also made insertional mutants (reversible) by cloning the internal fragments of nodB and nifA into a suicide vector and introducing the resulting plasmids to select for the single homologous recombination events (annotated as ::nodB, ::nifA) (Fig. 1A). We reasoned that under certain selective pressure, the revertants (the insertional mutants may revert to wild type through a second homologous recombination event) may persist and we could detect such revertants by screening for the loss of the kanamycin-resistant cassettes. The mutants we constructed had no detectable effects on in vitro growth in the regular rich medium (Fig. 1B).

Reversible and Irreversible nodB Mutants Present Distinct Nodulation Phenotypes

To examine the effect of nodB on nodulation, we immersed approximately 10^8 cells of wild type, ΔnodB, ::nodB, or a 1:1 mixture of wild type with either ΔnifA or ::nifA, with Phaseolus vulgaris seedlings. We examined the number of nodules on each plant after 30 days. Approximately 55 nodules/plant were formed when plants were inoculated with wild type (Fig. 2A, empty circles). As expected, no nodules were found on plants inoculated with ΔnodB mutants (empty squares), since nodB is essential for nodulation. Phenotypically, however, ::nodB mutants did not display any nodulation defects, as the number of nodules formed by ::nodB mutants was comparable to that of wild type (empty triangles). Interestingly, both mixed-inoculations (filled symbols) formed nodules similar in number to that of wild type. Additionally, the size of the nodules formed under different conditions was indistinguishable as the average weight of those nodules was similar to each other (Fig. 2B).

To analyze the competitiveness of nodB mutants, we examined the nodule occupancy in coinoculation experiments. From 300 nodules (total 78 plants) formed by mixed-inoculation of wild type and ΔnodB (Fig. 3A, left panel), none were occupied by ΔnodB alone, as expected. Interestingly, approximately 70% of the nodules were occupied by the wild type strain alone. The remaining 30% of nodules were a mixture of wild type and ΔnodB mutants. Among those nodules containing both strains, on average, the number of ΔnodB mutants was only 1/10 of the

![Figure 1. R. etli nodB and nifA mutation constructs. (A). The deletion constructs of nodB and nifA (left panel) were made by cloning flanking regions of nodB and nifA into a suicide vector harboring the sacB counter-selective marker. The resulting plasmids were then introduced into R. etli and double cross-over events were selected. The mutations generated by this method are permanent. The insertional mutants (right panel) were constructed by cloning internal fragments of nodB and nifA into a suicide vector. The resulting plasmids were then introduced into R. etli and single cross-over events were selected. The reversion to wild type may occur if the strains undergo a second cross-over event. CmR: chloramphenicol-resistant gene; TcR: tetracycline-resistant gene; KmR: kanamycin-resistant gene. (B). Growth of R. etli and its derivative mutants. The cultures were grown in PY medium at 28 C. At the time points indicated, OD600 was measured. Data are mean and s.d. of three independent experiments.](Image 58x212 to 262x455)

![Figure 2. Nodule formation by wild type R. etli and its derivative nodB mutants. (A). The number of nodules on Phaseolus vulgaris formed 30 days post-inoculation by wild type (empty circles), ΔnodB (empty squares), ::nodB (empty triangles), 1:1 mixed inoculation of wild type and ΔnodB (filled squares), and mixed inoculation of wild type and ::nodB (filled triangles). (B). Average biomass of the nodules. Data are the combination of six individual experiments. Statistical analysis was performed using the Student's t-test comparing nodule formation to that of wild type. NS: no significance; ***: p<0.0001; NA: not applicable.](Image 315x168 to 554x507)
number of wild type in the same nodule, thus the competitive index was \(\approx 0.1\) (Fig. 3B, circles). When we examined nodule occupancy for nodules formed by mixed inoculation of wild type and ::nodB mutants, we found that 6% nodules contained ::nodB mutants alone, whereas 63% contained only the wild type strain, and 31% a wild type and ::nodB mixture (Fig. 3A, right panel). Among the nodules containing both ::nodB and wild type, the competitive index of ::nodB(Sm\(^{R}\), Km\(^{R}\)) was \(\approx 0.06\) (Fig. 3B, squares). These data suggest that \(\Delta\)nodB mutants can only present in nodules with the help from wild type, whereas ::nodB revertants can do so independent of wild type.

**Reversible nodB Mutants Displayed Selective Benefit during Nodulation**

We speculated that the reason the reversible nodB mutant (::nodB) could form nodules but not the deletion nodB mutant (\(\Delta\)nodB) was because ::nodB mutant can revert to wild type. To test this, we first examined the reversion rate of ::nodB mutant ex planta. After 30-day growth of ::nodB mutants in culture broth (squares) or around **Phaseolus vulgaris** roots (triangles) in the absence of antibiotics, less than 3% of ::nodB mutants became kanamycin-sensitive (Fig. 4A). We confirmed by PCR analysis that those Km\(^{R}\) mutants were indeed revertants that contained the wild type copy of the nodB gene (data not shown). Next, we examined the reversion rate of ::nodB mutants isolated from 30-day-old nodules. Strikingly, from 386 nodules formed by single-inoculation with ::nodB mutants, 298 nodules contained 100% revertants, and on average, 97.9% of ::nodB had reverted to wild type (Fig. 4B, squares). In the nodules formed by mixed inoculation of wild type and ::nodB mutants, if nodules contained only ::nodB mutants, 97.2% of ::nodB mutants had reverted to wild type (Fig. 4B, empty triangles), explaining the data shown in Fig. 3A that “::nodB mutants” could exist alone in nodules (empty green bar). However, if nodules contained both wild type and ::nodB mutants, the reversion rate was significantly reduced (Fig. 4B, filled triangles). Furthermore, the competitive index of the ::nodB revertant was higher than those of \(\Delta\)nodB and ::nodB mutants (Fig. 3B, triangles). Taken together, these data suggest that due to the importance of NodB in nodulation, only those revertants are able to infect host and form nodules; however, while co-infected with wild type, the pressure for reversion is reduced. It is unclear whether the reversion happens prior to infection or during the infection.

**nifA Mutants have Advantages Over Wild Type in Nodules**

In addition to studying nodB, a gene critical for nodulation, we also examined nodulation of nifA mutants (both deletion and insertion) with or without co-infecting with wild type. We then inoculated wild type, \(\Delta\)nifA, ::nifA, or 1:1 mixture of wild type with either \(\Delta\)nifA or ::nifA, with **Phaseolus vulgaris** seedlings. We examined the number of nodules on each plant after 30 days. We found that single-inoculations of \(\Delta\)nifA and ::nifA both formed wild type levels of nodules (Fig. 5A, empty squares and triangles). Interestingly, mixed inoculations of wild type with either \(\Delta\)nifA or ::nifA induced plants to form statistically significantly more nodules than those of single inoculations (Fig. 5A, filled symbols). The size of nodules formed by different strains, however, was similar to each other (Fig. 5B). It is possible that ::nifA mutants save energy by not fixing nitrogen so that they have advantages for reproduction inside the nodules.

We further analyzed nodule occupancy and competitiveness of mixed infections of wild type and ::nifA mutants. Both \(\Delta\)nifA and ::nifA mutants displayed nodulation advantages over wild type. The number of nodules containing ::nifA mutants (approximately 48% nodules containing only ::nifA mutants) was significantly higher than that of wild type alone (less than 15%) (Fig. 6A). For those nodules containing both wild type and mutant strains, ::nifA mutants were at least two-fold more than those of wild type and the competitive index of both \(\Delta\)nifA and ::nifA was over 2 (Fig. 6B, circles and squares). These data suggest that ::nifA reversible and irreversible mutant constructs behaved similarly in the nodulation process and mutations in N\(_2\) fixation genes may have advantages over wild type in nodulation.
The Reversion Rate of Reversible nifA Mutants is Unchanged in planta

To further investigate the influence of the host on nifA mutant nodulation, we examined the reversion rate of \( \Delta \)nifA to wild type under different growth conditions. The reversion rate of \( \Delta \)nifA grown in the absence of antibiotic selection in rich medium (PY broth) for 30 days was less than 1%, whereas the reversion rate of \( \Delta \)nifA grown around host roots was approximately 3% (Fig. 7A). Interestingly, the reversion rate of \( \Delta \)nifA isolated from 30-day-old nodules was elevated to approximately 8% (Fig. 7B), though no difference was detected from nodules formed by single-inoculation of \( \Delta \)nifA mutants or co-infection of wild type and \( \Delta \)nifA mutants, suggesting that little selective pressure from the host was imposed on nifA mutants during this colonization duration.

Co-infection of nifA Mutants with Wild Type does not Alter Nitrogen Fixation Rate

One prominent feature of rhizobium-legume interaction is that rhizobia are able to fix nitrogen after nodules are formed. To examine whether nifA mutants affect \( N_2 \) fixation, we measured nitrogenase activity from 30-day-old nodules. Fig. 8 shows that, as expected, neither \( \Delta \)nifA-induced nor \( \Delta \)nifA-induced nodules had much nitrogenase activity, as NifA is essential for activating genes that are required for nitrogen fixation. However, the level of nitrogenase activity from the co-infection of nifA mutants with wild type was indistinguishable with nodules formed by wild type strains. These results are surprising as nodulation occupancy and competitive index of nifA

---

**Figure 4. The \( \Delta \)nodB reversion rate in vitro and in planta.** (A). The reversion in vitro. \( \Delta \)nodB mutants were grown in PY medium in the absence of kanamycin (squares) at 28°C and were subcultured to fresh medium every other day or grown in rhizosphere of Phaseolus vulgaris planted in autoclaved Vermiculite (triangles). Samples were withdrawn at the time points indicated and colony formation units (CFU) of \( \Delta \)nodB (Sm\(^R\), Km\(^R\)) and nodB revertants (Sm\(^R\), Km\(^S\)) were determined by plating onto PY medium plates containing streptomycin (Sm) only and streptomycin plus kanamycin (Km). The reversion rate was calculated by the percentage of nodB revertants relative to total Sm\(^R\) cell number. Data are mean and s.d. of three independent experiments. (B). The reversion in nodules. Nodules formed by \( \Delta \)nodB single inoculation (squares) or by \( \Delta \)nodB wild type mixed inoculation (triangles). Nodules formed by mixed inoculations contained either only nodB (empty triangles) or a mixture of nodB and wild type strains (filled triangles). Nodules were harvested 30-day post-inoculation, surface-sterilized, crushed, and resuspended in PY medium. Colony forming units (CFU) of \( \Delta \)nodB (Sm\(^R\), Km\(^R\)), nodB revertants (Sm\(^R\), Km\(^S\)), and wild type (Sm\(^R\), Rif\(^R\)) were determined by plating onto PY medium plates containing Sm only, Sm+Rif, or Sm+Km. The reversion rate was calculated by the percentage of nodB revertants (Km\(^S\)) to total number (Sm\(^R\), Rif\(^S\)) of cells. Statistical analyses were performed using the Student’s t-test comparing to reversion rate of \( \Delta \)nodB mutants ex planta. NS: no significance; **: p<0.005. doi:10.1371/journal.pone.0070138.g004

**Figure 5. Nodule formation by wild type R. etli and its derivative nifA mutants.** (A). The number of nodules on Phaseolus vulgaris formed 30 days post-inoculation by wild type (empty circles), \( \Delta \)nifA (empty squares), \( \Delta \)nifA (empty triangles), 1:1 mixed inoculation of wild type and \( \Delta \)nifA (filled squares), and mixed inoculation of wild type and \( \Delta \)nifA (filled triangles). (B). Average biomass of the nodules. The data are the combination of 6 individual experiments. Statistical analyses were performed using the Student’s t-test comparing to nodule formation in the wild type strain. NS: no significance; ***: p<0.0001. doi:10.1371/journal.pone.0070138.g005

\( \Delta \)nifA grown around host roots was approximately 3% (Fig. 7A). Interestingly, the reversion rate of \( \Delta \)nifA isolated from 30-day-old nodules was elevated to approximately 8% (Fig. 7B), though no difference was detected from nodules formed by single-inoculation of \( \Delta \)nifA mutants or co-infection of wild type and \( \Delta \)nifA mutants, suggesting that little selective pressure from the host was imposed on nifA mutants during this colonization duration.

---

The Reversion Rate of Reversible nifA Mutants is Unchanged in planta

To further investigate the influence of the host on nifA mutant nodulation, we examined the reversion rate of \( \Delta \)nifA to wild type under different growth conditions. The reversion rate of \( \Delta \)nifA grown in the absence of antibiotic selection in rich medium (PY broth) for 30 days was less than 1%, whereas the reversion rate of
mutants were higher than wild type (Fig. 6) and the \( \text{nifA} \) reversion rate was less than 10% (Fig. 7). It is unclear whether, under such conditions, wild type rhizobia produce elevated nitrogenase activity to compensate for the loss resulted from \( \text{nifA} \) mutants or for some other unknown reason.

**Discussion**

Social cheating can be common in microbial populations [25–28]. Cheating individuals, acting selfishly, can persist within a population despite being detrimental to the long-term survival of that population [29]. Similarly, “cheaters” can sometimes evade host pressure to cooperate in mutualistic relationships, particularly in rhizobium-legume interactions where mutualism efficiency, at least as defined in terms of nodule productivity, remains intact [5]. Here, we study induced cheating in the plant symbiont \( \text{Rhizobium etli} \) through two important processes thought necessary for success of the symbiosis: nodule formation and nitrogen fixation. Our

---

**Figure 6. Competitiveness of \( \text{nifA} \) mutants in nodulation.** (A). Nodule occupancy. Nodules formed by 1:1 mixed inoculation of wild type and \( \Delta\text{nifA} \) (left panel) or \( \text{::nifA} \) (right panel) were harvested 30-day post-inoculation, surface-sterilized, crushed, and sequentially stabbed on PY agar containing Sm-Rif, Sm-Tc, Sm-Km. After incubation at 28°C for 72 hrs, plates were inspected for the identity of strains. Nodule occupancy is based on 90 nodules isolated from 3 plants. Data are mean and s.d. of four independent experiments. Statistical analyses were performed using the Student's t-test comparing colonization to that of wild type. ***: \( p < 0.0001 \). (B). Competitive index. For nodules containing mixed strains, CFU of wild type (Sm\(^\text{R} \), Rif\(^\text{R} \)), \( \Delta\text{nifA} \) (Sm\(^\text{R} \), Tc\(^\text{R} \)), \( \text{::nifA} \) (Sm\(^\text{R} \), Km\(^\text{R} \)), \( \text{nifA} \) revertants (Sm\(^\text{R} \), Km\(^\text{S} \)) was determined by plating on PY medium plates containing streptomycin (Sm) only and streptomycin plus kanamycin (Km). The reversion rate was calculated as percentage of \( \text{nifA} \) revertants (Km\(^\text{S} \)) to total number of cells (Sm\(^\text{R} \)). Data are the mean and s.d. of three independent experiments. NS: no significance.

doi:10.1371/journal.pone.0070138.g006

**Figure 7. The \( \text{::nifA} \) reversion rate in vitro and in planta.** (A). The reversion in vitro. \( \text{::nifA} \) strains were grown in PY medium in the absence of kanamycin (squares) at 28°C and were subcultured to fresh medium every other day or grown in rhizosphere of \( \text{Phaseolus vulgaris} \) planted in autoclaved Vermiculite (triangles). Samples were withdrawn at the time points indicated and colony forming units (CFU) of \( \text{::nifA} \) (Sm\(^\text{R} \), Km\(^\text{R} \)) and \( \text{nifA} \) revertants (Sm\(^\text{R} \), Km\(^\text{S} \)) were determined by plating on PY medium plates containing streptomycin (Sm) only and streptomycin plus kanamycin (Km). The reversion rate was calculated as percentage of \( \text{nifA} \) revertants (Km\(^\text{S} \)) to total number of cells (Sm\(^\text{R} \)). Data are the mean and s.d. of three independent experiments. (B). The reversion in nodules. Nodules formed by \( \text{::nifA} \) single inoculation (squares), or \( \text{::nifA} \)-wild type mixed inoculations (triangles). Of the mixed inoculations, nodules either contained only \( \text{nifA} \) (empty triangles) or a mixture of mutants with wild type strains (filled triangles). Nodules were harvested 30-day post-inoculation, surface-sterilized, crushed, and resuspended in PY medium. CFU of \( \text{::nifA} \) (Sm\(^\text{R} \), Km\(^\text{S} \)), \( \text{::nifA} \) revertants (Sm\(^\text{R} \), Km\(^\text{R} \)), and wild type (Sm\(^\text{R} \), Rif\(^\text{R} \)) were determined by plating on PY medium plates containing Sm only, Sm+Rif, or Sm+Km. The reversion rate was calculated as percentage of \( \text{nifA} \) revertants (Km\(^\text{S} \)) in total number of cells (Sm\(^\text{R} \), Rif\(^\text{S} \)) cells. Statistical analyses were performed using the Student's t-test comparing reversion rate of \( \text{::nifA} \) mutants ex planta. NS: no significance.

doi:10.1371/journal.pone.0070138.g007
results indicate that both intra- and inter-specific cheating is facilitated by cooperating strains in this system.

Intra-specific cheating, occurring when individuals within a population seemingly parasitize other individuals of the same species, has been shown to occur in a variety of natural systems [25–28]. In R. etli-host interactions, this cheating is predicted in situations such as nitrogen fixation to be thwarted by differential host sanctions imposed on non-cooperating nodules, likely due to allocating fewer resources to nodules supplying the host plant with little or no nitrogen [8]; however, few studies have examined the role of intra-specific cheating during the colonization process, a context potentially preceding the appropriation of resources and thus having little influence from the host. Here, we show that it is possible for mutants deficient in nodulation factor (Nod factors) production to colonize host nodules only if co-colonizing with Nod factor producing individuals, effectively “hitch-hiking” during the colonization process. We tested this using nodB mutants created by two different mechanisms (Fig. 1A) and theorized that those created by double-crossover deletions should not have the potential to revert to wild type, whereas those created by single-crossover events could revert. In this context, deletion strains (ΔnodB) could not colonize host legumes by themselves, though mutants could co-exist in roughly 30% of nodules when co-inoculated with wild type strains (Fig. 3A), suggesting that host legumes can be primed for nodule production by strains producing Nod factors. The ability of nod deletion mutants to colonize only in the presence of wild type strains can imply somewhat of a redundancy to nodulation factor production in environments where wild type individuals are common.

Though deletion strains could not colonize by themselves, we investigated the colonization of nodB mutants created by single-crossover mutations (::nodB) because of the potential for these strains to revert to wild type through subsequent homologous recombination events. Nodule occupancy levels for experiments in which ::nodB was co-inoculated with wild type strains were similar as those seen in the ΔnodB-wild type co-inoculations (Fig. 3A). Though nodule formation was possible when legumes were inoculated with only these mutants (::nodB) (Fig. 2A), 97.2% of nodB mutants found in these nodules had reverted to wild type, which is predicted by the fact that nodulation factor production is necessary for the establishment of nodules on host legumes [9]. Taken together with the necessity for wild type co-colonization in ΔnodB mutants, it is likely that these reversion events in ::nodB mutants are again simply necessary to establish nodule formation. As a whole, these data suggest that nodB mutants can potentially forego producing nodulation factors and instead rely on the production from neighboring individuals. Depending on the cost of nodulation factor production to wild type strains, these findings could have implications for a tragedy of the commons in which no individuals are able to colonize hosts due to a lack of nodulation factor production, though we have not surveyed isolates of R. etli to investigate whether nod mutants are common in natural systems.

Within the host nodule, it has been predicted that non-cooperating R. etli can be readily controlled, likely due to differential host sanctions meant to punish nodules deficient in nitrogen fixation [5]. Studies have shown that both Rhizobia occupancy per nodule and Rhizobia occupancy per nodule mass have decreased when forcing cheating by substituting an atmospheric N₂O₂ mixture with Ar:O₂ [8], though these experiments effectively force the entire rhizobia nodule population to cheat and may not represent host sanctions in the event of a mutant lineage deficient in nitrogen-fixation arising within an otherwise cooperating population. To test the effects of this, we created nifA mutants, again by two different mechanisms, hypothesizing that those mutants created by double-crossover deletions (ΔnifA) should not have the potential for reversion, while those created by single-crossover events (::nifA) could possibly revert to wild type. Surprisingly, single inoculation experiments with either ::nifA or ΔnifA mutants produced nodule numbers and sizes similar to those seen in wild type controls, contrary to what is predicted by host sanctions (Fig. 5A). Similarly, single inoculation experiments showed that neither ::nifA nor ΔnifA single inoculation experiments resulted in significantly different nodule weights compared to wild type controls (Fig. 5B). Although it is unclear why the host does not select against non-cooperating nodules, it is possible that host-derived sanctions could be detected after a longer growth period.

Though host sanctions are typically studied in contexts where whole nodules are forced to cheat, legume plants are typically infected with several bacterial lineages [5]. Within individual nodules, it could be expected that mutant bacterial lineages deficient in nitrogen-fixation could endure a selective advantage due to diminished metabolic burden, sweeping to high frequencies or fixation within the population. To study this, legumes were co-inoculated with either wild type strains and their ::nifA reversible mutant derivatives or wild type and their ΔnifA deletion derivatives. Surprisingly, in both cases, co-inoculations formed more nodules per plant (Fig. 5A) compared to wild type and single-inoculations of each of the mutants, though the average weight of each nodule remained similar (Fig. 5B). Within the nodules, roughly 50% contained only the mutant strain and approximately 40% contained a mixed population of wild type and mutants (for both ::nifA and ΔnifA). From these mixed populations, competitive index was measured and both mutants were found to have selective advantages relative to wild type strains, as expected by the decreased metabolic costs endured by lacking nitrogenase activity. Similarly, in ::nifA reversible mutants, reversion rate was low both in single inoculations as well as in mixed inoculations, likely due to the costs associated with reverting. Interestingly, nitrogenase activity in co-inoculation experiments showed similar levels to those seen in wild type. While nodules inoculated with mutant strains by themselves could not reduce acetylene, the high levels of...
nitrogenase activity seen in mixed inoculations could suggest that wild type strains can increase fixation of atmospheric nitrogen, though we have not investigated expression levels of nifH in these instances. As host sanctions would likely be subjected to the nodule as a whole, this finding could imply that, in a mixed population of cooperating and non-cooperating strains, cooperating individuals could increase nitrogenase activity to account for the deficit due to cheaters, likely in attempts to avoid sanctions imparted on the nodule as a whole, thus punishing both cooperating and non-cooperating strains.

In this study, we show both that *Rhizobium* mutants can cheat during the nodule colonization process as well as suggest mechanisms for the stable persistence of these cheaters within the population. As it is likely that natural *Rhizobium*-plant mutualisms contain many distinct bacterial lineages, this study implies a potentially relevant manner in which to study social parasitism in the natural context, as well as a reason for the persistence of many lineages within a population.

**Supporting Information**

**Data S1**

(XLSX)

**Acknowledgments**

We thank Dr. Susana Brom for providing *R. etli* CE3 strain, and Dr. Menghua Yang for helping strain constructs.

**Author Contributions**

Conceived and designed the experiments: JL HZ DSK ZZ JZ. Performed the experiments: JL HZ DSK HW. Analyzed the data: JL HZ DSK. Wrote the paper: JL HZ DSK JZ.

**References**

1. Long SR (1989) Rhizobium-legume nodulation: life together in the under-ground. Cell 56: 203–214.
2. Gonzalez V, Bistos F, Ramirez-Romero MA, Medrano-Soto A, Salgado H, et al. (2003) The mosaic structure of the symbiotic plasmid of *Rhizobium etli* CFE124 and its relation to other symbiotic genome compartments. Genome Biol 4: R36.
3. Pooput R, Martinez-Romero E, Gautier N, Prone JC (1995) Wild type *Rhizobium etli*, a bean symbiont, produces acetyl-fucosylated, N-methylated, and carboxamylated noduleation factors. J Biol Chem 270: 6050–6055.
4. Kaneo T, Nakamura Y, Sato S, Asamizu E, Kato T, et al. (2000) Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. DNA Res 7: 331–338.
5. Denison RF (2000) Legume sanctions and the evolution of symbiotic cooperation by rhizobia. American Naturalist 156: 567–576.
6. West SA, Kiers ET, Simms EL, Denison RF (2002) Sanctions and mutualism stability: why do rhizobia fix nitrogen? Proc Biol Sci 269: 685–694.
7. Smith JM (1989) Evolution: generating novelty by symbiosis. Nature 341: 284–285.
8. Kiers ET, Rouseau RA, West SA, Denison RF (2003) Host sanctions and the legume-rhizobium mutualism. Nature 425: 78–81.
9. M M, E.L S (1987) Occurrence and nature of mixed infections in nodules of field-grown soybeans (Glycine max). Biology and Fertility of Soils 5: 112–114.
10. Rolfe BG, Gresshoff PM (1980) Rhizobium-Trifolii Mutant Interactions during the Establishment of Nodulation in White Clover. Australian Journal of Biological Sciences 33: 491–504.
11. Dumas Z, Kummerf R (2012) Cost of cooperation rules selection for cheats in bacterial metapopulations. J Evol Biol 25: 473–484.
12. Noel KD, Sanchez A, Fernandez L, Leemans J, Cevallos MA (1984) Rhizobium Phaseoli symbiotic mutants with transposon Tn5 insertions. J Bacteriol 158: 140–155.
13. Songbook J, Russell DW (2001) Molecular cloning: a laboratory manual: CSHL press.
15. Rutter AE, Masters D (2003) The mosaic structure of the symbiotic plasmid of *Rhizobium etli* CFE124 and its relation to other symbiotic genome compartments. Genome Biol 4: R36.
16. Poulot R, Martinez-Romero E, Gautier N, Prone JC (1995) Wild type *Rhizobium etli*, a bean symbiont, produces acetyl-fucosylated, N-methylated, and carboxamylated noduleation factors. J Biol Chem 270: 6050–6055.
17. Kalogeraki VS, Wamun SC (1997) Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. Gene 188: 69–75.
18. Jinackern S, Sadowsky MJ (2008) Nodulation gene regulation and quorum sensing control density-dependent suppression and restriction of nodulation in the Bradyrhizobium japonicum-soybean symbiosis. Appl Environ Microbiol 74: 3749–3756.
19. Fahraeus G (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J Gen Microbiol 16: 374–381.
20. Hunt S, Layzell DB (1993) Gas exchange of legume nodules and the regulation of nitrogenase activity. Annual review of plant biology 44: 483–511.
21. Silvester WB, Sollins P, Verhoeven T, Clune SF (1982) Nitrogen fixation and acetylene reduction in decaying conifer boles: effects of incubation time, aeration, and moisture content. Canadian Journal of Forest Research 12: 646–652.
22. John M, Rohrig H, Schmidt J, Wienceke U, Schell J (1993) Rhizobium NodB protein involved in nodulation signal synthesis is a chitosodgossaccharide-deacetylase. Proc Natl Acad Sci U S A 90: 625–629.
23. Fischer HM (1994) Genetic regulation of nitrogen fixation in rhizobia. Microbiol Rev 58: 352–386.
24. Valderrama B, Davalos A, Girard L, Moster E, Mor J (1996) Regulatory proteins and co-acting elements involved in the transcriptional control of Rhizobium etli reiterated nifH genes. J Bacteriol 178: 3119–3126.
25. Gregor D, Travišano M (2004) The Prisoner’s Dilemma and polymorphism in yeast SUC genes. Proc Biol Sci 271 Suppl 3: S25–S26.
26. Velicer GJ, Kroos L, Lenski RE (2000) Developmental cheating in the social bacterium *Mycobacterium xanthus*. Nature 404: 598–601.
27. Dao DN, Kessin RH, Ennis HL (2000) Developmental cheating and the evolutionary biology of Dictyostelium and Myxococcus. Microbiology 146 (Pt 7): 1505–1512.
28. Sandos KM, Mitzinberg SM, Schuster M (2007) Social cheating in Pseudomonas aeruginosa quorum sensing. Proc Natl Acad Sci U S A 104: 15876–15881.
29. Fiega F, Velicer GJ (2003) Competitive fates of bacterial social parasites: persistence and self-induced extinction of Myxococcus xanthus cheats. Proc Biol Sci 270: 1527–1534.