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Host control over infection and proliferation of a cheater symbiont

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Abstract

Host control mechanisms are thought to be critical for selecting against cheater mutants in symbiont populations. Here, we provide the first experimental test of a legume host's ability to constrain the infection and proliferation of a native-occurring rhizobial cheater. *Lotus strigosus* hosts were experimentally inoculated with pairs of *Bradyrhizobium* strains that naturally vary in symbiotic benefit, including a cheater strain that proliferates in the roots of singly infected hosts, yet provides zero growth benefits. Within co-infected hosts, the cheater exhibited lower infection rates than competing beneficial strains and grew to smaller population sizes within those nodules. *In vitro* assays revealed that infection-rate differences among competing strains were not caused by variation in rhizobial growth rate or interstrain toxicity. These results can explain how a rapidly growing cheater symbiont – that exhibits a massive fitness advantage in single infections – can be prevented from sweeping through a beneficial population of symbionts.

Introduction

Bacterial symbionts offer fitness benefits to many plant and animal species (Sprent *et al.*, 1987; Nyholm & McFall-Ngai, 2004; Dethlefsen *et al.*, 2007; Zhang *et al.*, 2007). Yet, cheater mutants – symbiont genotypes that exploit hosts but fail to reciprocate benefits – are predicted to invade these populations. Selection models predict the evolutionary spread of exploitative mutants in infectiously acquired symbiont populations, as horizontal transmission can promote the fitness of strains that exploit resources from current hosts to maximize their spread to new hosts (Bull & Rice, 1991; Frank, 1996; Sachs *et al.*, 2004; Sachs & Simms, 2006, 2008). To counteract symbiont exploitation, hosts must evolve mechanisms to selectively exclude cheaters at the point of infection and/or sanction the uncooperative symbionts after infection has occurred (Bull & Rice, 1991; Denison, 2000; Simms & Taylor, 2002; West *et al.*, 2002; Sachs *et al.*, 2004). Yet, few experiments have challenged hosts with related symbiont strains that naturally vary in symbiotic quality (Sachs & Wilcox, 2006; Simms *et al.*, 2006; Bever *et al.*, 2009; Heath & Tiffin, 2009) to test whether hosts can select against uncooperative mutants.

The legume–rhizobium mutualism is an excellent model system to study symbiotic cooperation. Rhizobia are soil bacteria that form nodules on the roots of legumes and differentiate into specialized endosymbiotic cells called bacteroides, which fix atmospheric nitrogen in exchange for photosynthates provided by the plant (Sprent *et al.*, 1987). Rhizobial transmission among legumes is infectious (Sprent *et al.*, 1987), and individual plants are commonly nodulated by multiple rhizobial genotypes (e.g. Sachs *et al.*, 2009). Both of these characteristics, infectious transmission and a multiplicity of infection, are predicted to promote the evolution of parasitic strains that drain host resources without providing nitrogen in return (Bull & Rice, 1991; Sachs *et al.*, 2004; Sachs & Simms, 2008). Moreover, rhizobia fix nitrogen for hosts at a high metabolic cost (Trainer & Charles, 2006), so nonfixing rhizobial mutants can gain a fitness advantage over beneficial strains. Consistent with this theory, both nonfixing and poorly fixing rhizobia (symbiotically ineffective) exist alongside nitrogen-fixing rhizobia in agricultural and natural soils (Quigley *et al.*, 1997; Moawad *et al.*, 1998; Burdon *et al.*, 1999; Chen

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et al., 2002; Collins *et al.*, 2002; Sachs *et al.*, 2010). A key question is to discern what host control mechanisms keep such a cheater genotype from sweeping through the symbiont population.

It has been widely predicted that ineffective rhizobia are cheaters, exhibiting an adaptive uncooperative strategy (Denison, 2000; Denison & Kiers, 2004) by redirecting plant carbon towards selfish ends (Hahn & Studer, 1986; Lopez *et al.*, 1995; Denison, 2000; Denison & Kiers, 2004) instead of channelling it into the energetically expensive nitrogen fixation pathway (Trainer & Charles, 2006). An alternative hypothesis is that many ineffective rhizobia are maladapted to the agricultural hosts that they were tested on and experience little or no benefits from these infections (Sachs & Simms, 2008). Only recently has a naturally occurring rhizobial cheater been discovered. The cheater *Bradyrhizobium* strain proliferates in *Lotus strigosus* nodules when hosts are singly infected and offers no growth benefit to *L. strigosus* hosts (Sachs *et al.*, 2010). Unlike any other ineffective rhizobia studied to date, this *Bradyrhizobium* exhibits superior fitness during infection of native hosts, which is five- to 10-fold higher than beneficial strains.

Host control over symbiotically ineffective rhizobia has been modelled to occur at two potential time points: at the initiation of the symbiosis via selective infection (e.g. 'partner choice'; Simms & Taylor, 2002; Sachs *et al.*, 2004) and during the maintenance of the symbiosis via 'post-infection sanctions' of uncooperative strains (Denison, 2000). Partner choice can clearly protect legumes from some types of uncooperative rhizobia, such as lineages of toxin-producing rhizobia that are blocked from nodulation relative to nontoxin-producing strains (Devine *et al.*, 1990; Devine & Kuykendall, 1996). However, legumes appear to have little ability to detect differences in nitrogen fixation at the point of infection. For instance, partner choice experiments that used nonfixing mutants (generated in labs) have found that these strains infect hosts (Kuykendall & Elkan, 1976) and compete successfully for nodulation with beneficial strains (Amarger, 1981; Hahn & Studer, 1986), inconsistent with partner choice theory. Only Champion and colleagues (1992) found evidence that such a nonfixing mutant exhibited lowered infection compared to related beneficial strains, and the difference was rather small. Among experiments that have examined naturally occurring rhizobia, Simms and colleagues (2006) found that strains that offer mediocre benefits competed successfully for nodulation against highly beneficial strains, and Sachs and colleagues (2010) discovered native strains that offered little or no benefits to hosts, yet nodulated hosts (in single infections) as efficiently as beneficial strains, both inconsistent with partner choice theory. In contrast, Heath & Tiffin (2009) found evidence that hosts preferentially exclude strains that provide poor benefits to those specific host genotypes, but they did not rule out the effects of interstrain competition or interstrain toxicity on host infection rates.

There is currently more support for the post-infection sanctions hypothesis than for partner choice. Tests of the post-infection sanctions hypothesis using soya beans and lupines have found that hosts can promote the growth of nodules that house more effective strains (Singleton & Stockinger, 1983; Singleton & Van Kessel, 1987) and cause nodules with nonbeneficial rhizobia to be smaller and bear fewer bacteria (Kiers *et al.*, 2003; Simms *et al.*, 2006). Yet, only Simms and colleagues (2006) used naturally occurring strains, and more recent soya bean experiments have cast doubt on earlier findings. Experiments by Marco and colleagues (2009) co-infected multiple soya bean genotypes with effective and ineffective rhizobia and measured rhizobial fitness via real-time PCR as well as by culturing, but found no evidence that ineffective rhizobia were sanctioned. Finally, studies in other legume species have also found no evidence consistent with sanctions (Atkins *et al.*, 1984; Pate *et al.*, 1984; Heath & Tiffin, 2009; Sachs *et al.*, 2010).

Here, we offer the first test of both the partner choice and post-infection sanctions models of legume host control using a naturally occurring cheater *Bradyrhizobium* (in which exploitation of sympatric *Lotus* hosts has been empirically demonstrated; Sachs *et al.*, 2010). We infected *L. strigosus* hosts with single and dual genotype *Bradyrhizobium* infections that varied in symbiotic benefit and simultaneously estimated host and symbiont fitness. To rule out the effects of interstrain competition and/or interstrain toxicity on host infection rates, we also estimated bacterial fitness in single and dual strain inocula without the host present. Unlike any previous study, we sampled beneficial and cheater rhizobia as well as their hosts from the same environment, so we can test the evolutionary response of our host population to locally encountered uncooperative strains (Sachs *et al.*, 2009, 2010). The three main goals of the experiment were to (i) test whether naturally occurring uncooperative *Bradyrhizobium* mutants nodulate hosts at reduced rates when competing against cooperative strains (partner choice hypothesis), (ii) examine whether the uncooperative bradyrhizobia exhibit reduced population sizes within host nodules (post-infection sanctions hypothesis) and (iii) distinguish the effects of interstrain competition and interstrain toxicity versus host control in the relative fitness of rhizobial strains.

Materials and methods

Bradyrhizobium test cultures

Six strains were selected from previous genotypic and phenotypic surveys of wild *Bradyrhizobium japonicum* (strains #'s 2, 18, 24, 37, 38, 49; Sachs *et al.*, 2010) based on variation in their fitness effects on hosts (infected host growth relative to uninoculated controls), their own

fitness within hosts during infection (population size within nodules) and our ability to differentiate strains in culture via antibiotic resistance. In a previous study (Sachs *et al.*, 2010), four of the strains were classified as highly beneficial on *L. strigosus* hosts (#'s 18, 24, 37, 49), one provided relatively poor benefit (#38) and one strain provided zero growth benefits to *L. strigosus* (#2). Hereafter, strains #2 and #38 are referred to as uncooperative. Growth effects on hosts ranged from a 500% growth benefit (#24, #49) to a 6% growth reduction (#2) compared to uninoculated controls. Strain #2 was identified as a cheater mutant because it induced zero or negative host growth (compared to uninoculated controls) and proliferated rapidly in *Lotus* host tissues, reaching per mass densities within nodules that were almost an order of magnitude above any of the beneficial strains tested previously (Sachs *et al.*, 2010).

In vitro rhizobial growth assays

We measured relative growth rates of the rhizobial strains in clonal and competitive cultures. As numerical dominance of rhizobia on host roots can be a key determinant of infection rate (Vlassak & Vanderleyden, 1997), we used growth rate to predict a null hypothesis of rhizobial infection rates on plants. We estimated each rhizobial strain's clonal growth rate over the time course and conditions of greenhouse inoculations leading to nodulation (approximately 29 °C, 90 h to initiate infection; Haynes *et al.*, 2004). Clonal liquid cultures of each strain were initiated (2 μL of archived stocks in 30 mL Modified Arabinose Gluconate Media (MAG); Sachs *et al.*, 2009), and once at log phase, cultures were diluted into 12 replicate cultures in vials (4 mL, 4×10^5 cells) and incubated for 90 h in a shaking incubator (29 °C, 180 rpm) before cell density was estimated (using optical density). To estimate each strain's growth rate in mixed inocula and thus test for the effects of interstrain toxicity, we followed the same procedure as mentioned earlier, but the 12 replicate cultures were composed of equal mixes of each of the co-infecting strain combinations (e.g. #'s 2, 18; 2, 24; 2, 37; 2, 49; 18, 38; 24, 38, 37, 38, 38, 49; 4 mL, 2×10^5 cells each). After 90 h of growth, cell densities were estimated. Each coculture was plated at low density (10 cells mL^{-1}), and at least 100 of the resultant colonies were replica subcultured on the appropriate antibiotic treatments to resolve the relative concentrations of the cocultured strains.

Inoculation protocol and design

We followed the host infection protocol of Sachs and colleagues (2009). Briefly, *L. strigosus* fruits were collected at Bodega Marine Reserve, CA, USA from the same population as the tested *Bradyrhizbium* strains (within 100 m). Seed production of *L. strigosus* is low at this field site (20–200 seeds per plant), so we gathered seeds from hosts throughout the site and created a random mix of seeds in which parental plants were equally represented throughout the field site. Because the efficiency of legume control over uncooperative rhizobia can vary with host genotype (Kiers *et al.*, 2007), we chose not to focus our experiment on a small handful of host genotypes but instead we sought to assay the mean host response from this small population. Seeds were surface sterilized in bleach, rinsed in sterile ddH_2O and nick scarified and germinated in sterile ddH_2O. Seedlings were planted into bleach-sterilized pots filled with autoclaved quartzite sand and incubated in a growth chamber (20 °C, 80% relative humidity, 12:12 day/night cycle, 2× daily misting, 14 days) before being transferred to a greenhouse under approximately 50% shade for hardening (14 days, 2× daily misting). Once in the greenhouse, plants were fertilized weekly with Jensen's nitrogen-free solution (Somasegaran & Hoben, 1994), beginning with 2 mL per seedling, increasing by 1 mL each week until reaching 5 mL per plant, which was used thereafter. *Bradyrhizobium* cultures were initiated from approximately 2 μL of original frozen stock inoculated into 250 mL of liquid MAG media and incubated to logarithmic phase growth in a shaking incubator (29 °C, 180 rpm, 3–5 days). Bacterial concentrations were estimated via optical density (OD) using a Klett colorimeter and the following curve: rhizobial population $mL^{-1} = ((4.576 \times 10^6)\ (OD^{culture}–OD^{blank}) – (4.632 \times 10^7))$. Grown cultures were lightly centrifuged (5000 *g*, 20 min) and resuspended in prewarmed sterile ddH_2O to concentrations of 10^8 cells mL^{-1}. Single inoculation treatment plants were infected with 5 mL of a single strain added directly to the soil, and co-inoculated plants received an equal mix of two strains adding up to the same volume and bacterial concentration. Control plants were inoculated with 5 mL of sterile ddH_2O.

Each inoculation experiment was performed with a replicated, blocked design (Table 1). Sterile-grown *L. strigosus* seedlings were arranged by size and divided into blocks. Four inoculation experiments consisted of 108 plants each (six replicate plants per treatment, six treatments, three replicate blocks). Within each block, sets of six size-matched sterile-grown seedlings were randomly assigned to one of six different treatments: three single-strain inoculation treatments (two beneficial strains, one uncooperative strain), two co-inoculation treatments (pairing each of the beneficial strains separately with the uncooperative strain) and one control treatment. We did not co-inoculate different beneficial strains, as we were only interested in whether hosts could exert control over strains that offered little or no benefits to hosts. Co-infected strains could be differentiated in plated cultures by their antibiotic resistance profiles. Antibiotic resistance of the strains are as follows (plus = resistant, minus = sensitive): #2 (100 ug mL^{-1} streptomycin$^+$), strain #18 (100 ug mL^{-1} streptomycin$^-$, 50 ug mL^{-1} ampicillin$^+$), #24 (100 ug mL^{-1} streptomycin$^-$, 50 ug mL^{-1}

Table 1 Summary of inoculation design.

Expt.	Date	Inoculation treatments	Blocks	*N*
I	23 January 2009	2; 18; 24; 2 + 18; 2 + 24; ctl	3	108
II	26 January 2009	38; 18; 24; 38 + 18; 38 + 24; ctl	3	108
III	2 April 2009	2; 37; 49; 2 + 37; 2 + 49; ctl	3	108
IV	2 April 2009	38; 37; 49; 38 + 37; 38 + 49; ctl	3	108
V	2 April 2009	2; 24; 2 + 24; ctl	3	72

Inoculation dates, inoculation treatments and block and plant replicates are shown. Expt = experiment number, Date = inoculation date, Inoculation treatments = rhizobial strains and strain combinations that were infected into six plants each per block with the less beneficial strain first (Sachs *et al.*, 2010) followed by the two beneficial strains and the co-infections, Blocks = number of replicated blocks randomly placed in the greenhouse, *N* = total number of plants in each experiment, ctl = control plants that were inoculated with sterile ddH_20.

ampicillin$^+$), #37 (100 ug mL^{-1} streptomycin$^-$, #25 ug mL^{-1} carbenicillin$^+$), #38 (50 ug mL^{-1} ampicillin$^-$) and #49 (100 ug mL^{-1} streptomycin$^-$, 50 ug mL^{-1} ampicillin$^+$). Inoculation experiments were initiated on 23 January 2009 (beneficial strains #18, #24, uncooperative strain #2), 26 January 2009 (beneficial strains #18, #24, uncooperative strain #38) and 2 April 2009 (beneficial strains #37, #49, uncooperative strain #2; beneficial strains #37, #49, uncooperative strain #38). Finally, to study potential differences caused by changes in day length, one smaller experiment (72 plants) was also initiated on 2 April 2009 and included only the beneficial strain 24 and the uncooperative strain 2 plus co-infections and controls. This added study carries information about season because it repeats a strain combination tested in January. All five experiments included a total of 504 plants.

Plant harvest

One replicate block from each experiment was harvested 5 weeks after inoculation, and the remaining two blocks were harvested 3 weeks later. At harvest, plants were unpotted, separated into roots and shoots, and shoots were oven-dried and weighed to measure growth rate, a component of host plant fitness. All plants were examined for evidence of nodulation, which was not found in any controls. All nodules were dissected off the roots of each plant and were counted and photographed on 1 mm grid graph paper to assess nodule area (mm^2), a correlate of nodule mass. Nodule size is one commonly measured component of rhizobial fitness (Simms *et al.*, 2006; Heath & Tiffin, 2009; Sachs *et al.*, 2010).

Assays of rhizobial fitness: nodule occupancy and population size within nodules

Culturing of nodules allows us to estimate the number of viable rhizobia within each nodule, which are ultimately released from *Lotus* nodules into the soil (Mergaert *et al.*, 2006). Details of the nodule-culturing protocol can be found in Sachs *et al.* (2010). Briefly, two plants from each inoculated treatment were randomly selected for nodule culturing from both the 5-week and 8-week harvests. We dissected six randomly chosen nodules from each of two replicate plants from each tested treatment. Each nodule was surface sterilized, rinsed and crushed with a sterile pestle, and the nodule rhizobia were plated on solid MAG media with replicated serial dilutions of 10^{-3} and 10^{-5}. For the nodules of co-infected plants, at least 100 plated rhizobial colonies per nodule culture were replica subcultured on an antibiotic treated plate and a control plate (MAG media) to estimate the relative proportion of each inoculated strain. We ruled out evidence of horizontal transfer of antibiotic resistance between strains by additionally genotyping a subset of colonies as well as distinguishing a subset of the strains using significant differences in colony size (Sachs, unpublished).

Data analysis

Relative plant growth effects of the single-strain infection treatments were calculated by dividing the shoot dry mass of each inoculated plant by the mass of its size-matched (uninfected) control. Per plant nodule occupancy of each co-infecting strain was estimated as the proportion of nodules that each strain infected, irrespective of relative strain density within the nodule. Within nodule population size of each co-infecting strain was calculated as the total rhizobial population per nodule multiplied by the relative proportion of the focal strain within the nodule. The proportional measures within each nodule are nonindependent, so to analyse these data we randomly assigned the population density estimates for each strain within a nodule to one of two independent datasets and tested hypotheses separately in each data subset (using adjusted alpha values of 0.025 for repeated tests). Whole plant rhizobial fitness was calculated by multiplying the mean strain population per nodule times the number of nodules, as well as by multiplying mean bacterial strain per nodule area times the total nodule area of the plant. Both methods gave similar results and we report only the former. We used analysis of variance (ANOVA) or nested ANOVAS in which

replicate blocks were treated as random effects. We used post hoc pairwise Student's *t*-tests to compare among treatment effects (experiments I–IV and *in vitro* growth assays). In experiment V, which only involved two strains, we used *t*-tests to analyse treatment effects. For the *in vitro* growth assays of rhizobial strains, we calculated doubling time of each strain using a least squares fitting exponential (Eric. W. Weisstein, From MathWorld – A Wolfram Web Resource. http://mathworld.wolfram.com/LeastSquaresFittingExponential.html.)

Results

In vitro growth rates of rhizobia

In vitro growth rates of clonal rhizobia were significantly different among strains ($F_{5,71} = 357.9$, $P < 0.0001$), ranging from a doubling time of approximately 7.2 h (strain #49) to approximately 8.3 h (strain #38; Fig. 1). Strain #2 (the cheater strain; Sachs *et al.*, 2010) was the second fastest growing strain (doubling time approximately 7.4 h). Competitive *in vitro* growth assays (that estimated individual rhizobial growth rates in dual-inoculated cultures) revealed no emergent effects of interstrain competition or toxicity: the differences in growth rates that we found among strains (in the clonal infections) perfectly predicted the relative growth of the same strains when in competition with each other (Fig. 1; Table S1). Assuming that numerical dominance (caused by differences in growth rate) is a major determinant of infection rate (Vlassak & Vanderleyden, 1997), we predict the following null hypothesis for infection rates of the uncooperative strains: the cheater strain #2 outcompetes cooperative strains #'s 18, 24 and 37, but is outcompeted by #49, whereas the relatively ineffective strain #38 is outcompeted by all the cooperative strains.

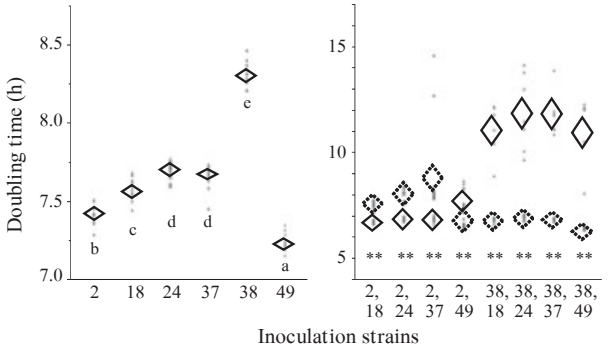

Fig. 1 *In vitro* growth rates for clonal cultures are shown on the left and cocultures on the right. X axes indicate the strain(s) used within each (co-)culture, with the uncooperative strains listed previously for cocultures (Sachs *et al.*, 2010). The Y axes indicate the doubling time of the strain(s) calculated using a least squares fitting exponential (E. W. Weisstein, Mathworld Wolfram Web Resource; http://mathworld.wolfram.com/LeastSquares FittingExponential.html). Diamonds indicate mean doubling time for each strain ± standard error, with uncooperative strains indicated with solid diamonds (right only). Superscript letters (left) indicate significant differences among treatments using a Student's *t*-test. Double asterisks (right) indicate coculture experiments with significant growth differences among the competing strains ($P < 0.025$, both two-tailed *t*-tests).

Plant growth effects of single-strain inoculations

The single-strain inoculations on *L. strigosus* confirmed previous results (Sachs *et al.*, 2010), that strains #2 and #38 are relatively uncooperative rhizobia (our *a priori* assumption; Fig. 2). The growth effects of rhizobial strains were less pronounced in the 5-week than 8-week harvests, so only the latter is reported. Strain #2 (the cheater strain; Sachs *et al.*, 2010) caused zero growth (experiments I, III) or negative growth (experiment V) compared to uninfected controls. Strain #2 was less beneficial (in terms of host growth) than all the strains it was paired with. Strain #38 provided relatively poor host growth benefits that were significantly worse than strains 37 and 49 (experiment IV) but could not be distinguished from strains 18 and 24 (experiment II) even though the trend was the same (experiment I: $F_{2,33} = 11.8$, $P = 0.0002$; experiment II: $F_{2,34} = 0.1$, $P = 0.94$; experiment III: $F_{2,32} = 13.1$, $P < 0.0001$; experiment IV: $F_{2,35} = 11.6$, $P = 0.0002$; experiment V: $F_{1,23} = 148.9$, $P < 0.0001$). Strain #2 caused significantly more nodules than other strains in two of three experiments and generated smaller nodules in all three experiments (see Table S2).

Rhizobial nodule occupancy in co-infected plants

Of the 190 nodules from co-inoculated plants that we tested for strain occupancy, only five were occupied solely by the strains that were *a priori* defined as less beneficial (Sachs *et al.*, 2010), whereas 94 of the nodules were co-infected and 91 were occupied solely by the more beneficial strain (Fig. 3, Table S3), consistent with the partner choice hypothesis. We found significantly different rates of nodule occupancy between paired strains (beneficial versus uncooperative) in each of the experiments except II, which was also the only experiment where we failed to detect any growth effect differences among rhizobial strains (experiment I: $F_{3,15} = 5.6$, $P = 0.0119$; experiment II: $F_{3,15} = 0.5$, $P = 0.71$; experiment III: $F_{3,15} = 68.3$, $P < 0.0001$; experiment IV: $F_{3,15} = 4.3$, $P = 0.028$; experiment V: $F_{1,7} = 120.2$, $P < 0.0001$). Rhizobial strains that were *a priori* defined as more beneficial, occupied (or co-occupied) all tested nodules, except in experiment II. Of the seven paired rhizobial strains that caused significantly different effects on host growth (Table S3), nodule occupancy was significantly higher for the more beneficial strain in five of these (strains 2, 18; 2, 37; 2, 49; 38, 49; and 2, 24 from experiment V), whereas we could

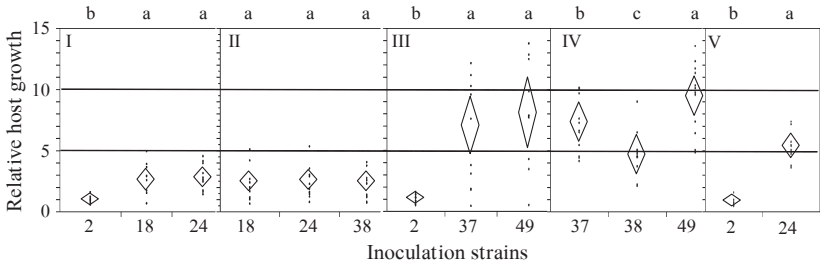

Fig. 2 Host growth effects of single inoculations are shown for the 8-week harvests (experiments I–V). Relative growth is measured as biomass of inoculated plants divided by their size-matched control plants. Diamonds indicate mean relative growth ± standard error. Superscript letters indicate which treatments differed significantly within each experiment (experiments I–IV: ANOVA and post hoc Student's *t*-tests; experiment V: two-tailed *t*-test).

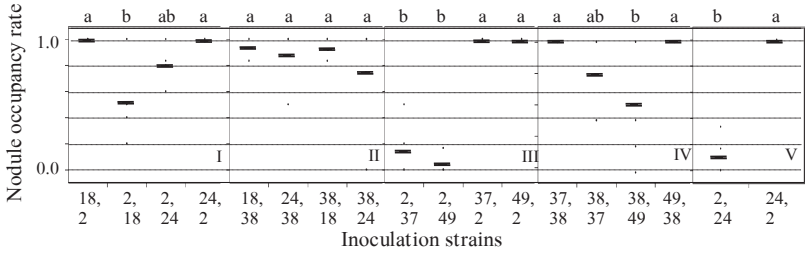

Fig. 3 Mean nodule occupancy rates are shown for co-infection experiments (5-, 8-week harvests combined; experiments I–V). *X* axes indicate the inoculation strains, with focal strain listed previously for each co-infection. Bars indicate mean nodule occupancy rate for each focal strain. Superscript letters indicate which treatments differed significantly within each experiment (experiments I–IV: ANOVA and post hoc Student's *t*-tests; experiment V: two-tailed *t*-test).

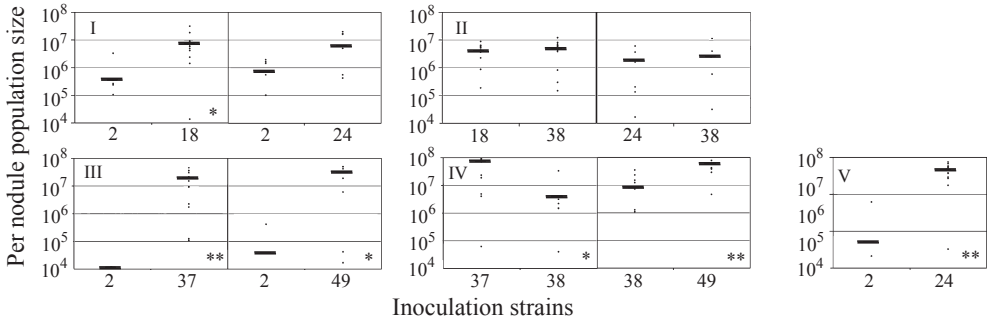

Fig. 4 Mean per nodule rhizobial population sizes are shown for co-infection experiments (8-week harvest; experiments I–V). *X* axes indicate the inoculation strains. Bars indicate mean nodule population size for each focal strain. Single and double asterisks indicate significant growth differences among the competing strains ($P < 0.025$, significant in one- or two-tailed *t*-tests, respectively).

not detect significant nodule occupancy differences in the others (strains 38, 18; 38, 24; 38, 37; and 2, 24 from experiment I). In all nonsignificant cases but one (38, 18), the more beneficial strain still exhibited higher nodule occupancy rates.

Within nodule rhizobial population size

Rhizobial population sizes within nodules generally increased between the five- and 8-week harvests (Table S4). The differences among strains were more pronounced in the latter harvest, which we discuss here. In the 8-week harvest, we found significant differences among strains in each of the experiments except II. Consistent with the post-infection sanctions hypothesis, we found that of the seven paired rhizobial strains that caused significantly different effects on host growth in single infections (Fig. 4; Table S4), rhizobial population size was significantly higher for the more beneficial strain in six of these (strains 2, 18; 2, 37; 2, 49; 38, 37; 38, 49; and 2, 24 from experiment V). We could not detect significant population size differences in the other paired strains (strains 2, 24, from experiment I), although the trend was still the same.

Discussion

Hosts of beneficial bacteria most often acquire their symbionts environmentally (Nyholm & McFall-Ngai, 2004) and thus are predicted to evolve mechanisms to counteract infection by cheater mutants. Hosts can select against symbiont cheater at the point of infection (partner choice: Bull & Rice, 1991; Simms & Taylor, 2002; Sachs *et al.*, 2004) or via post-infection sanctions (Denison, 2000). For instance, homeostasis in the human gut-floral community is promoted by antimicrobial responses within the host epithelium (Vaishnava *et al.*, 2008). In bobtail squids that host the bioluminescent bacterium *Vibrio fisheri* (Nyholm & McFall-Ngai, 2004), mutant symbionts that fail to produce light are eliminated from the host's light organ (Visick *et al.*, 2000). Fungus-growing ants harbour antibiotic-producing symbiotic bacteria that protect their gardens from parasites (Currie *et al.*, 1999) and actively choose their native beneficial symbiont over related strains (Zhang *et al.*, 2007). All of these systems appear consistent with host control mechanisms. Yet, only rarely have experimenters directly tested whether hosts can actively select for beneficial partners over uncooperative mutants (Sachs & Simms, 2008), as opposed to the more general pattern of host–symbiont lineage specificity. Only in the squid system mentioned earlier did experimenters challenge hosts with related symbiont strains that varied in symbiotic quality, and in this case the uncooperative mutants were generated in the laboratory (Visick *et al.*, 2000).

Our experiments here provide the strongest evidence to date that a legume host can bias infection rates towards beneficial rhizobia over uncooperative strains (partner choice hypothesis). In particular, we found that *Bradyrhizobium* strains with high symbiotic quality nodulate *L. strigosus* at significantly higher rates than uncooperative competitors (e.g. experiments I, III, IV, V) but that hosts do not differentiate among rhizobia that provide nondetectable differences in growth benefits (experiment II). Competition among strains for nodulation can be mediated mainly by numerical differences among competing strains (Vlassak & Vanderleyden, 1997), but our data suggests that nodulation preference is more important. Our *in vitro* rhizobial fitness analyses allow us to reject the null hypothesis that variation in nodulation rates was caused by differences in rhizobial growth rates or interstrain toxicity. Strain #2, the most uncooperative strain was also among the fastest growing and most competitive *in vitro*, but nonetheless infected nodules at relatively low rates in competition.

Past experiments may have failed to find such strong nodulation preferences for multiple reasons. When lab-generated nonfixing mutants were used, it could be that the hosts could not differentiate these from nearly isogenic cooperative strains (Amarger, 1981; Hahn & Studer, 1986; Champion *et al.*, 1992). When strains that naturally varied in symbiotic quality were used, it may have been that the natural differences in symbiotic quality were too slight (Simms *et al.*, 2006). Moreover, our data stand in contrast to agricultural studies that have often found that the ability of rhizobia to compete for nodulation is unrelated to symbiotic quality (e.g. Vasquez-Arroyo *et al.*, 1998; Bloem & Law, 2001; Hafeez *et al.*, 2001). These studies used non-native rhizobia and/or hosts, so differences in adaptation to local soils or different hosts can confound the results. In contrast to all previous studies, we sampled beneficial and cheater rhizobia as well as their hosts from the same environment, thus our results likely represent the evolutionary response of our host population to locally encountered uncooperative strains that are genetically diverged from the cooperative strains (Sachs *et al.*, 2009, 2010).

Our data also add further support for the post-infection sanctions hypothesis (Denison, 2000), specifically that (*L. strigosus*) hosts can selectively reward beneficial rhizobia within growing nodules and punish poorly beneficial strains (e.g. Kiers *et al.*, 2003; Simms *et al.*, 2006). Population sizes of beneficial strains within nodules were often 10–100 times higher compared to the relatively uncooperative strains that they were paired with (which has a much more severe effect than that has been previously discovered). Models of post-infection sanctions often predict that a high prevalence of mixed-infection nodules can reduce the efficiency of sanctions at the nodule level (Denison, 2000). In our experiment, nearly half of nodules tested were found to be co-infected, which suggests to us that the host sanctions may have occurred at the bacteroid level (within nodules; e.g. Denison, 2000).

Legumes potentially exhibit multiple mechanisms to reward beneficial rhizobia and/or to punish uncooperative strains. Our phenotypic data suggests that these mechanisms function during initial rhizobial infection as well as the later development of the *Lotus–Bradyrhizobium* symbiosis. Legumes can first gain some control over rhizobia via nodulation specificity, which is now known to be expressed by multiple host genes (e.g. Endre *et al.*, 2002; Madsen *et al.*, 2003) that likely evolved to protect legumes from rhizobia that are poorly matched or provide insufficient benefits (Devine & Kuykendall, 1996). However, only in the case of host recognition of toxin-producing rhizobia (Devine *et al.*, 1990; Devine & Kuykendall, 1996), has it been obvious that legumes are specifically blocking uncooperative rhizobia in favour of more beneficial strains. If legumes initially recognize symbiotically ineffectiveness via genotype specific signals, such as nod factors or cell-surface molecules, these signals must be reliably correlated with symbiotic benefit to provide fitness benefits to hosts (Simms & Taylor, 2002). In the population of *Bradyrhizobium* that we studied, the uncooperative rhizobial strains were always found to be genetically diverged from the beneficial strains at both housekeeping and symbiotic loci (Sachs *et al.*, 2009, 2010), but this may not always be the case. Experiments that have manipulated nitrogen fixation

ability via introducing mutations into these loci suggest that legumes cannot detect nitrogen fixation ability *per se* at the point of infection. Nonetheless, we found that strain 38 (the marginally beneficial strain; Sachs *et al.*, 2010) achieved relatively high nodule occupancy when it was paired with strains that caused similar host growth (experiment II), but exhibited low nodule occupancy when paired with significantly more beneficial strains (experiment IV). These data are consistent with early host recognition of symbiotic quality, but we are not aware of the mechanistic basis for such detection. Although legumes can clearly detect and react to rhizobia that exhibit poor symbiotic quality (Kiers *et al.*, 2003; Simms *et al.*, 2006; data herein), it is not apparent whether these hosts react differently to cheaters compared to other uncooperative rhizobia. As we have only isolated one strain that fulfils the definition of a cheater (Sachs *et al.*, 2010), experiments would be need to be performed that include other exploitative strains to further explore the relationship between exploitation of the host and host response.

Conclusion

In earlier work, Sachs and colleagues (2010) discovered a naturally occurring cheater *Bradyrhizobium*. In single-strain infections, the cheater proliferates within *L. strigosus* nodules, attains higher density than beneficial strains, but provides no growth benefits to hosts. Here, we conducted dual inoculation experiments using *Bradyrhizobium* strains that vary in symbiotic quality on *L. strigosus*, ranging from the nonbeneficial cheater to highly beneficial strains. We found that *L. strigosus* hosts can constrain the infection and later proliferation of this cheater in dual strain infections. *In vitro* competition experiments allowed us to reject the alternative hypothesis that variation in nodulation rates is caused by differences in rhizobial growth rates or interstrain toxicity.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Measures of rhizobial fitness in clonal and competitive *in vitro* cultures.

Table S2 Single-strain inoculation phenotypes.

Table S3 Total and average nodule occupancy rates.

Table S4 Nodule-level rhizobial strain fitness (Separate Harvests).

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