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Nonnodulating *Bradyrhizobium* spp. Modulate the Benefits of Legume-Rhizobium Mutualism

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ABSTRACT

Rhizobia are best known for nodulating legume roots and fixing atmospheric nitrogen for the host in exchange for photosynthates. However, the majority of the diverse strains of rhizobia do not form nodules on legumes, often because they lack key loci that are needed to induce nodulation. Nonnodulating rhizobia are robust heterotrophs that can persist in bulk soil, thrive in the rhizosphere, or colonize roots as endophytes, but their role in the legume-rhizobium mutualism remains unclear. Here, we investigated the effects of nonnodulating strains on the native *Acmispon-Bradyrhizobium* mutualism. To examine the effects on both host performance and symbiont fitness, we performed clonal inoculations of diverse nonnodulating *Bradyrhizobium* strains on *Acmispon strigosus* hosts and also coinoculated hosts with mixtures of sympatric nodulating and nonnodulating strains. In isolation, nonnodulating *Bradyrhizobium* strains did not affect plant performance. In most cases, coinoculation of nodulating and nonnodulating strains reduced host performance compared to that of hosts inoculated with only a symbiotic strain. However, coinoculation increased host performance only under one extreme experimental treatment. Nearly all estimates of nodulating strain fitness were reduced in the presence of nonnodulating strains. We discovered that nonnodulating strains were consistently capable of coinfecting legume nodules in the presence of nodulating strains but that the fitness effects of coinfection for hosts and symbionts were negligible. Our data suggest that nonnodulating strains most often attenuate the *Acmispon-Bradyrhizobium* mutualism and that this occurs via competitive interactions at the root-soil interface as opposed to *in planta*.

IMPORTANCE

Rhizobia are soil bacteria best known for their capacity to form root nodules on legume plants and enhance plant growth through nitrogen fixation. Yet, most rhizobia in soil do not have this capacity, and their effects on this symbiosis are poorly understood. We investigated the effects of diverse nonnodulating rhizobia on a native legume-rhizobium symbiosis. Nonnodulating strains did not affect plant growth in isolation. However, compared to inoculations with symbiotic rhizobia, coinoculations of symbiotic and nonnodulating strains often reduced plant and symbiont fitness. Coinoculation increased host performance only under one extreme treatment. Nonnodulating strains also invaded nodule interiors in the presence of nodulating strains, but this did not affect the fitness of either partner. Our data suggest that nonnodulating strains may be important competitors at the root-soil interface and that their capacity to attenuate this symbiosis should be considered in efforts to use rhizobia as biofertilizers.

Rhizobia are heterotrophic soil bacteria with diverse lifestyles. Some rhizobial lineages have acquired the capacity to form nodules on legume roots and fix atmospheric nitrogen for these hosts (1). Nodulating rhizobia are attracted to flavonoids released by legumes. In response, the rhizobia secrete nod factors that provoke morphological changes to the roots, enabling the bacteria to enter root cortical cells, become encased by a plant-derived membrane, differentiate into bacteroids, and fix nitrogen (2, 3). Among nodulating rhizobia, nodulation genes are typically carried on symbiosis plasmids (4, 5) or on a genomic island (the “symbiosis island”) (6–9). However, soil populations consistently include rhizobia that do not individually nodulate legume hosts (10–18), often because they lack key loci that are needed to induce nodulation (19, 20).

Rhizobial strains that nodulate host roots can have dramatic effects on legume fitness, but these nodulating symbionts must compete with other inhabitants of rhizosphere communities and their relative abundance compared to that of other microbes can vary (21). More specifically, the relative frequency of nonnodulat-

ing versus nodulating rhizobia also varies, but nonnodulating genotypes typically dominate and can encompass as much as 99% of the total rhizobial population (10–18). Nonnodulating strains can reduce the number of nodules formed by nodulating strains on legume hosts (22, 23) and can invade nodule tissues in the presence of closely related nodulating strains (24, 25). This suggests

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that they may be able to reduce nodulating strain fitness through competitive exclusion at the root surface. Nonnodulating strains have also been shown to promote plant growth on nonlegume hosts (26–28), but the direct effects of nonnodulating rhizobia on legume host performance remain unclear, either in isolation or when in competition with nodulating strains. Moreover, it is unknown whether nonnodulating rhizobia affect legume host performance, while in the rhizosphere or by gaining access to host resources *in planta* as endophytes.

Here, we investigated the effects of nonnodulating strains on native hosts and symbionts of the *Acmispon-Bradyrhizobium* mutualism in California. We inoculated *Acmispon strigosus* (formerly *Lotus strigosus*) hosts with sympatric *Bradyrhizobium* isolates to examine the effects of nonnodulating strains on both host and symbiont performance. Experimental treatments included clonal inoculations with either nonnodulating or nodulating strains, mixed inoculations of nodulating and nonnodulating strains, and inoculations with water as a control. Strain treatments were organized into 16 unique sympatric coinoculation strain pairs, with 8 pairs each sourced from independent host populations in Northern and Southern California. To investigate factors that might influence the fitness outcomes of interstrain competition and the ability of nonnodulating strains to coinfect legume nodules, coinoculation strain pairs varied in terms of genetic relatedness between competing strains and in terms of estimated abundance of each strain in the sampled populations (10, 14). We conducted separate experiments with different coinoculation ratios. One matched empirical estimates of nodulating versus nonnodulating strain abundance in the *A. strigosus* rhizosphere (10, 14). The other was extremely biased toward nonnodulating strains to maximize the potential for observing nodule coinfection and the effects of interstrain competition on modulating the benefits of the legume-rhizobium mutualism. Our goals in these experiments were to examine (i) the growth effects of nonnodulating strains on hosts in isolation, (ii) the effects of competing nonnodulating strains on host performance and nodulating symbiont fitness, and (iii) the genotype-specific effects of the nodulating versus nonnodulating strains in determining the fitness outcomes of interstrain competition.

MATERIALS AND METHODS

Selection of *Bradyrhizobium* strains and inbred *Acmispon* hosts. *Bradyrhizobium* isolates were previously collected from the nodules and the soil-root interface of *A. strigosus* host plants at Bodega Marine Reserve (BMR) in Northern California and the University of California Riverside (UCR) in Southern California (10, 14). All isolates were previously tested for nodulation ability in greenhouse inoculation assays and were genotyped at multiple loci, including genes present on the chromosome (i.e., present in all *Bradyrhizobium*) and genes carried on the symbiosis island to confirm its presence or absence (10, 14). Strains for this study were chosen in order to examine the effects of (i) field site of origin (BMR versus UCR), since the sites varied in the relative frequencies of nodulating versus nonnodulating strains (10, 14), (ii) relatedness between competing strains (identity at chromosomal loci versus unrelated), and (iii) strain abundance of each strain tested in their sampled habitat (i.e., rare versus abundant) (10). Strains were also selected in order to vary antibiotic resistance profiles, which were used to identify coinfecting strains *in vitro* (29).

From each field site, 8 sympatric strains were chosen, composed of 3 nodulating and 5 nonnodulating strains, resulting in a total of 16 *Bradyrhizobium* strains (some strains were used in more than one coinoculation strain pair). Strains from each field site were grouped into 8 sympatric

strain pairs to be experimentally coinoculated, each comprising one nodulating strain and one nonnodulating strain (Table 1). Since the primary focus was to investigate the effects of nonnodulating strains, we did not test coinoculation pairs containing only nodulating or only nonnodulating strains. The antibiotics used to differentiate nodulating and nonnodulating strains within each pair included chloramphenicol (100 µg/ml), carbenicillin (100 µg/ml), gentamicin (100 µg/ml), kanamycin (100 µg/ml), and streptomycin (100 µg/ml). Four of the strain pairs had identical genotypes at two chromosomal loci (*recA* and *glnII*) but differed in nodulation ability, modulated by the presence or absence of symbiosis island loci (10). The 12 remaining pairs consisted of diverged nodulating and nonnodulating strains that varied in the number of chromosomal single nucleotide polymorphisms (SNPs) and in local abundance (Table 1). Results of a pilot study found no evidence of horizontal gene transfer of the symbiosis loci between nodulating and nonnodulating strains; recovered nonnodulating genotypes were resequenced to confirm their identity, and PCR on these isolates consistently failed to amplify symbiosis loci (*nolL* and *nodDA*), suggesting that they did not incorporate the symbiosis island.

Inbred lines of *A. strigosus* were generated from each field site following published protocols (14), except that seedlings were transplanted into 1-gallon pots with enriched soil (UCR number 3 soil). Plants were grown for 5 months (19 November 2013 to 17 April 2014) in UCR greenhouse 11 (33.972798, -117.323548), and fruits were picked as they developed (~1,500 seeds per plant). No supplemental lighting was used to alter day length. We chose one inbred line of hosts per site for inoculation (BMR04.02; UCR09.03). All *Bradyrhizobium* strains were inoculated onto sympatric hosts.

Preparation of *Bradyrhizobium* inocula. Each *Bradyrhizobium* strain was initiated from ~2 µl of frozen stock and streaked onto plates with modified arabinose gluconate medium (MAG) (14). A single colony of each strain was spread onto 5 MAG plates and incubated until lawns formed (29°C, ~8 days). Bacteria were scraped from each plate and resuspended in liquid MAG, and concentrations were estimated via optical density (30). The resuspended cells were centrifuged (4,000 rpm, 20 min) to remove media and resuspended again in sterile double-distilled water (ddH₂O) at 10⁸ cells ml⁻¹.

Inoculation experiments. Seed preparation and planting followed previously published methods (14). Inoculated plants received a total of 5 × 10⁸ rhizobial cells in 5 ml of ddH₂O (equivalent by mass to ~10⁶ cells g⁻¹ soil), which is higher than most estimates of natural rhizobial soil populations (up to 10⁵ nodulating cells g⁻¹ soil) (31–33), but compensates for rhizobial attrition that occurs during the stressful inoculation process (10, 14, 20, 30, 34–36).

Two separate experiments were conducted with different coinoculation ratios. The “ecological experiment” used coinoculation ratios that matched the empirical population estimates of nodulating versus nonnodulating strain abundance in *A. strigosus* rhizospheres (i.e., 1:3 at BMR; 1:95 at UCR) (10, 14). The “extreme experiment” used a coinoculation ratio of 1:500, nodulating to nonnodulating rhizobia, for both host population sources to maximize the potential for competition and nodule coinfection by nonnodulating rhizobia.

For each host population, axenic *A. strigosus* seedlings were separately arranged by size and size-matched seedlings were randomly assigned to sympatric inoculation treatments and greenhouse locations. For each coinoculation pair, bacterial treatments consisted of (i) clonal inoculation of the nodulating strain, (ii) clonal inoculation of the nonnodulating strain, (iii) coinoculation of both strains, and (iv) inoculation with water (ecological experiment: 4 treatments per pair × 16 strain pairs × 4 replicate plants per treatment combination × 2 harvest points, 256 plants per host population; extreme experiment: 4 treatments per pair × 16 strain pairs × 4 replicate plants per treatment combination, 128 plants per host population). Plants were inoculated on 3 October 2014 (BMR) and 9 October 2014 (UCR).

TABLE 1 Summary of strain features and antibiotic resistance^a

Host population	Pair no.	No. of SNPs between strains	Strain	Genotype	Genotype abundance	Nodulation ability	Antibiotic resistance ^b
BMR	1	0	45	G01_R01	24	+	CHL ^r
			15			–	CHL ^s
	2	0	37	G05_R02	24	+	CHL ^s
			80			–	CHL ^r
	3	83	49	G03_R01	355	+	GEN ^r
			1	G17_R17	8	–	GEN ^s
	4	40	49	G03_R01	355	+	GEN ^r
			41	G112_R09	1	–	GEN ^s
	5	58	49	G03_R01	355	+	STR ^s
			64	G16_R16	2	–	STR ^r
	6	64	37	G05_R02	24	+	GEN ^r
			1	G17_R17	8	–	GEN ^s
	7	17	37	G05_R02	24	+	GEN ^r
			41	G112_R09	1	–	GEN ^s
	8	48	37	G05_R02	24	+	STR ^s
			64	G16_R16	2	–	STR ^r
UCR	9	0	107	G03_R01	355	+	GEN ^r
			98			–	GEN ^s
	10	0	131	G11_R07	62	+	CAR ^r
			110			–	CAR ^s
	11	73	87	G03_R01	355	+	KAN ^s
			102	G36_R35	20	–	KAN ^r
	12	108	87	G03_R01	355	+	GEN ^r
			109	G42_R47	5	–	GEN ^s
	13	12	87	G03_R01	355	+	CAR ^r
			112	G04_R21	1	–	CAR ^s
	14	77	131	G11_R07	62	+	KAN ^s
			102	G36_R35	20	–	KAN ^r
	15	110	131	G11_R07	62	+	GEN ^r
			109	G42_R47	5	–	GEN ^s
	16	12	131	G11_R07	62	+	GEN ^r
			112	G04_R21	1	–	GEN ^s

^a The numbers of SNPs between strains and genotype abundances were previously determined by Hollowell and colleagues (10).

^b CHL, chloramphenicol; GEN, gentamicin; STR, streptomycin; CAR, carbenicillin; KAN, kanamycin.

Harvest and coinfection analysis. During harvest, plants were removed from the pots, and soil was separated from the roots by washing with tap water. Nodules were dissected, counted, and photographed. Roots, shoots, and nodules were separated and oven dried (60°C, >4 days) prior to weighing. We harvested all plants prior to flower formation since this is when nodule senescence in *A. strigosus* often begins in the greenhouse (i.e., around 9 weeks postinoculation, but this can range from 8 to 24 weeks in the field depending on rainfall) (20). Additionally, results of a pilot study suggested that coinfection varied with plant developmental stage. To maximize the potential to observe coinfection, half of the plants from each host population in the ecological experiment were harvested 4 weeks after inoculation ($n = 128$ per host population) and the remaining half at 8 weeks ($n = 128$ per host population). All plants in the extreme experiment from both host populations were harvested 6 weeks after inoculation ($n = 128$ per host population). Therefore, at each harvest point, 16 plants per coinoculation pair (4 plants per inoculation treatment) were harvested.

The frequencies of coinfecting nodules and the relative proportion of each rhizobial strain within the nodules were estimated for each coinoculation strain pair at each harvest point. Two (of 4) plants were randomly selected for nodule culturing for each strain pair and harvest week ($n = 96$). For each sampled plant, 4 randomly selected nodules were chosen for bacterial culturing. We cultured bacteria from a total of 24 nodules per coinoculation strain, 8 at each harvest week, from 96 test plants ($n = 384$). The proportion of plants per treatment and the number of nodules per

plant selected for culturing were chosen in order to complete harvests in a timely manner and to be consistent with those in our previous studies (30, 35). Nodules were surface sterilized following previously described methods, crushed, and spread onto 3 MAG plates (30). To estimate the relative proportions of the nodulating and nonnodulating strains within the nodule, 100 randomly selected colonies were replica plated onto MAG plates containing the appropriate antibiotic with plain MAG plates as controls for growth (Table 1; detection limit of nonnodulating strains of 1%) (30, 35). If fewer than 100 colonies were present, they were all tested for resistance traits.

To confirm that all plated colonies came from the internal portions of the nodules, nodule surface sterilization efficiency was confirmed experimentally. Briefly, 8 *A. strigosus* nodules (collected from 3 separate plants) were dissected from plant roots, and each unsterilized nodule was individually rolled over 1 MAG plate using a sterile loop. Nodules were then surfaced sterilized in undiluted bleach (6% sodium hypochlorite) for 2 min, rinsed 3 times in sterile water, and subsequently rolled over a second MAG plate to confirm the absence of surface contaminants. Nodules were then crushed using a sterile glass rod, and bacteria were plated onto a third MAG plate to confirm rhizobial viability within each surface sterilized nodule. Original surface contaminants were present on all nodules tested (i.e., growth on the first MAG plate) and effectively removed in all cases (i.e., no growth on the second MAG plate). Rhizobial viability was confirmed in 7 (of 8) nodules (i.e., growth on the third MAG plate).

TABLE 2 Effects of nonnodulating strains in altering host and symbiont fitness^a

Host population and pair	F ratio for ecological experiment, wk 8			F ratio for extreme experiment, wk 6		
	Shoot biomass	Nodule no.	Total nodule biomass	Shoot biomass	Nodule no.	Total nodule biomass
BMR						
Net ^b	119.12 ^{***c}	185.36 ^{***c}	69.36 ^{***}	44.73 ^{***d}	164.25 ^{***c}	90.50 ^{***}
1	20.52 ^{**}	32.76 ^{***c}	49.79 ^{***c}	3.67 [*]	15.09 ^{**}	4.40 [*]
2	12.67 ^{**}	13.72 ^{**}	6.46 ^{**}	3.16	15.87 ^{**+c}	9.94 ^{**c}
3	4.60 [*]	16.79 ^{**}	9.71 ^{**}	2.41	11.69 ^{**}	32.78 ^{***}
4	38.16 ^{***}	22.72 ^{***}	31.91 ^{***}	10.64 ^{**}	20.28 ^{***}	11.86 ^{**}
5	25.34 ^{***}	18.72 ^{***}	18.23 ^{***}	2.49	33.81 ^{***}	14.10 ^{**}
6	12.78 ^{**}	15.79 ^{**}	14.16 ^{**}	6.69 ^{**}	34.16 ^{***}	8.97 ^{**}
7	43.93 ^{***}	43.50 ^{***}	29.75 ^{***}	9.13 ^{**}	27.94 ^{***c}	15.20 ^{**}
8	12.95 ^{**}	13.26 ^{**}	4.33 [*]	8.14 ^{**c}	19.91 ^{***}	18.68 ^{***}
UCR						
Net ^e	130.82 ^{***c}	192.01 ^{***c}	140.82 ^{***c}	77.23 ^{***c}	191.68 ^{***c}	62.75 ^{***c}
9	29.34 ^{***c}	267.68 ^{***c}	32.22 ^{***c}	12.82 ^{**}	13.50 ^{**}	5.91 [*]
10	42.92 ^{***}	28.26 ^{***}	144.57 ^{***}	8.27 ^{**}	34.76 ^{***}	10.94 ^{**}
11	18.27 ^{***c}	18.87 ^{***}	29.10 ^{***}	13.67 ^{**}	95.90 ^{***c}	9.46 ^{**}
12	15.64 ^{***c}	22.29 ^{***c}	10.67 ^{***c}	8.39 ^{**}	13.79 ^{**}	12.84 ^{**}
13	17.38 ^{***c}	64.27 ^{***c}	18.78 ^{***c}	7.29 ^{**}	14.01 ^{**c}	6.17 ^{**}
14	23.98 ^{***}	52.08 ^{***c}	33.12 ^{***c}	7.86 ^{**}	87.32 ^{***c}	12.34 ^{**}
15	6.52 ^{**}	13.36 ^{**}	10.25 ^{**}	26.54 ^{***c}	37.96 ^{***c}	56.20 ^{***c}
16	6.06 ^{**}	6.23 ^{**}	5.45 [*]	19.04 ^{***c}	18.37 ^{***}	8.819 ^{**}

^a F ratio statistics are reported from one-way ANOVAs comparing effects of inoculation treatments (df = 3) by harvest week and net source host population (n = 128) and within coinoculation (n = 16). Asterisks indicate significant F ratio statistics (*, P < 0.05; **, P < 0.01; ***, P < 0.0001). To determine significant differences among inoculation treatments, indicated in boldface type, ANOVAs were followed by pairwise t tests comparing inoculation treatments (P < 0.05).

^b Refers to plants from all pairs sourced from BMR merged within treatment.

^c Refers to cases where the clonal nodulating treatment results are significantly higher than the coinoculation treatment results.

^d Refers to cases where the clonal nodulating treatment results are significantly lower than the coinoculation treatment results.

^e Refers to plants from all pairs sourced from UCR merged within treatment.

Data analysis. We used shoot biomass as our primary estimate of plant performance, which is the most commonly reported plant fitness component (37). We used nodule number and mass as proxies for nodulating strain fitness in our experiments. Previous work by Sachs and colleagues (20) demonstrated that both of these parameters are positively correlated with beneficial rhizobial population sizes in *A. strigosus*, similar to results for other systems (38–41).

The effects of inoculation treatments on host performance and nodulating strain fitness were analyzed separately for each host population at each harvest week using one-way analyses of variance (ANOVAs) (df = 3) (42). ANOVAs with significant F ratio statistics were followed by pairwise t tests (42) to test for differences among treatments. To examine the effects of nonnodulating strains on host performance, hosts receiving clonal inoculations of nonnodulating strains were compared to uninoculated controls. To test if competing nonnodulating strains altered host and symbiont fitness during symbiosis, host performance and symbiont fitness were compared between clonal inoculations of nodulating strains and coinoculation treatments. Net fitness effects were determined by combining plant data from all pairs within an inoculation treatment for each host population and harvest week (n = 128). Effects within each pair were analyzed using plants only from each respective pair for each host population and harvest week (n = 16).

Nodules and plants were scored as coinfecting if ≥1 replica plated colonies were identified as a nonnodulating strain. Although several factors can influence the ability of a nonnodulating strain to coinfect legume nodules, the primary objectives in our coinfection analyses were to (i) estimate the coinfection ability of each nonnodulating strain, (ii) detect any patterns in coinfection ability, and (iii) determine if there are any host performance costs to coinfection. Thus, the coinfection ability for each nonnodulating strain was assessed using data from all harvest weeks and coinoculation ratios where each nonnodulating strain was present in the inoculum. Since half of the coinoculated plants were selected for culturing

and bacteria from 4 nodules per plant were replica plated, we regard our estimates of coinfection as conservative.

Strain variations in the capacity to coinfect nodules or block coinfection were analyzed using one-way ANOVAs followed by Tukey's honestly significant difference (HSD) test among nonnodulating and nodulating strains, respectively. To test if the proportion of coinfection for chromosomally identical pairs differed from that for chromosomally diverged pairs (0, >10 SNPs) we used a generalized linear mixed model with genetic divergence (identical or diverged) as a fixed effect and coinoculation strain pair as a random effect (fit model platform in JMP 10.0). To examine if the coinfection frequency scales with the genetic distance, a correlation analysis was performed between the proportion of nodules coinfecting per plant and the number of SNPs between each coinoculation strain pair.

To examine the effects of coinfection, host performance and symbiont fitness were compared using one-way ANOVAs between coinoculated plants without evidence of coinfection and coinfecting plants (where nodule subculturing data were available) separately for each host population and harvest week (df = 1; n = 16).

RESULTS

Effects of nonnodulating strains on hosts in isolation. None of the nonnodulating strains formed nodules or any detectable features on roots when inoculated in isolation (see Table S1 in the supplemental material). In no case did clonal inoculation with a nonnodulating strain affect host growth compared to that of uninoculated control plants (Table 2).

Effects of competing nonnodulating strains on host growth and symbiont fitness. In the ecological experiment, coinoculation of nodulating and nonnodulating strains reduced host growth and measures of nodulating strain fitness in both host populations.

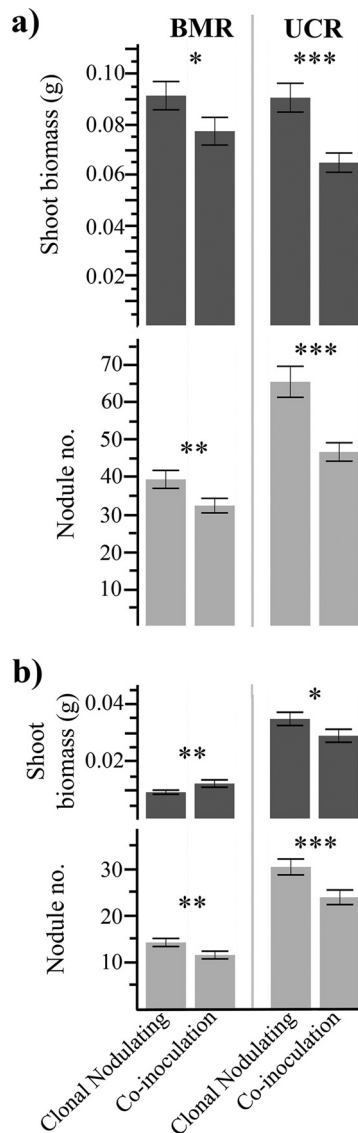


FIG 1 Coinoculation alters host growth and symbiont fitness. Shoot biomass and total nodule number data were merged within treatment from all pairs from the same host population. (a) Shoot biomass and the total nodule number from each host population in the ecological ratio experiment 8 weeks postinoculation. (b) Shoot biomass and the total nodule number from each host population in the extreme ratio experiment 6 weeks postinoculation. Asterisks denote significant differences between net clonal inoculations of nodulating strains and net coinoculations with nonnodulating strains within each host population (one-way ANOVAs: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). Error bars represent 1 standard error.

For the BMR population, the coinoculated treatments reduced net host growth by $\sim 15\%$, and net nodulating strain fitness was significantly decreased in terms of total nodule number ($\sim 18\%$ reduction), but net total nodule biomass was not significantly affected (Fig. 1a; Table 2). Nodulating strain fitness proxies, but not host growth, varied based on strain combinations for BMR hosts (Table 2). For the UCR population, net host performance was significantly reduced by $\sim 28\%$ in coinoculated treatments. Net nodulating strain fitness was also decreased in coinoculated treatments at UCR, with $\sim 29\%$ and $\sim 32\%$ reductions in total nodule

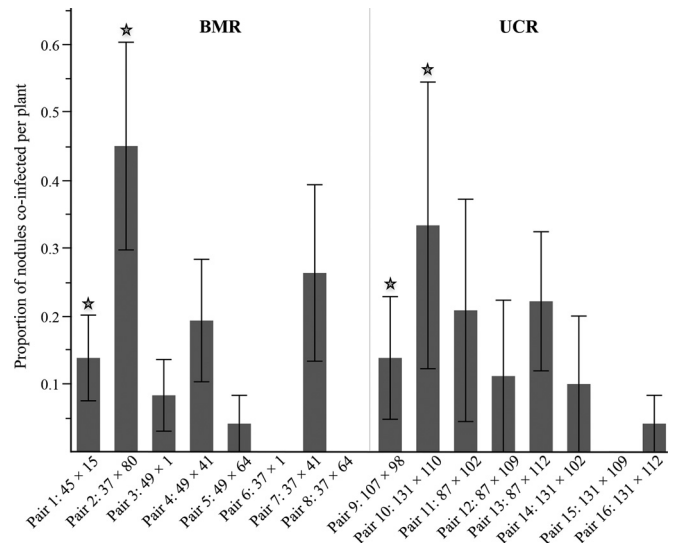


FIG 2 Proportion of coinfected nodules by strain combination. Coinfection proportions for each strain pair were averaged across all harvest weeks and inoculation ratios within each source host population. Stars represent coinoculation pairs where strains are genetically identical at the chromosomal level. Error bars represent 1 standard error.

number and total nodule biomass, respectively (Fig. 1a; Table 2). Host performance and nodulating strain fitness proxies varied based on strain combinations for UCR hosts (Table 2). The mean numbers of nodules per plant, per strain pair, and per harvest week are reported in Table S2 in the supplemental material.

Extreme coinoculation ratios of nodulating to nonnodulating strains resulted in various effects for each host population. Net BMR host growth was significantly increased by $\sim 24\%$ under extreme coinoculation conditions compared to that of clonally inoculated plants, yet the net total nodule number was reduced by $\sim 19\%$ (Fig. 1b; Table 2). Similar to inoculation with an ecologically relevant ratio, net total nodule biomass was not significantly different among treatments (Table 2). All metrics of net host growth and nodulating strain fitness were significantly decreased in the UCR hosts (Fig. 1b; Table 2). Coinoculation with an extreme ratio of nonnodulating strains resulted in an $\sim 17\%$ reduction in net host growth, an $\sim 21\%$ reduction in net total number of nodules, and an $\sim 23\%$ reduction in net total nodule biomass (Fig. 1b; Table 2). Host growth and nodulating strain fitness proxies varied based on strain combinations for both host populations under an extreme coinoculation ratio (Table 2).

Effects of nonnodulating strain coinfection on host and symbiont fitness. Data from 296 nodules from which nodule occupancy was successfully estimated by subculturing were analyzed (Table 1; see also Table S1 in supplemental material). The coinfection ability and estimated within-nodule population proportion of each nonnodulating strain were determined using data from all harvest weeks and coinoculation ratios. All nonnodulating strains were able to colonize the nodule tissue of at least one *A. strigosus* nodule, and evidence of coinfection was uncovered in all coinoculation pair combinations tested, except for pairs 6, 8, and 15 (Fig. 2 and 3a). Nonnodulating strains exhibited variations in both their ability to coinfect *A. strigosus* nodules and their within-nodule population estimates (Fig. 3a and b). However, the capac-

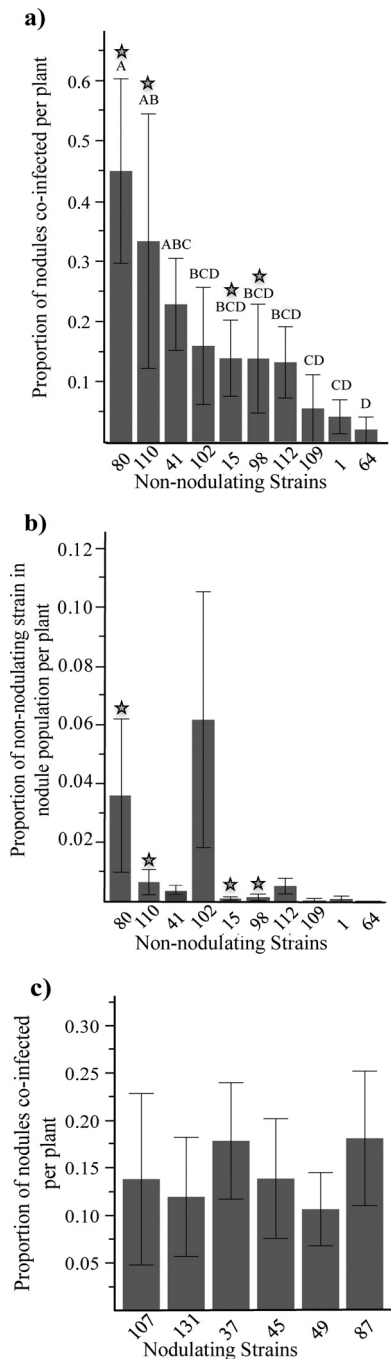


FIG 3 Proportion of coinfecting nodules by individual strain and within-nodule proportion estimates. Coinfection proportions were averaged for each strain using data from all strain combinations. Stars represent nonnodulating strains that are genetically identical to coinoculated nodulating strains at the chromosomal level. (a) Proportion of coinfecting nodules for each nonnodulating strain. Letters are significant differences among nonnodulating strains (Tukey's HSD test, $P < 0.05$). (b) Mean proportion of each nonnodulating strain within the total nodule population per plant. (c) Proportion of coinfecting nodules for each nodulating strain. Error bars represent 1 standard error.

ity to coinfect nodules was not correlated with strain genotype abundance ($R^2 = 0.0015$, $P = 0.7081$).

Nonnodulating strains that were paired with genetically identical nodulating strains (strains 15, 80, 98, and 110) coinfecting

TABLE 3 Effects of coinfection on host and symbiont fitness^a

Experiment and host population	Harvest wk	F ratio			
		Shoot biomass	Root biomass	Nodule no.	Total nodule biomass
Ecological					
BMR ^b	4	0.001	0.182	0.187	0.568
	8	0.005	0.090	0.404	1.341
UCR ^c	4	0.020	0.066	0.021	0.845
	8	0.223	0.039	2.505	0.302
Extreme					
BMR ^b	6	4.090	0.656	0.024	1.280
UCR ^c	6	0.192	0.385	0.301	1.553

^a F ratio statistics are reported from one-way ANOVAs comparing effects of coinfection (df = 1) by harvest week and net source host population ($n = 16$).

^b Refers to plants from all pairs sourced from BMR merged within treatment.

^c Refers to plants from all pairs sourced from UCR merged within treatment.

significantly more nodules per plant than nonnodulating strains in genetically diverged coinoculation pairs (generalized linear mixed model: $F_{1,96} = 6.21$, $P = 0.0258$ and SNP correlation analysis $R^2 = -0.0719$, $P = 0.0086$, $n = 96$) (Fig. 2 and 3a). However, we did not find any significant differences in terms of estimated within-nodule populations (Fig. 3b). None of the nodulating strains were able to prevent coinfection, and the proportion of coinfecting nodules per plant was not significantly different among nodulating strains (Fig. 3c).

No significant effects of coinfection were found on host or nodulating strain fitness in either host population in the ecological and extreme ratio experiments (Table 3). Coinfection was reduced over time in the ecological experiment in both host populations (harvested at 4 and 8 weeks), although this trend was not significant for BMR host plants (Fig. 4).

DISCUSSION

Rhizobia are increasingly being understood to have multifarious lifestyles, including root-nodule symbiosis, colonization of plant roots in the rhizosphere or as root endophytes, and independent growth in the soil or other habitats (24–28, 32). Yet, these lifestyles can be transient and are only partially dependent on the presence or absence of symbiosis loci, which have been a major focus of research. Nodulating strains with the canonical nodulation loci, for instance, are not capable of forming nodules on all hosts (e.g., host specificity) (14, 43, 44). Furthermore, nonnodulating strains (lacking key nodulation genes) can coinfect legume nodules, expropriating a symbiotic role (24, 25). Regardless of what factors determine lifestyle, strains that do not form nodules comprise the majority of rhizobial populations that have been sampled (10–18). Our data here suggest that nonnodulating rhizobia are not passive players in the host rhizosphere. Instead, nonnodulating rhizobia can have considerable effects on the legume-rhizobium symbiosis, most often by reducing plant performance and attenuating nodulating symbiont fitness.

Our results from clonal inoculations corroborate the results from previous work, which found no effect of nonnodulating strains on legume host growth in isolation (14, 25, 45). However, under parameters that model relative abundances of nonnodulating strains within the rhizosphere, our data set revealed substantial costs to host growth and symbiont fitness in both host populations examined (except for total nodule biomass at BMR) (Fig. 1a;

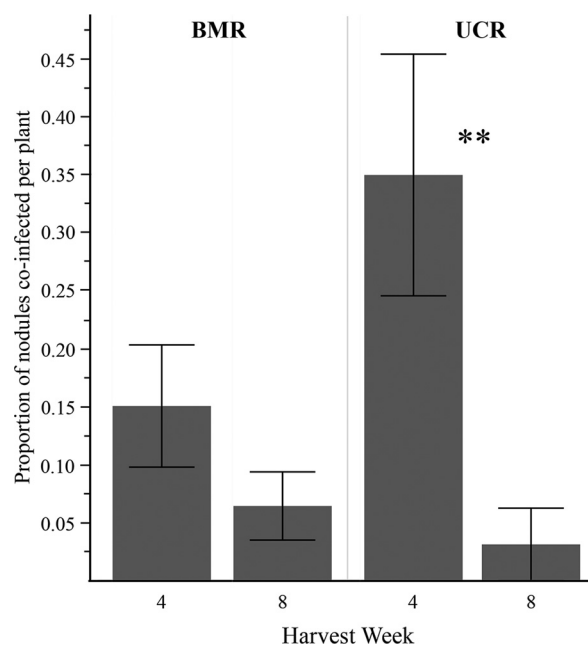


FIG 4 Proportion of coinfection in the ecological ratio experiment at 4 and 8 weeks postinoculation for each host population. The proportion of nodules coinfecting per plant was calculated by averaging all coinoculated treatment plants from all pairs in the same host population (one-way ANOVA: **, $P < 0.01$). Error bars represent 1 standard error.

Table 2). Our data are consistent with the results of previous reports of a reduction in nodulating strain fitness, measured by numbers of nodules formed by nodulating strains (22, 23), but reveal that this does not always result in a significant decrease in all rhizobial fitness estimates (i.e., total nodule biomass). The higher ratio of nonnodulating strains in ecologically relevant UCR coinocula might explain this difference and further suggests a competitive role for nonnodulating strains at the root-soil interface, but we are unable to disentangle the effects of coinoculation ratios from any differences due to host genotype.

The host growth responses to extreme ratios of nonnodulating strains differed between host populations. Coinoculation resulted in a significant increase in net BMR host growth (although this trend was only significant for strain pair 8) (Table 2), yet reductions in nodule numbers and effects on total biomass were similar compared to ecologically relevant ratios (Fig. 1a and b; Table 2). Legumes have finely tuned mechanisms to regulate nodule numbers (46), but the number of nodules formed in any interaction is nonetheless a product of the host and rhizobium genotypes (38, 47). This suggests that host control over nodule numbers is incomplete (34). Just as ineffective rhizobia form many nodules on hosts without benefiting the host (30), effective rhizobium strains might often produce more nodules on a host than is optimal for host growth. Thus, the reduction of nodule numbers by nonnodulating strains might actually increase host growth if the nodulation strains present are prolific nodule producers. However, this hypothesis cannot be explicitly tested with our data since there was no significant variation in nodule numbers for BMR populations. All estimates of host performance and nodulating strain fitness were reduced under extreme coinoculation conditions at UCR (Fig. 1b; Table 2), although they were not as pronounced as those

for coinoculations with ecologically relevant ratios. Differences in host growth among treatments are more distinct as plants approach flowering (closer to the harvest at 8 weeks in the ecological experiment); thus, the magnitudes in the reductions in host performance and nodulating strain fitness might have been more comparable if the extreme experiment was harvested at a later date.

Legume nodules can harbor multiple lineages of bacteria (48–56), yet few studies have considered the capacity of rhizobial strains lacking nodulation loci to invade and persist within nodule tissue (but see references 24 and 25). To our knowledge, ours is the first study to explore the potential for coinfection using native combinations of strains on sympatric hosts. All of the nonnodulating strains that we tested were able to coinfect nodules (Fig. 3a and b), and this was true in nearly every strain combination tested (Fig. 2). This suggests that coinfection with nonnodulating strains is likely to be at least as common as coinfection with nonrhizobial bacteria. These data lend support to past reports of rhizobia that were isolated from legume nodules but were subsequently found to be unable to form nodules in inoculation tests (57, 58). Our data also imply that the coinfection ability can vary, depending on both rhizobial and plant factors. First, the estimated natural abundance of nonnodulating strains did not appear to impact coinfection ability, but genetic relatedness between strains did have a significant effect. The proportion of coinfecting nodules was increased in strain pairs that were more closely related (0 SNPs) (Table 1), compared to strain pairs that were more distantly related (>10 SNPs) (Table 1). One possible explanation for this result is that the similarity of critical signaling molecules during root colonization is more important than abundance for nonnodulating strains (e.g., exopolysaccharides [EPS]) (25). Recently Zgadzaj and colleagues (25) found symbiotic rhizobia with compatible EPS had an advantage over coinfecting endophytes with different EPS molecules. In *Bradyrhizobium*, EPS genes are chromosomally carried (not within the symbiosis island) (8). Hence, it is possible that chromosomally identical (i.e., 0 SNPs) coinoculation pairs make similar, if not identical EPS, explaining the higher coinfection rates than for strain pairs that are genetically unrelated (i.e., >10 SNPs). Second, evidence of coinfection decreased over time since inoculation (Fig. 4). Previous work has shown that legume hosts can actively sanction ineffective rhizobial strains (nonfixing), reducing nodule growth rate and within-nodule rhizobial population sizes (30, 35, 40, 59). Since the presence of nonnodulating strains within legume nodules did not increase host growth in this study (Table 3), we can consider infections by nonnodulating strains to be similar to ineffective infections. Sanction mechanisms could be one reason for the observed decline of coinfection over time, but we are unable to discern if the host is controlling nonnodulating strain population sizes via sanctions or if nonnodulating strains are poorly adapted to survival and proliferation within the nodule environment. Last, while coinfection is prevalent, the lack of any measurable effects on host growth suggests that it might not play a critical role in terms of host fitness (Table 3).

Theoretical and empirical work on the legume-rhizobium symbiosis has generally assumed that legume fitness is predominantly regulated by which rhizobial strains successfully nodulate host roots (20, 30, 35, 40, 59–62). Investigations have sought to uncover the mechanisms of competition among nodulating strains of various levels of symbiotic effectiveness (i.e., nitrogen

fixation capacity) and to understand how the outcomes of this competition affect host fitness. Although effective nodulating strains can be competitive for nodulation (37, 63, 64), competitive ability may not be correlated with beneficial quality (41, 61, 65–67). Researchers attempting to apply highly effective rhizobial strains to improve legume crop commonly find that these strains nodulate hosts at low rates and that the inoculant strains get out-competed by less efficient symbionts (60–62, 68). Despite the prevalence and dominance of nonnodulating strains (10–18), such studies have neglected the impact of endemic nonnodulating strains as potential negative competitors on the mutualism. Our work illustrates that endemic nonnodulating rhizobial strains often coinfect legume nodules and, more importantly, may play an active role in modulating the legume-rhizobium mutualism. Our results also show that nonnodulating rhizobia lack effects on host growth in isolation and during nodule coinfection, suggesting that the key fitness effects of nonnodulating strains are mediated by interstrain competition at the root-soil interface before nodulation occurs. Further research is necessary to understand the specific mechanisms of interstrain competition within the microbiota of the rhizosphere, but the overall competitive effects of nonnodulating rhizobial strains and other nonnodulating rhizosphere microbes should be promptly considered both in bioinoculant development and in research.

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