doi:10.1111/j.1558-5646.2012.01788.x

TRADE-OFFS, SPATIAL HETEROGENEITY, AND THE MAINTENANCE OF MICROBIAL DIVERSITY

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Received April 6, 2012
Accepted August 9, 2012
Data Archived: Dryad doi:10.5061/dryad.g1t69

Specialization and concomitant trade-offs are assumed to underlie the non-neutral coexistence of lineages. Trade-offs across heterogeneous environments can promote diversity by preventing competitive exclusion. However, the importance of trade-offs in maintaining diversity in natural microbial assemblages is unclear, as trade-offs are frequently not detected in artificial evolution experiments. Stressful conditions associated with patches of heavy-metal enriched serpentine soils provide excellent opportunities for examining how heterogeneity may foster genetic diversity. Using a spatially replicated design, we demonstrate that rhizobium bacteria symbiotic with legumes inhabiting contrasting serpentine and nonserpentine soils exhibit a trade-off between a genotype's nickel tolerance and its ability to replicate rapidly. Furthermore, we detected adaptive divergence in rhizobial assemblages across soil type heterogeneity at multiple sites, suggesting that this trade-off may promote the coexistence of phenotypically distinct bacterial lineages. Trade-offs and adaptive divergence may be important factors maintaining the tremendous diversity within natural assemblages of bacteria.

KEY WORDS: **Adaptation, genetic variation, life-history evolution, symbiosis, trade-offs.**

In heterogeneous environments the stable coexistence of lineages can be promoted by the evolution of specialization and concomitant trade-offs (Fry 1996; Kassen and Rainey 2004). Evolutionary trade-offs are expected between costly traits when adaptation to increase investment in one trait diminishes the ability to allocate to another (Levins 1968; Stearns 1989; Agrawal et al. 2010). This general principle can apply to both intra- and interspecific diversity (Bell 1991; Chesson 2000; Kassen 2002; Sears and Chesson 2007). Reciprocal transplants of macroorganisms and microbial selection experiments demonstrate that trade-offs and strong gradients within spatially heterogeneous environments can help to maintain phenotypic diversity via specialization (Elena and Lenski 2003) or local adaptation (Hereford 2009). However, few studies have demonstrated the importance of trade-offs and environmental heterogeneity for promoting diversity in natural microbial assemblages (Kassen and Rainey 2004).

Naturally co-occurring microbial lineages display tremendous phenotypic diversity and explaining the maintenance of this diversity remains an evolutionary challenge (Hughes Martiny et al. 2006; Prosser et al. 2007; Fierer and Lennon 2011). In part, this challenge stems from the fact that natural assemblages of microbes may exhibit weak evolutionary trade-offs. Examination of microbial adaptation under laboratory conditions suggests that trade-offs are often undetectable (Novak et al. 2006; Bennett and Lenski 2007; Hughes et al. 2007; Jasmin and Kassen 2007; Jessup and Bohannan 2008; Lee et al. 2009). Natural populations of microbes could be selected for adaptations that do not entail strong trade-offs (Lee et al. 2009) due to large populations, rapid generation times, high gene flow between environments, or fluctuating selection. Few studies, however, have rigorously tested natural microbial assemblages for ecologically important trade-offs or for whether spatial heterogeneity may promote the

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maintenance of diversity (Gudelj et al. 2010) (but see Belotte et al. 2003; Lennon 2007; Miller et al. 2009; Edwards et al. 2011). It is unclear whether such patterns are robust across multiple assemblages because there are few spatially replicated studies in microbial evolutionary ecology (Prosser 2010; Lennon 2011).

Heterogeneity in soil conditions, particularly for heavy-metal enrichment, has been implicated broadly in fostering phenotypic diversity (Haase and Bouchet 1998; Kruckeberg 2002; Espeland et al. 2008; Branco 2010). Stressful conditions associated with both anthropogenic mine spoils (McNeilly 1968) and naturally heavy-metal enriched serpentine soils (Brady et al. 2005; Harrison and Rajakaruna 2011), have confirmed the importance of trade-offs for plant diversity. Plant lineages adapted to metals typically exhibit metal tolerance (Brady et al. 2005) measured as the degree to which lineages maintain fitness despite the stress associated with elevated metal concentrations (Agrawal et al. 2010). Plant adaptation to chronic abiotic stress often trades-off with maximal growth rate (Grime 1977; Chapin and Shaver 1985; Grime 1988) in that metal-tolerant plants display reduced growth relative to nontolerant plants on nonstressful soils, thus putting them at a competitive disadvantage (Kruckeberg 1954; McNeilly 1968; Brady et al. 2005). The trade-off between tolerance and growth rate can thus promote the persistence of both stress tolerant and rapid growth phenotypes. The bacteria symbiotic with plants can play a critical role in modulating plant fitness across heterogeneous conditions (Friesen et al. 2011). However, whether a similar pattern of trade-offs and differentiation across metal-enriched soil conditions promotes the maintenance of diversity in these mutualistic microbes is unknown.

In the legume-rhizobium mutualism, symbiotic rhizobium bacteria housed in root nodules provide the plant with reduced nitrogen in exchange for photosynthates (Sprent 2007; Oldroyd et al. 2009). Rhizobia vary in the symbiotic benefits they provide to plants, and these benefits can be context dependent (Heath and Tiffin 2007). Between symbiotic plant generations, rhizobia are free living in the soil during which time they may be subject to intense edaphic selection unbuffered by the host (Denison and Kiers 2011), although the degree to which buffering within the nodule reduces abiotic selection during symbiosis is not well understood. Coexisting lineages of rhizobia exhibit great phenotypic diversity for abiotic tolerance and growth rate, which may underlie differences in their ability to persist in contrasting soil environments (Lakzian et al. 2002).

To understand the maintenance of heritable phenotypic diversity within host-associated assemblages, this study considers rhizobia symbiotic with either a native or an invasive legume species across serpentine soil boundaries. Although the rhizobia associated with a given host species may be composed of multiple species, such assemblages are an evolutionarily cohesive unit of microbial biodiversity because they often exhibit high frequencies of horizontal gene transfer, particularly for loci conferring physiological tolerance (Sullivan et al. 1995; van Berkum et al. 2003; Bailly et al. 2007; Lakzian et al. 2007; Prosser et al. 2007; Li et al. 2009; Philippot et al. 2010; Sachs et al. 2010; Tian et al. 2010). The legume *Acmispon wrangelianus* is native to California and associates with rhizobia in the genus *Mesorhizobium* (Porter, unpubl. ms.). *Medicago polymorpha* is a legume native to Mediterranean Eurasia, is invasive in California, and associates with rhizobia in the genus *Ensifer* (Charman and Ballard 2004; Silva et al. 2007, Porter, unpubl. ms.). Both hosts are ecologically similar winter-annual legumes inhabiting both nonserpentine and serpentine soils, the latter of which is enriched in toxic levels of the heavy-metal nickel and exhibits unusual ionic ratios (Harrison 1999; Harrison et al. 2003) due to its origin in the earth's mantle (Brady et al. 2005).

In this study, we examine levels of genetic variation among rhizobia for both tolerance to nickel and growth in the absence of nickel. We then ask whether the data are consistent with a nickel tolerance-growth trade-off among rhizobium isolates. Next, we determine whether the soil heterogeneity resulting from serpentine soil outcrops maintains phenotypic diversity via the adaptive differentiation of rhizobial assemblages across soil boundaries. We examine these patterns across spatially replicated serpentine/nonserpentine soil type boundaries.

Methods

COLLECTIONS

Rhizobia were isolated from field-collected legume root nodules. Legumes were collected from three reserves in California, USA that contain both serpentine and nonserpentine soil grasslands: (1) the McLaughlin Natural Reserve (McLaughlin); (2) the Hopland Research and Extension Center (Hopland); and (3) the Jasper Ridge Biological Preserve (Jasper Ridge) (Fig. S1; Table 1).

The physical structure of serpentine outcrops and distribution of host legumes varied among reserves. McLaughlin contains fine-scale spatial heterogeneity with numerous small (~6–25×10^2 m^2) patches of serpentine in a matrix of nonserpentine soil. *Acmispon wrangelianus* (Fisch. & C.A. Mey) D.D. Sokoloff (formerly *Lotus wrangelianus*) and *M. polymorpha* L. occur at multiple, small serpentine outcrops. At McLaughlin we sampled both legumes along 15–25 m transects spanning a high-density patch of plants at three small serpentine outcrops and as well as three nonserpentine areas used in previous studies (Harrison 1999; Harrison et al. 2003) (Tables 1, S1). At Hopland and Jasper Ridge, only *A. wrangelianus* was sampled due to the absence of *M. polymorpha*. Outcrops of serpentine at Hopland and Jasper Ridge are larger (~8 and 13×10^4 m^2, respectively) than those occupied by *A. wrangelianus* at McLaughlin; so, at each reserve we sampled along a single 65–80 m transect across one serpentine

Table 1. Description of sampled rhizobial assemblages.

Host	Reserve	Soil	Site	Ca:Mg (ppm)	Nickel (mg/kg)	Isolates (*n*)	Mean nickel tolerance (SE)	Mean growth rate (SE)
Aw	McL	NS	43	4.4	2.0	31	−0.19 (0.03)	0.53 (0.05)
.	.	.	54	5.7	0.8	32	−0.20 (0.04)	0.49 (0.04)
.	.	.	103	1.3	6.8	31	−0.24 (0.04)	0.42 (0.04)
.	.	S	2	0.2	3.1	28	−0.07 (0.03)	0.53 (0.04)
.	.	.	32	0.8	9.1	28	−0.16 (0.03)	0.47 (0.03)
.	.	.	48	0.5	5.8	24	−0.12 (0.03)	0.48 (0.06)
.	Hop	NS	NH1	2.6	1.6	30	−0.29 (0.03)	0.50 (0.02)
.	.	S	SH1	0.3	21.8	28	−0.05 (0.05)	0.42 (0.03)
.	Jas	NS	NJ1	2.2	1.2	31	−0.36 (0.05)	0.56 (0.04)
.	.	S	SJ1	0.6	86.5	30	0.03 (0.03)	0.46 (0.03)
Mp	McL	NS	37	1.7	3.2	26	−0.30 (0.03)	0.61 (0.02)
.	.	.	54	2.8	0.6	28	−0.33 (0.03)	0.65 (0.03)
.	.	.	58	1.0	3.5	28	−0.35 (0.03)	0.60 (0.03)
.	.	S	24	1.1	4.5	24	−0.34 (0.04)	0.61 (0.04)
.	.	.	416	0.5	28.7	26	−0.31 (0.03)	0.59 (0.03)
.	.	.	43	0.5	7.2	26	−0.36 (0.04)	0.58 (0.04)

McL = McLaughlin; Hop = Hopland; Jas = Jasper Ridge; NS = nonserpentine soil; S = serpentine soil; *Aw* = *Acmispon wrangelianus*; *Mp* = *Medicago polymorpha*.

outcrop through a high-density patch. A similar transect was sampled through the closest high-density patch on nonserpentine soil (Tables 1, S1). Soil was collected from three points within the patch and evenly bulked into a single sample for soil chemistry analyses (A and L Western Analytical Labs) including DTPA extractable nickel and calcium:magnesium ratios (Table 1).

ISOLATION OF RHIZOBIA

Legumes with intact nodules were collected at least 30 cm apart, except for rare instances where plants were not sufficiently spaced. One rhizobium isolate from one randomly selected, surface sterilized nodule per plant was axenically cultured via three restreakings from single colonies (Vincent 1970), and preserved in 50% glycerol at −80°C. Rhizobia therefore experienced generations of growth in a common laboratory environment. We used DNA sequencing and BLAST searches in Genbank to confirm that isolate sequences at target loci were consistent with those expected for rhizobia (Table S2). 16S ribosomal DNA sequences (Gaunt et al. 2001) indicated *A. wrangelianus* symbionts are primarily *Mesorhizobium* spp., with rare instances of *Rhizobium* spp. and that *M. polymorpha* symbionts are primarily *Ensifer medicae,* with rare instances of *Rhizobium* spp.

EXPERIMENTAL GROWTH ASSAY

Assays were used to contrast the growth of each isolate in the presence and absence of nickel. Each assay contained six replicate 96-well cell culture plates (200 μl wells). Up to 48 samples were cultivated on each plate and included up to 46 experimental isolates as well as at least two uninoculated control samples. Assays were sequentially initiated as inoculum cultures became turbid after nodule isolations. Therefore, a variable number of wells in the 96-well plates were left uninoculated in addition to the uninoculated control samples. No uninoculated control wells showed evidence of contamination during the experiment. Each isolate was grown in 100 μl of tryptone yeast (TY) media (Somasegaran and Hoben 1994) in the presence (1 mM NiCl) and absence (0 mM NiCl) of nickel in adjacent wells to minimize the effects of spatial heterogeneity within a plate on a given isolate. Although the nickel concentration in serpentine soils may vary seasonally, a 1 mM nickel solution is equivalent to dissolving the DPTA extractable nickel from 1 kg of dry Hopland serpentine soil in 370 mL of media, effectively simulating ∼30% gravimetric soil water content. Liquid media growth assays avoid the problem of nickel forming a complex within a solid substrate, which can alter the concentration of nickel available to cells (Hartley et al. 1997; Goncalves et al. 2009). Wells received 1.25 μl of actively growing inoculum in TY adjusted to ∼5000 CFU and incubated at 30°C at 200 rpm. Cell density was checked after 72 h for *A. wrangelianus* rhizobia and 48 h for the faster-growing *M. polymorpha* rhizobia; preliminary experiments determined that most strains were in the exponential growth phase at this time. Two spectrophotometric readings of optical density (OD_{600}) were taken per plate and averaged. Optical density of a culture is well correlated with cell density (Somasegaran and Hoben 1994) and was therefore used as a fitness proxy. This design compensates for interstrain differences in the relationship between optical density and cell

density because the growth of each strain is compared to itself across treatments.

In all, 19 growth assays were conducted on a total of 450 isolates, including 292 from *A. wrangelianus* and 158 from *M. polymorpha*; yielding six replicates of each of the strain by nickel environment treatment combinations (6 replicates × 450 strains × 2 nickel environments) (Table S2). To determine the repeatability of the growth assays, 65 *A. wrangelianus* isolates and 116 *M. polymorpha* isolates were assessed a second time using identical protocols. Growth assay results were strongly predictive of those from repeated assays (*A. wrangelianus:* $F_{1,63} = 23.27$, $P < 0.0001$; *M. polymorpha:* $F_{1,114} = 32.49$, $P < 0.0001$), so values from the original 19 assays were pooled in the analyses of variance below.

ANALYSIS

Genetic variation for tolerance and growth rate

For each isolate, tolerance to nickel was calculated by subtracting the fitness of an isolate in the absence of nickel from its fitness in the presence of nickel within each of the six replicate plates, following Tiffin and Rausher (1999). Negative tolerance values indicate that nickel reduces fitness relative to growth in the absence of nickel. Analysis of variance (ANOVA) (GLM Procedure, SAS Institute, 2006) tested for genetic variation in tolerance and growth in the absence of nickel among rhizobia symbiotic with *A. wrangelianus* and with *M. polymorpha*. Isolate identity as a fixed effect was the only factor in this model.

Tolerance-growth trade-offs

The trade-off between tolerance and growth was estimated as the covariance between tolerance and fitness in the absence of nickel (Mauricio et al. 1997; Tiffin and Rausher 1999; Roff and Fairbairn 2006). A negative covariance indicates a trade-off. This calculation introduces a statistical bias because the same data are used to estimate both tolerance and fitness in the absence of nickel, artificially inflating the estimate of covariance. To compensate, we used a statistical correction developed specifically for this problem (Mauricio et al. 1997; Tiffin and Rausher 1999) to correct the inflated component of covariance. To evaluate statistical significance, we calculated 99% confidence intervals for the corrected covariance using 10,000 bootstraps to determine if this interval overlapped zero (Stinchcombe 2002; Agrawal et al. 2004; Stinchcombe 2005) for the host-associated rhizobia at each reserve.

To examine soil chemistry as a potential source of selection for nickel tolerance, simple linear regression (GLM procedure, SAS Institute, 2006) was used to determine if the concentration of nickel in an isolate's home soil was a significant predictor of nickel tolerance. Regressions were performed for *A. wrangelianus* rhizobia across all isolates from the three reserves, as well as for both *A. wrangelianus* and *M. polymorpha* rhizobia across sites within McLaughlin alone.

Adaptive differentiation

Acmispon wrangelianus rhizobia at multiple reserves. A mixed model ANOVA (Mixed Procedure, SAS Institute, 2006) in a factorial, repeated measures structure tested for spatially replicated patterns of adaptive divergence. Specifically, did serpentine rhizobial isolates exhibit greater growth in nickel-enriched media than did nonserpentine isolates, and conversely did nonserpentine isolates exhibit greater growth in the absence of nickel than did serpentine isolates? Multiple sites were sampled for both soil types at McLaughlin, and here these isolates were pooled by soil type. Reserve identity (reserve), serpentine or nonserpentine soil type (soil), and the presence or absence of nickel in the growth assay (nickel) were treated as fixed effects. There were six repeated measures of optical density for both levels of nickel for each strain. Strain (nested in region and soil) and the nickel-by-strain interaction (nested in region and soil) were treated as random effects. Because of the hierarchical error structure in the model, different factors were used as replication for testing effects at different hierarchical levels. Reserve, soil, and nickel were analyzed as a factorial combination of treatments, with reserve, soil, and their interaction tested over the mean squared error of strain (i.e., strain as the unit of replication). Nickel and its interactions with reserve and soil were tested over the mean squared error of the nickel-by-strain interaction (i.e., the nickel-by-strain interaction as the unit of replication). Because transformations of the data did not provide fully homogeneous variances among factors, raw optical density values were weighted by the inverse of the variance for the highest significantly heteroscedastic interaction, the nickel-by-soil-by-region interaction (Stanton and Thiede 2005; Baythavong et al. 2009). Least squares mean (LSM) comparisons were used to evaluate the significance and direction of significant effects. A significant effect of soil on response to nickel would be consistent with adaptive differentiation in rhizobial assemblages. Because this interaction varied among reserves, three protected ANOVAs were conducted separately, one for each reserve, to evaluate the effect of soil on the response to nickel.

Medicago polymorpha and A. wrangelianus rhizobia at a single reserve. A similar mixed model ANOVA was used to test whether the growth of isolates collected from replicated sites of both soil types within the McLaughlin reserve differed when cultured in two nickel environments. Serpentine or nonserpentine soil type (soil) was treated as a fixed effect, and replicate serpentine or nonserpentine soil sites within the reserve (site) were treated as a random effect nested within soil type; sites were selected at random from a collection of representative patches within the reserve. Nickel and strain factors were treated as described above.

Observations were weighted by the inverse of the variance for nickel, the term driving the heteroscedasticity in the model, to satisfy assumptions of homogeneity of variance.

Results

GENETIC VARIATION FOR TOLERANCE AND GROWTH

Genetic variation for both tolerance and growth is indicated by significant differences among isolates for both nickel tolerance (*A. wrangelianus*: $F_{291,1466} = 31.09$, $P < 0.0001$; *M. polymorpha*: $F_{157,947} = 21.20$, $P < 0.0001$) and growth rate in the absence of nickel (*A. wrangelianus*: $F_{291,1466} = 37.84$, $P < 0.0001$; *M. polymorpha*: $F_{157,947} = 21.28$, $P < 0.0001$).

TOLERANCE-GROWTH TRADE-OFF

Acmispon wrangelianus rhizobia exhibited a trade-off between tolerance and growth at all reserves. Rhizobia from Jasper Ridge had the strongest trade-off between nickel tolerance and growth, with a corrected covariance of −0.0412 (99% CI: −0.0533 to −0.0289) (Figs. 1A, S2A); Hopland and McLaughlin rhizobia exhibited similar trade-offs, with corrected covariances of −0.0225 (99% CI: −0.0270 to −0.0176) and −0.0179 (99% CI: −0.0268 to −0.0100), respectively (Figs. 1A, S2B, C). Rhizobia from *M. polymorpha*, at McLaughlin also showed a trade-off, with a corrected covariance of −0.0152 (99% CI: −0.0210 to −0.0100) (Figs. 1A, S2D); indistinguishable from that of *A. wrangelianus* rhizobia at this reserve.

Isolates from sites with greater soil nickel concentration showed greater average nickel tolerance. Across all sites, the magnitude of tolerance for *A. wrangelianus* rhizobia was significantly predicted from nickel concentration in the origin soil ($F_{1,291} = 24.85$, $P < 0.0001$, R-squared = 0.14) (Fig. 1B). However, within McLaughlin alone, neither *A. wrangelianus* ($F_{1,172} = 0.03$, $P = 0.8658$) nor *M. polymorpha* ($F_{1,156} = 0.12$, $P = 0.7326$) rhizobia displayed this pattern.

ADAPTIVE DIFFERENTIATION

Acmispon wrangelianus rhizobia at multiple reserves

Acmispon wrangelianus rhizobial assemblages exhibited adaptive differentiation between soil types (Table S3); rhizobia from serpentine soils have higher fitness in the presence of nickel (LSM, $P < 0.0001$), and rhizobia from nonserpentine soils have higher fitness in the absence of nickel (LSM, $P = 0.0426$). Overall, nickel inhibited growth (Table S3). The greater overall growth of serpentine rhizobia (Table S3) was driven by an asymmetric trade-off in which the growth advantage of serpentine rhizobia in the presence of nickel was greater than was the growth advantage of nonserpentine rhizobia in the absence of nickel (Fig. 2).

The magnitude of adaptive differentiation varied among the reserves (significant soil-by-nickel-by-reserve interaction, Table S3). Therefore, separate ANOVAs were conducted for each reserve. At Jasper Ridge, serpentine rhizobia grew 144% faster than nonserpentine rhizobia in the presence of nickel (LSM, $P < 0.0001$) and nonserpentine rhizobia grew 21% faster than serpentine rhizobia in the absence of nickel (LSM, $P = 0.0483$; Table S3, Fig. 2A). At Hopland, serpentine rhizobia grew 78% faster than nonserpentine rhizobia in the presence of nickel (LSM, $P < 0.0001$) and nonserpentine rhizobia grew 19% faster than serpentine rhizobia in the absence of nickel (LSM, $P = 0.0232$; Table S3, Fig. 2B). At McLaughlin, serpentine rhizobia grew 43% faster than nonserpentine rhizobia in the presence of nickel (LSM, $P = 0.0009$; Table S3, Fig. 2C), although nonserpentine rhizobia did not demonstrate a growth advantage in the absence of nickel.

Medicago polymorpha and A. wrangelianus rhizobia at a single reserve. *Medicago polymorpha* rhizobia at McLaughlin showed no evidence for adaptive differentiation between soil types (no soil-by-nickel interaction, Table S4, Fig. 2D). The presence of nickel imposed a physiological stress on these rhizobia by inhibiting growth (nickel main effect, Table S4; Fig. 2D). Analysis of the *A. wrangelianus* rhizobia from McLaughlin in a model analogous to that used for the *M. polymorpha* rhizobia yielded results with a strong correspondence to those generated in the protected ANOVA (Table S4).

Discussion

Despite abundant research on patterns of environmental microbial diversity, few studies document the ecological and evolutionary forces that maintain this diversity. This study is the first to demonstrate a trade-off in bacteria between serpentine tolerance and growth. The striking patterns of replicated adaptive divergence across serpentine soil boundaries in native rhizobia is one of the few demonstrations of this phenomenon in wild microbes (for others see Belotte et al. 2003; Sikorski and Nevo 2005) because few studies use spatially replicated designs to test for adaptive divergence in wild microbial assemblages (Prosser 2010; Lennon 2011). These findings are consistent with those from smaller experiments (6–10 isolates) that suggest symbiotic microbes from serpentine and nonserpentine soils may differ in nickel tolerance (ectomycorrhizae: Goncalves et al. 2007, 2009; rhizobia: Petgel 1980). However, the large number of isolates (450 total) in this study allow detection of a trade-off between tolerance and growth as well as a spatially replicated, population-level examination of differentiation in nickel tolerance across two host-associated rhizobium assemblages.

Trade-offs, such as the one we detected between nickel tolerance and growth rate, are a critical component of theoretical work predicting the stable coexistence of diverse lineages (Dieckmann and Doebeli 1999; Kneitel and Chase 2004). Although artificial

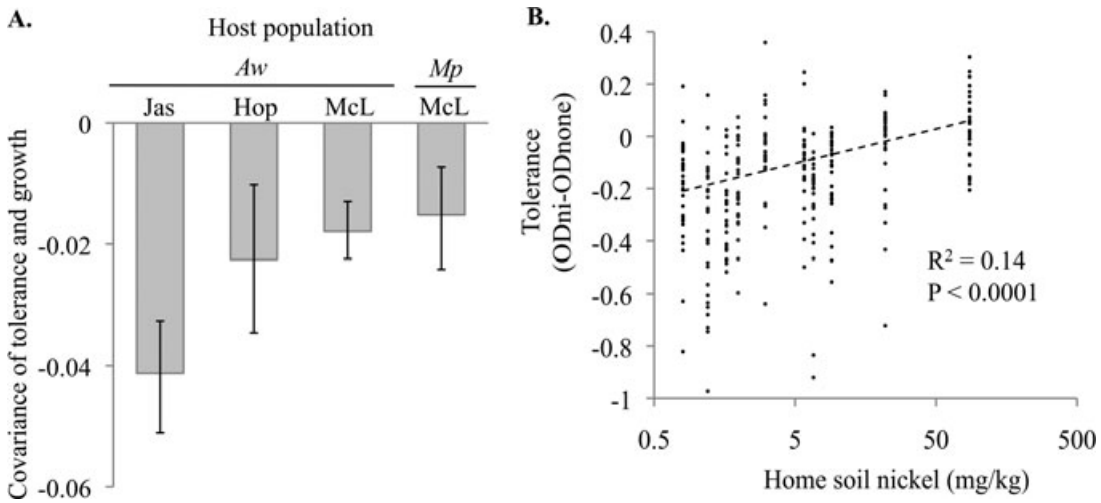

Figure 1. (A) Magnitude of the trade-off between nickel tolerance and growth rate for rhizobial isolates from hosts *Acmispon wrangelianus* (*Aw*) and *Medicago polymorph* (*Mp*) from Jasper Ridge (Jas), Hopland (Hop), and McLaughlin (McL). Trade-off is measured as the adjusted covariance between nickel tolerance and growth in the absence of nickel. Bars are bootstrapped 99% confidence intervals around observed mean adjusted covariances. (B) Regression of nickel tolerance of isolates from *A. wrangelianus* on the concentration of nickel in the home soil. Dashed line indicates a regression, points indicate individual isolates. Note logarithmic scale.

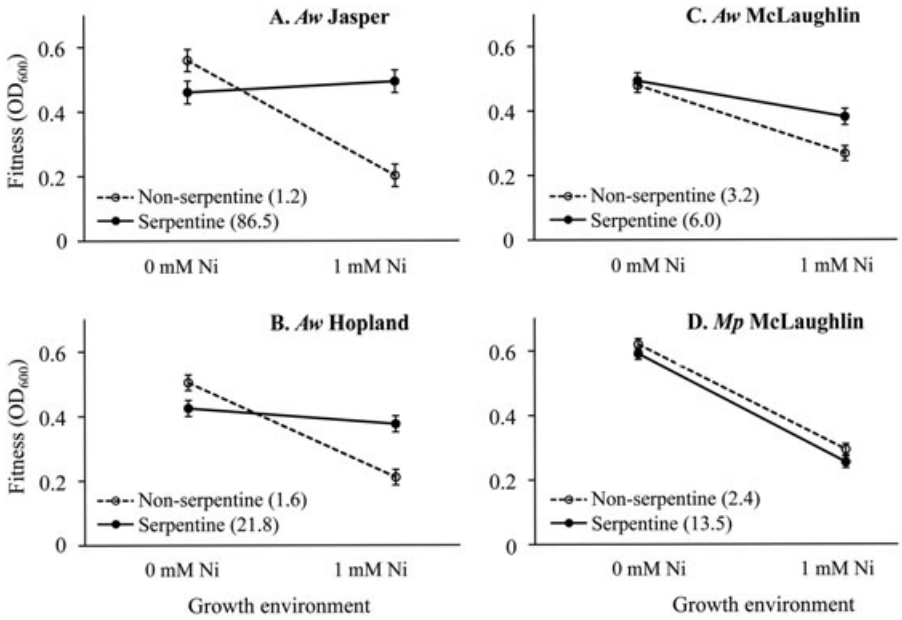

Figure 2. Fitness of rhizobial isolates from nonserpentine (dashed line) and serpentine soils (solid line) grown with 1 mM or 0 mM nickel. Rhizobial isolates from: *Acmispon wrangelianus* (*Aw*) at Jasper Ridge (A), *Aw* at Hopland (B), *Aw* at McLaughlin (C), and *Medicago polymorpha* (*Mp*) at McLaughlin (D). The mean concentration of nickel in the soils from which strains were collected at a reserve is shown in parentheses (mg/kg soil) for both serpentine (S) and nonserpentine (N) soils. Fitness was measured as the optical density of cultured cells after a period of growth; plotted values are ANOVA least squares means (LSM). Bars are LSM ± standard error.

evolution experiments demonstrate that bacterial adaptation frequently does not entail a strong trade-off (Novak et al. 2006; Bennett and Lenski 2007; Hughes et al. 2007; Jasmin and Kassen 2007; Jessup and Bohannan 2008; Lee et al. 2009), the native and invasive rhizobial assemblages in this study exhibited significant trade-offs. This trade-off is asymmetric in that the cost paid by nickel-adapted serpentine strains in the absence of nickel is less than that paid by nickel-sensitive nonserpentine strains grown in the presence of nickel. Asymmetric trade-offs are predicted in some models of coexistence and are often observed under artificial selection (Jasmin and Kassen 2007; Lee et al. 2009).

Our findings support current models based on trade-offs predicting the coexistence of microbial lineages in heterogeneous environments (Bell 1991; Chesson 2000; Kassen 2002; Sears and

Chesson 2007). Coexistence of rhizobia associated with native *A. wrangelianus* results from adaptive differentiation across serpentine soil boundaries. *Acmispon wrangelianus* isolates from naturally metaliferous serpentine soil outperform those from nonserpentine soil in the presence of nickel, but tend to be outperformed by nonserpentine isolates in the absence of nickel. Robust growth in the presence of nickel appears to be an important adaptation to serpentine soil; in contrast, rapid growth in the absence of nickel may be an adaptation to nonserpentine soil where conditions may select for greater competitive ability. Thus, as demonstrated in plants (Kruckeberg 1954; Brady et al. 2005), heterogeneity in soil chemistry may maintain phenotypic diversity in bacteria via adaptive divergence. Although rhizobia in this study exhibit trade-offs and adaptive divergence in a manner similar to plants, these assemblages are difficult to classify as populations or communities in the classical sense because bacteria are not easily sorted into reproductively isolated species (Green et al. 2008; Hanson et al. 2012). Because theory based upon trade-offs applies to biodiversity ranging from populations within species undergoing local adaptation to communities of species undergoing differentiation, this is an especially useful area for exploring evolutionary mechanisms promoting coexistence in natural microbial assemblages where limits to gene flow are unclear.

Adaptive differentiation and nickel tolerance in *A. wrangelianus* symbionts were greater at reserves with higher soil nickel concentrations. This suggests that soil chemistry generates a continuum of selection not fully captured by the serpentine/nonserpentine dichotomy, as has been found for plants (Berglund et al. 2001; Berglund et al. 2004). Higher nickel concentrations would intensify selection for nickel tolerance, which trades off with growth in the absence of nickel. Jasper Ridge rhizobia show the greatest differential between the growth of serpentine and nonserpentine strains in the presence of nickel, followed by Hopland rhizobia, and then McLaughlin rhizobia, in a pattern consistent with soil nickel concentrations. Greater diversity is maintained as the disparity in conditions between environmental patches increases in artificial microbial selection experiments (Kassen and Bell 2000; Jasmin and Kassen 2007), suggesting reserves with more nickel-enriched serpentine could support greater levels of diversity via greater differentiation. The trade-off between nickel tolerance and growth in *A. wrangelianus* rhizobia was stronger at Jasper Ridge than at McLaughlin. This spatial variation in the magnitude of rhizobial trade-offs demonstrates that the strength of evolutionary trade-offs varies among these natural populations (Hereford 2009), which could elicit different evolutionary responses to selection (Lenski 1988a,b; Novak et al. 2006; Bennett and Lenski 2007; Hughes et al. 2007; Lee et al. 2009). The serpentine soil at Jasper Ridge and Hopland occurs in larger outcrops than at McLaughlin. Larger outcrops could provide a more consistent selective environment across rhizobial generations undergoing dispersal, which, in turn, could result in stronger adaptive differentiation. Detangling the relative contributions of selection intensity versus habitat patch size to adaptive differentiation will contribute greatly to a better understanding of mechanisms affecting natural microbial diversity.

Differentiation of these bacterial assemblages is consistent with the observation that mycorrhizal fungi (Panaccione et al. 2001; Schechter and Bruns 2008; Branco 2010; Branco and Ree 2010) and nonsymbiotic bacteria (Oline 2006) can form distinct serpentine soil communities, albeit in taxonomically, not functionally defined communities. In other cases, however, distinct communities have not been detected (Moser et al. 2009; Fitzsimons and Miller 2010). The specific abiotic stressors that can drive differentiation in these assemblages have not previously been well understood. Mechanisms of microbial resistance to nickel have been examined in bacteria and can include inducible efflux systems which pump nickel cations out of the cytoplasm (Mengoni et al. 2010); nickel-tolerant serpentine *Bradyrhizobia* harbor putative components of two distinct pathways for such nickel tolerance (Chaintreuil et al. 2007).

Although serpentine rhizobia symbiotic with native *A. wrangelianus* at McLaughlin exhibit adaptation to nickel, those associated with the invasive *M. polymorpha* do not, despite sharing a similar trade-off between nickel tolerance and growth. The lack of nickel adaptation in these presumably recent invaders could result from too few generations of selection across soil boundaries. However, this seems unlikely given the rapid evolution of other rhizobia to anthropogenic stressors such as heavy metals (Wu and Lin 1990; Lakzian et al. 2002; Delorme et al. 2003; Pereira et al. 2006; Sa-Pereira et al. 2009). Moreover, the host, *M. polymorpha,* invaded McLaughlin ~150 years ago (Lau 2008) and has evolved soil-specific ecotypes (Porter et al. 2011). Diversity in the invasive rhizobial assemblage could be maintained via trade-offs between other costly traits underlying tolerance of other axes of environmental heterogeneity, or by alternative mechanisms such as bet-hedging strategies via dormancy (Jones and Lennon 2010). Both host species from this study are adaptively differentiated across soil types (Porter et al. 2011; Porter, unpubl. ms.) and differences in partner selectivity by host ecotypes could influence rhizobial differentiation across serpentine soil boundaries.

We know little about how alternation between free-living and symbiotic lifestyles affects microbial evolution under context-dependent selection. Determining whether abiotic tolerance and symbiotic performance are decoupled will be key to understanding evolutionary feedbacks between hosts and symbionts in heterogeneous environments. This study has taken a first step by examining rhizobial trade-offs and differentiation in heterogeneous environments in the unassociated life phase. Little evidence suggests that in symbiosis rhizobia tolerant of abiotic stress ameliorate the

effects of abiotic stress on hosts (Thrall et al. 2008; Friesen et al. 2011), unlike tolerant mycorrhizae (Van Tichelen et al. 2001; Adriaensen et al. 2004; Adriaensen et al. 2005; Adriaensen et al. 2006; Krznaric et al. 2009). However, if rhizobium abiotic tolerance and symbiotic performance are found to trade-off, this constraint could further promote mutualistic microbial diversity in heterogeneous environments and lead to complex coevolutionary outcomes.

ACKNOWLEDGMENTS

Thanks to J. Luong and P. Cherng for laboratory assistance, J. Piovia-Scott, M. Friesen, and N. Willits for advice, J. Stinchcombe for assistance calculating trade-offs, and to the McLaughlin, Hopland, and Jasper Ridge reserves. ML Stanton, DR Cook, EL Simms, and two anonymous reviewers helped improve the manuscript. Funding was provided by a GRFP and DDIG from the National Science Foundation, and a University of California Mathias Grant.

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Associate Editor: J. Wernegreen

Supporting Information

The following supporting information is available for this article:

Table S1. Coordinates for sample sites.

Table S2. Mixed model ANOVA for rhizobial isolates from *Acmispon wrangelianus* originating from serpentine and nonserpentine soils at three reserves, grown with and without excess nickel.

Table S3. Mixed model ANOVAs for rhizobial isolates from *Acmispon wrangelianus* (*Aw)* and *Medicago polymorpha* (*Mp*) originating from serpentine and nonserpentine soils at McLaughlin, grown with and without excess nickel.

Figure S1. Locations of the reserves sampled (stars).

Figure S2. The relationship between nickel tolerance and fitness in the absence of nickel for rhizobial isolates from: (A) *Acmispon wrangelianus* (*Aw*) at Jasper Ridge, (B) *Aw* at Hopland, (C) *Aw* at McLaughlin, and (D) *Medicago polymorpha (Mp)* at McLaughlin.

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

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