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Special Issue Article

Function is a better predictor of plant rhizosphere community membership than 16S phylogeny

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Summary

Rhizobacterial communities are important for plant health but we still have limited understanding of how they are constructed or how they can be manipulated. High-throughput 16S rRNA sequencing provides good information on taxonomic composition but remains an unreliable proxy for phenotypes. In this study, we tested the hypothesis that experimentally observed functional traits would be better predictors of community membership than phylogenetic origin. To test this hypothesis, we sampled communities on four plant species grown in two soil types and characterized 593 bacterial isolates in terms of antibiotic susceptibility, carbon metabolism, resource use and plant growth-promoting traits. In support of our hypothesis we found that three of the four plant species had phylogenetically diverse, but functionally constrained communities. Notably, communities did not grow best on complex media mimicking their host of origin but were distinguished by variation in overall growth characteristics (copiotrophy/oligotrophy) and antibiotic susceptibility. These data, combined with variation in phylogenetic structure, suggest that different classes of traits (antagonistic competition or resource-based) are more important in different communities. This culture-based approach supports and complements the findings of a previous high-throughput 16S rRNA analysis of this experiment and provides functional insights into the patterns observed with culture-independent methods.

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Introduction

The rhizosphere is a hotspot for microbial activity, driving the evolution of a diverse set of microbes (Reinhold-Hurek *et al*., 2015). At a fundamental level rhizosphere microbes have to be able to exploit the nutrients in root exudates and also tolerate the secretion of any plant secondary metabolites (Grayston *et al*., 1998; Bertin *et al*., 2003; Marschner *et al*., 2004; Kumar *et al*., 2007; Berendsen *et al*., 2012). Plants have the ability to adjust root exudate composition (Haney and Ausubel, 2015; Panke-Buisse *et al*., 2015), and this composition varies between plant species and with environmental conditions (el Zahar Haichar *et al*., 2008; Compant *et al*., 2010; Bever *et al*., 2012; Philippot *et al*., 2013; Chaparro *et al*., 2014; Pérez-Jaramillo *et al*., 2016; Pérez-Izquierdo *et al*., 2019).

However, there is a great deal we do not know about the rhizosphere community assembly, and in particular how the ecology of these communities varies between host plant species. For example, rhizosphere microbes need to be efficient competitors in addition to having the basic metabolic functions for exploiting plant nutrients (Martínez-Granero *et al*., 2006). Competitive interactions can occur in the rhizosphere via either resource-based or antagonistic interactions (Whipps, 2001; Raaijmakers *et al*., 2009; Beneduzi *et al*., 2012) but the broad level importance of competitive traits in the rhizosphere is poorly characterized. If competition can limit colonization by plant pathogenic microbes (Berendsen *et al*., 2012), it is also likely to limit colonization of anthropogenic applications of rhizobacteria. Improving the functional understanding of plant colonization would help us manipulate microbial succession and community assembly in the rhizosphere (Ho *et al*., 2013; Krause *et al*., 2014).

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Understanding functional traits is therefore important, but we remain sceptical of approaches that infer phenotypes from 16S rRNA phylogenies (Ma *et al*., 2015; Lopes *et al*., 2016; Utturkar *et al*., 2016). These techniques assume that functional traits are tightly associated with 16S rRNA phylogeny, and given that genes involved in bacterial secondary metabolism are often horizontally mobile, these phylogenies are likely to be unreliable predictors of function (Kroll *et al*., 2017). Shotgun sequencing of DNA from entire communities can be more instructive for inferring function and has suggested important roles for functional categories including Fe and N transport and metabolism, secretion systems, chemotaxis and motility (Sessitsch *et al*., 2012; Mendes *et al*., 2014; Ofek *et al*., 2014; Bulgarelli *et al*., 2015). Nevertheless, this approach is challenging due to the high diversity of bacteria living in and around roots, abundant plant DNA, and the relatively poor annotation of genes identified in the plant rhizosphere, where 59% of the microbial genes have no known function (Ofek *et al*., 2014).

On the other hand, sequencing data can be incorporated into phylogenetic analysis of rhizosphere communities and this can help to generate hypotheses about the different ecological processes in community assembly, a technique widely used in the eukaryote literature (Vamosi *et al*., 2009). For example, the fact that rhizosphere bacteria represent a limited pool of the available soil microbes implies that there is a functional filter (e.g. metabolic adaptation to root exudates) for community membership. The fact that members are phylogenetically 'clustered' into a few major groups also indicates that these functional attributes can be vertically inherited. Conversely, if members are distributed evenly across phylogenetic trees this is consistent with a pattern of competitive exclusion in which coexistence of near relatives is prevented by competitive interactions between organisms with similar niches (Vamosi *et al*., 2009). Ultimately, specific hypotheses about the importance of different processes (competition, functional filters) in community assembly can be tested by measuring traits (Vamosi *et al*., 2009).

We can make further tests of community assembly hypotheses by growing plants in controlled 'common garden' designs that manipulate the source of microbes, physical growing conditions, and/or the plant species used to capture microbial species (Hacquard *et al*., 2015). The extent to which plants capture similar communities between replicates and across growing conditions allows us to test if plants are robustly associated with particular bacterial groups. A previous culture-independent study (using high-throughput 16S rRNA sequencing) examined community structure across two soil types and four crop plant species (Matthews *et al*., 2019). Here, we used a culture-based approach to make a functional characterization of bacterial isolates from the same experiment in order to complement the culture-independent approach. Cultured isolates were also characterized by 16S sequencing so we could examine the phylogenetic structure of different communities and make comparisons between traits and communities that take the non-independence caused by evolutionary history into account (Orme *et al*., 2012).

Given the importance of horizontal gene transfer for ecologically important bacterial traits, our key hypothesis was that functional traits would be better able to predict community membership than simple phylogeny. This hypothesis, therefore, predicts that phylogeny will be a weak predictor of morphological function at least for some traits, or, more formally, that traits can have low phylogenetic signal. Another prediction that derives from this hypothesis is that different communities would vary phenotypically in terms of their mean functional trait values. We also planned to use phylogenetic analyses of communities to examine broad-scale differences in structure between distinct ecological communities, i.e. to test for differences in clustering and evenness that might indicate the different importance of vertically inherited traits and competition in community structure (Vamosi *et al*., 2009).

We explored the relative importance of competition, metabolism and host plant through our choice of functional traits, selecting four broad trait classes that are proposed to contribute to rhizosphere colonization. First, antagonistic competition should select for high tolerance of antimicrobials, so we scored differences in susceptibility to a panel of antibiotics. We also examined resource-based ecological attributes by quantifying enzymatic activity on carbon sources commonly produced by plant roots and by examining growth on complex resources. The latter could test for variation in growth rate generally, for specialization on resources derived from a subset of host plant species, as well as the extent to which isolates were adapted to high nutrient (copiotrophy) or low nutrient environments (oligotrophy). Finally, plant growth-promoting (PGP) traits (phosphate solubilization, heat tolerance, auxin production) have been widely used to screen rhizobacterial isolates for plant beneficial attributes and remain good candidates for functional traits that are important for host manipulation or selection. As a culture-based methodology, this comes with limitations. Nevertheless, we argue that the coupling of culture-dependent approaches with high-throughput sequencing (Matthews *et al*., 2019) can be a powerful means of exploring the specific functions involved in colonization of the rhizosphere (Lugtenberg *et al*., 2002).

Results

Study context

Four crop species were grown in controlled experimental conditions in soils originating from two different habitats

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(grassland and woodland), recovered from Silwood Park, UK (Matthews *et al*., 2019). In order to determine how these two environmental conditions interact to determine the make-up of communities, we used a split-plot design with 40 pots and each of the four species grown in a single pot, subdivided by plastic inserts. Three 40 L soil samples were collected from subsites across both habitats then, independently sifted, mixed and decanted, using approximately 5 L per 7.5 L pot. Per subsite seven pots were filled to a total of 42 pots, 21 per soil type. Pots were directly sown with 20 onions, six peas, 10 tomatoes, and six sweet corn using surface sterilized seeds. Approximately 2 months later pots were sampled destructively over 3 weeks (Matthews *et al*., 2019). The provenance of isolates in this study is known in terms of their soil origin: grassland or woodland soils; and their isolation host species: *Allium fistulosum* L. var Ishikura (onion); *Pisum sativum* L. var Twinkle (pea); *Solanum lycopersicum* L. var MicroTom (tomato); *Zea mays* L. var Minipop (sweet corn). While previous work explored variation in bacterial community structure via 16S amplicon sequencing, here we used culture on standard media to generate a large collection (593) of bacterial isolates to examine both functional and phylogenetic variations.

Culturable isolate diversity was surveyed in three steps. First, we isolated six distinct morphotypes from each bacterial cell enrichment produced from each species, per pot, for each of 40 pots. Of these 960 isolates, 593 were isoclonal, experimentally tractable and provided satisfactory 16S rDNA sequence data. Subsequently, a phenotypic survey was used to characterize 19 traits and these data were combined in a comparative phylogenetic analysis focusing on the associations between taxa and traits with hosts and soils.

General description of communities

Trait data, phylogeny and taxonomy of 593 isolates are summarized at genus level in Fig. 1A. A genus-level analysis comparing phylogenetic and functional classifications shows that phylogeny and function are relatively uncoupled, confirming our prediction that 16S rRNA sequences would be poor predictors of phenotype (Fig. 1B). This genus-level analysis trades off taxonomic resolution against sensitivity to trait variance (which can be high isolate to isolate, Fig. S1). Communities recovered from different plants and soils vary with respect to the total numbers of isolates recovered and their taxonomic composition (Fig. 1C). Bacterial isolates were recovered from 37 genera in four Phyla; predominantly γ-Proteobacteria. The γ-Proteobacteria alone comprise 68% of the culturable bacteria and are dominated by *Pseudomonas* and *Enterobacter* spp.

Bacterial alpha diversity at the genus level shows no difference between soils (Table S1). On average grassland communities comprise 8.58 genera, woodland communities 8.25 genera, Welch Two Sample *t*-test ($t = 0.2$, $df = 20.36$, $p = 0.78$). Likewise, there is no significant difference in alpha diversity between hosts. Communities where taxa are clumped on the phylogeny tend to have a lower phylogenetic diversity (PD), because the isolates in these communities capture a smaller part of the total PD. For instance, PD is higher in *Solanum* than *Pisum* communities, and lower in *Zea* compared to *Allium* or *Solanum* communities. These patterns occur in spite of *Zea* communities comprising more isolates in both soils (Fig. 1C).

Factors shaping community composition

We tested whether community membership (plant and soil origins of isolates) explained variation in phylogenetic or functional traits. In support of our main hypothesis, functional traits proved to be ecologically more informative than phylogeny in shaping communities. Although phylogenetic and trait composition data were correlated ($r = 0.77$, $p = 0.001$) the multivariate analysis of communities by functional traits (Fig. 2A, ADONIS AIC 59.2) has greater explanatory power than that of phylogeny (Fig. 2B, ADONIS AIC 97.8). The communities are shown clustered by non-metric multidimensional scaling (NMDS) of phylogenetic distance (MNTD) (Fig. 2A) and Bray–Curtis dissimilarity of Euclidian trait distances (Fig. 2B). In both analyses, host species account for the highest variation (ANOSIM-Phylogenetic: $R = 0.31$, $F = 2.9$, $p = 0.03$; ANOSIM-Traits: $R = 0.44$, $F = 5.3$, $p = 0.001$). In contrast, relatively little variation between the isolate communities is explained by differences between soil origin (ANOSIM-Phylogenetic: $R = 0.01$, $F = 0.2$, $p = 0.7$), (ANOSIM-Traits: $R = 0.05$, $F = 1.2$, $p = 0.36$). There is no support for an interaction between soil and host (ANOSIM associated $p > 0.7$) and soil subsite has a variable contribution to phylogenetic or trait structure (Table 1). Thus, phylogenetically and functionally, host plant has a stronger impact on isolate community structure than soil type.

The relationship between phylogeny and function

Although phylogeny does not reliably predict phenotype (Fig. 1B), traits typically showed phylogenetic signal and were not randomly distributed across the phylogenetic tree (Table 2, see Fig. S2 for branch length analysis). However, phylogenetic signal did vary between traits and was often weak, while there were traits with near-random distributions. For example, estimates of Pagel's λ approached 0 for traits such as erythromycin and

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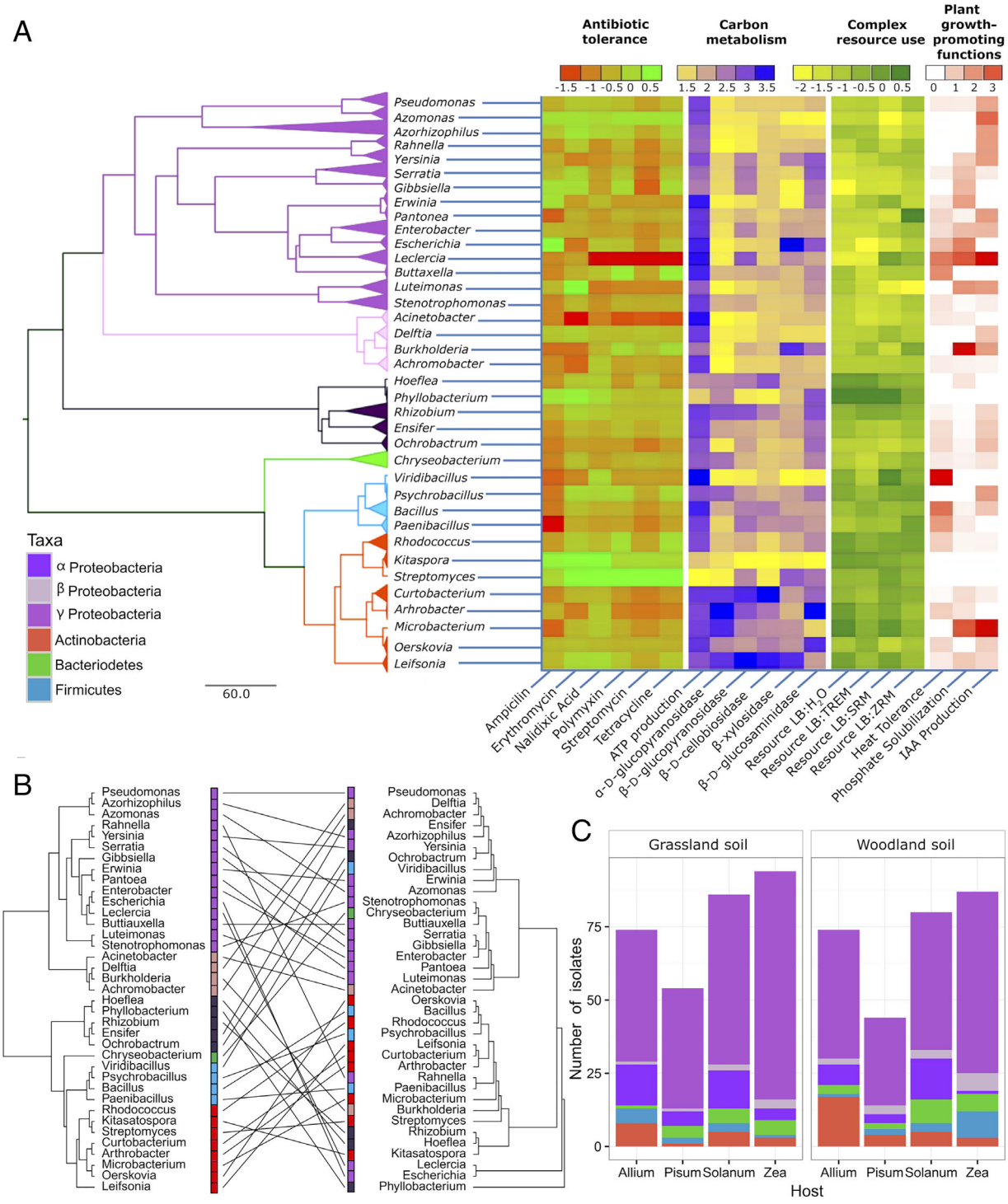

Fig 1. Summary of trait and isolate abundance.

A. Shows a genus-level summary of the isolate phylogeny coloured by Class. To the right genus level means of trait data are shown in heat maps grouped by type of trait.

B. Represents a comparison of the clustering of bacterial genera based on 16S rRNA gene phylogeny (left) and taxa re-sorted according to functional traits (right). The consensus phylogenetic tree was made using a partial 16S rRNA gene alignment with clades collapsed at the level of genus. Hierarchical clustering of traits was based on a Bray–Curtis dissimilarity matrix of mean genus trait values. The grey lines connect genera, with coloured bands indicating taxa groups coloured by Class.

C. Summarizes the abundance of isolates at the level of Class from different treatments: Grassland or Woodland soils isolated from four host species: *Allium fistulosum*, *Pisum sativum*, *Solanum lycopersicum*, *Zea mays*.

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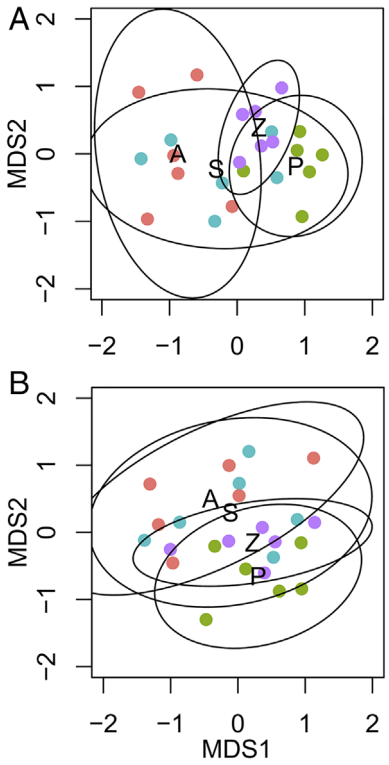

Fig 2. Phylogenetic and functional characterization of root-associated culturable bacterial communities. Two-dimensional projections are shown clustered by a non-metric multidimensional scaling analysis based on (A) Euclidian trait distance and (B) phylogenetic mean nearest taxon distance (MNTD). The data are abundance scaled and in both cases values indicating the degree of fit between the original distances in the matrix and the reproduced distances within the ordination plots are acceptable (stress = 0.2). Each point represents a single community, hosts are represented A = *Allium* (red) P = *Pisum* (green) S = *Solanum* (blue) Z = *Zea* (purple). Ellipses represent 95% confidence intervals of host groups. Note that data coloured by hosts are more obviously clustered by dissimilarities in functional traits (A) than phylogenetic composition (B).

Table 1. ANOSIM test results showing comparisons for culturable communities originating from two different soil types, grassland and woodland, and associated with: *Allium*, *Pisum*, *Solanum* and *Zea* plant roots.

Factor	*df*	*f*	R^2	*p*
Trait dissimilarity (Bray–Curtis)				
Soil	1.23	1.22	0.05	0.326
Host	3.23	5.32	0.44	0.001
Soil subsite	5.23	1.29	0.26	0.264
Host × soil	7.23	0.52	0.04	0.791
Phylogenetic dissimilarity (MNTD)				
Soil	1.23	0.23	0.01	0.722
Host	3.23	2.96	0.31	0.032
Soil subsite	5.23	2.50	0.41	0.042
Host × soil	7.23	0.57	0.07	0.67

polymyxin susceptibility, suggesting no phylogenetic signal (Table 2). In contrast, β-D-glucosaminidase, streptomycin susceptibility and phosphate solubilization had strong phylogenetic signal (Table 2). Most carbon-metabolism traits and all PGP traits showed phylogenetic signal, although parameter estimates were often modest. By inspection of phylogenies, we also observed that trait patterns were highly variable within clades and not always divergent between clades (Fig. 1A).

However, a significant phylogenetic structure sometimes derives from signal in a limited region of the tree. To address this, we explored how phylogenetic signal varied among five taxonomic ranks. Of these ranks, family explained the majority of variation in most traits (Fig. S3) and genus explained the least, i.e. the majority of variation in traits lies between families. For example, the Streptomycetaceae exhibited low antibiotic susceptibility (Moran's *I* range 0.41–0.52), while the Acinetobacteriaceae and Leclerciaceae are approximately 10 times more susceptible to antibiotics (Moran's *I* range 0.04–0.06). Moran's *I* at a family level broadly agreed with estimates of Pagel's λ, for example both Moran's *I* and Pagel's λ identified a near-random distribution for β-xylosidase and *Zea* root medium (ZRM) (Table 2).

Comparing phylogenetic structure between communities

Bacterial trait diversity (mean Euclidian distance) at the genus level showed no difference between soils, being 0.25 in grassland soil and 0.2 in woodland soil (Welch Two Sample *t*-test $t = 0.1$, $df = 20.4$, $p = 0.90$). Bacterial communities from *Solanum* roots are comparatively phylogenetically even, meaning that individual members are more distantly related from each other than expected by chance [standardized effect sizes (SES) >0], while communities from *Zea* were phylogenetically clustered, being more closely related than expected by chance (SES <0). In contrast to PD, trait diversity was more correlated between hosts (ANOVA $F_{3,20} = 3.374$, $p = 0.0387$). Trait diversity was lower than expected in *Zea* root communities (SES-MPD <0), although these communities had above average species richness (SR). PD was also significantly higher in *Zea* than in *Allium* root communities (*diff* = 3 EDU post hoc Tukey HSD adjusted $p = 0.025$).

Ecological relationships between traits and communities

We also sought to explore how communities differed from each other in terms of individual traits, to complement multivariate and clustering analyses above. These analyses used normalized means, which for growth data were the slope of productivity changes in response to

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Table 2. Variation in phylogenetic signal associated with 19 rhizobacterial traits.

Trait	λ	LR test $\lambda = 0$	*I*
Ampicillin (a)	0.013	4.93*	0.03**
Erythromycin (b)	6.6×10^{-6}	−0.01	0.07***
Nalidixic acid (c)	0.05	41.5***	0.16***
Polymyxin (d)	6.6×10^{-5}	−0.02	0.06***
Streptomycin (e)	0.34	88.5***	0.20***
Tetracycline (f)	0.024	13.1***	0.14***
ATP production (g)	0.0024	0.29	0.10***
α-D-glucopyranosidase (h)	0.071	28.6***	0.39***
β-D-glucopyranosidase (i)	0.079	24.6***	0.27***
β-D-cellobiosidase (j)	0.059	14.0***	0.23***
β-xylosidase (k)	6.6×10^{-5}	−0.02	0.01
β-D-glucosaminidase (l)	0.41	16.8***	0.08***
Resource LB:H_2O (m)	0.069	16.5***	0.26***
Resource LB:TREM (n)	0.055	39.2***	0.25***
Resource LB:SRM (o)	0.008	1.90	0.15***
Resource LB:ZRM (p)	0.00075	2.07	0.02
Heat tolerance (q)	0.021	4.78*	0.15***
Phosphate solubilization (r)	0.071	72.2***	0.23***
IAA production (s)	0.058	46.3***	0.07***

Pagel's λ varies from 0 to 1, with 0 indicating that traits are distributed randomly across trees; the associated likelihood ratio tests of the null hypothesis that $\lambda = 0$. Moran's *I* is an autocorrelation index, assessing the relationship between trait values and phylogenetic distance, tests here use values calculated at the family rank. Tests are based on a phylogenetic tree of all isolates ($n = 593$). All associated *p*-values summarized as: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

changes in concentration (Fig. 3). Comparisons used a phylogenetic-generalized least squares approach (PGLS - see Methods), which accounts for the fact that lineages may share similar values through recent evolutionary history (non-independence). PGLS revealed significant variation between communities in terms of antibiotic susceptibility and in terms of growth in complex media and PGP function for a number of traits; metabolic traits were relatively similar between communities (Fig. 3).

Consistent with diversity analyses above, host was often the most important factor shaping trait variation. Host plant significantly affected three of the six antibiotic susceptibility traits: nalidixic acid (trait Nal, Fig. 3; $F_{3,593} = 5.9$, $p < 0.001$), streptomycin (Strep, Fig. 3; $F_{3,593} = 2.6$, $p < 0.05$), tetracycline (Tet, Fig. 3; $F_{3,593} = 3.1$, $p < 0.05$) while typically *Solanum* communities were less susceptible to antibiotics overall (Fig. 3). In the case of nalidixic acid, for example, *Solanum* communities were significantly less susceptible than *Pisum* ($t = 3.4$, $p < 0.005$, $df = 585$) or *Zea* communities ($t = 3.3$, $p < 0.01$, $df = 585$; Fig. 3). Both ampicillin and erythromycin (Amp, Ery) had comparable weaker effects on microbial productivity at the highest dose tested (Fig. 3B).

After accounting for phylogeny, isolate communities differed significantly in three of the six metabolic traits. ATP production differed with both host, and host interacted with soil (Host, $F_{4,593} = 5.2$, $p = 0.002$; Host × Soil, $F_{5,593} = 3.4$, $p = 0.02$). A similar pattern was shown by β-xylosidase activity (Host, $F_{3,593} = 5.8$, $p < 0.001$: Host × Soil interaction $F_{8,593} = 3.3$, $p < 0.05$, Soil $p = 0.1$). For β-D-glucosaminidase activity, host, soil and their interaction contributed relatively equally as predictors (Host, $F_{3,593} = 3.1$, $p < 0.05$; Soil, $F_{3,593} = 4.7$, $p < 0.05$; Host × Soil $F_{8,593} = 3.2$, $p < 0.05$).

Host plant association affected growth on two of the four complex resource traits: tomato root exudate medium (TREM) and ZRM ($F_{4,593} = 6.8$, $p < 0.0001$; $F_{4,593} = 5$, $p < 0.005$ respectively), although there were similar trends in all these traits in that *Pisum* and *Zea* communities were the most sensitive to declining nutrient conditions, a pattern indicating copiotrophy (Fig. 3A). The oligotrophy/copiotrophy axis was most strongly tested with TREM media as this supported least growth of all the complex media (Fig. 3B). *Zea* root media supported similar levels of growth to Lysogeny Broth (LB) (Fig. 3B). Here *Zea* communities did not suffer reduced growth as LB was replaced with ZRM (ZRM slope = 0.45), but the *Pisum* community did and was more sensitive to ZRM than the *Solanum* community ($t = 3.4$, $p < 0.005$, $df = 585$) and the *Zea* community ($t = 3.5$, $p < 0.005$, $df = 585$), see Fig. 3A. *Pisum* communities also showed declining productivity with increased *Solanum* root media concentration (Fig. 3, trait SRM) although this was not significant in the PGLS comparison.

All three PGP traits presented significant community variation in the PGLS analysis. Heat tolerance (Htol) was correlated with host ($F_{4,593} = 3.43$, $p = 0.02$) but not with soil ($p > 0.1$). Heat tolerance was significantly greater in *Pisum* than *Solanum* communities ($t = 2.9$, $p = 0.02$,

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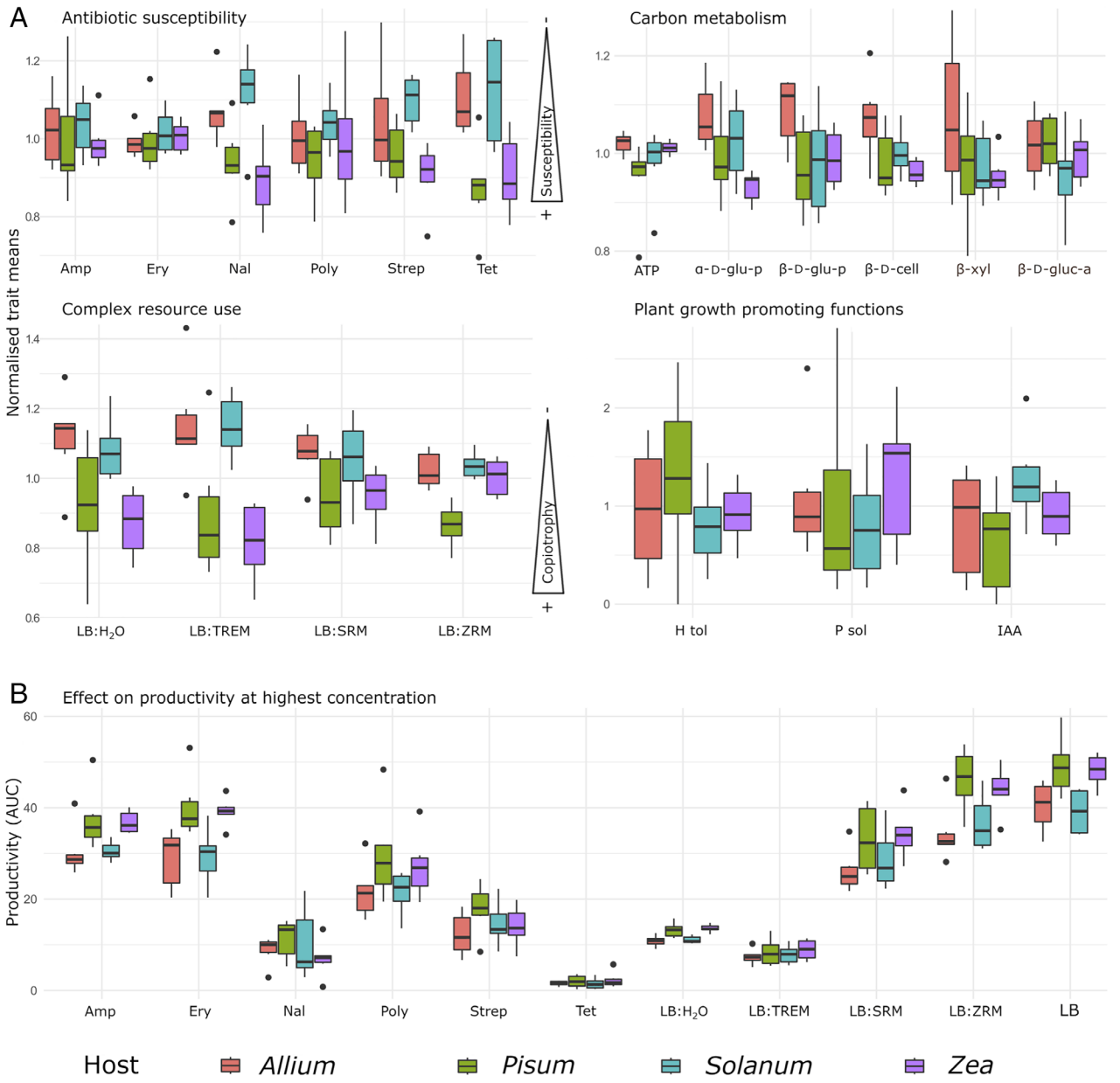

Fig 3. Variation in trait values between communities.
A. Communities are represented as pooled means of isolates from independent soil samples used to set up seven experimental pots. Data are normalized trait means, communities <1 were below the global mean, community means >1 above it.
B. Community productivity at the highest concentration tested for antibiotic susceptibility and complex resource use traits. The boxplots are coloured by host plant, with means shown as thick horizontal bars, the body of the box is the lower and upper quartiles (25% and 75%), the whiskers show the 5%–95% range and outliers are black points.

df = 585). Phosphate solubilization (Psol) showed a phylogenetically significant interaction between ecological groups (Host × Soil, $F_{8,593} = 4.6$, $p < 0.005$). This interaction was largely due to increased phosphate solubilization in Grassland *Zea* compared to Woodland *Pisum* communities ($t = 3.3$, $p = 0.025$, $df = 585$). Indole acetic acid (IAA) production, like phosphate solubilization, also showed a relatively strong interaction between host plant species and soil (Host × Soil $F_{8,593} = 3.9$, $p < 0.01$) but weaker main effects of host and soil (Host, $F_{4,593} = 2.6$, $p = 0.052$; Soil, $F_{3,593} = 4.0$, $p = 0.0456$).

Correlations between traits

In order to explore whether there were common ecological or physiological factors affecting similar traits among

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communities, we performed a multivariate analysis of trait covariance on all functional data. Pairwise correlations among traits were not always significant after accounting for phylogenetic effects. For example, antibiotic susceptibility traits were for the most part positively correlated with each other after accounting for phylogeny (Fig. 4). In contrast, the raw data suggested a positive correlation between metabolic traits associated with carbon metabolism, but this was not robust after accounting for phylogenetic effects (Fig. 4).

In addition, antibiotic susceptibility traits tended to be negatively correlated with ATP production (ATP), β-xylosidase (β-xyl) and β-D-glucosaminidase activity (β-D-gluc-a). Two antibiotic susceptibility traits were also negatively correlated with growth on *Solanum* root media (SRM) (Fig. 4, Fig. S4). We also observed correlations between heat tolerance and some antibiotic and metabolic traits (Fig. 4). A principal component analysis (PCA) analysis of trait covariation showed that metabolism and antibiotic susceptibility traits correlate closely with PC1, the first axis of trait covariance, which explained 22% of variation (Figs S4 and S5).

Discussion

Here we analysed differences in bacterial abundance, diversity and functional traits in rhizosphere communities. Bringing ecological, genotypic and functional trait information together, we aimed to identify trade-offs among traits and the degree to which they covary with rhizosphere community ecology. Our main finding is that functional traits predict root-associated bacterial communities better than phylogenetic identity alone, suggesting that phenotypes and functional specificity are important for rhizosphere community assembly. Thus, isolates from similar communities tend to share similar functional trait

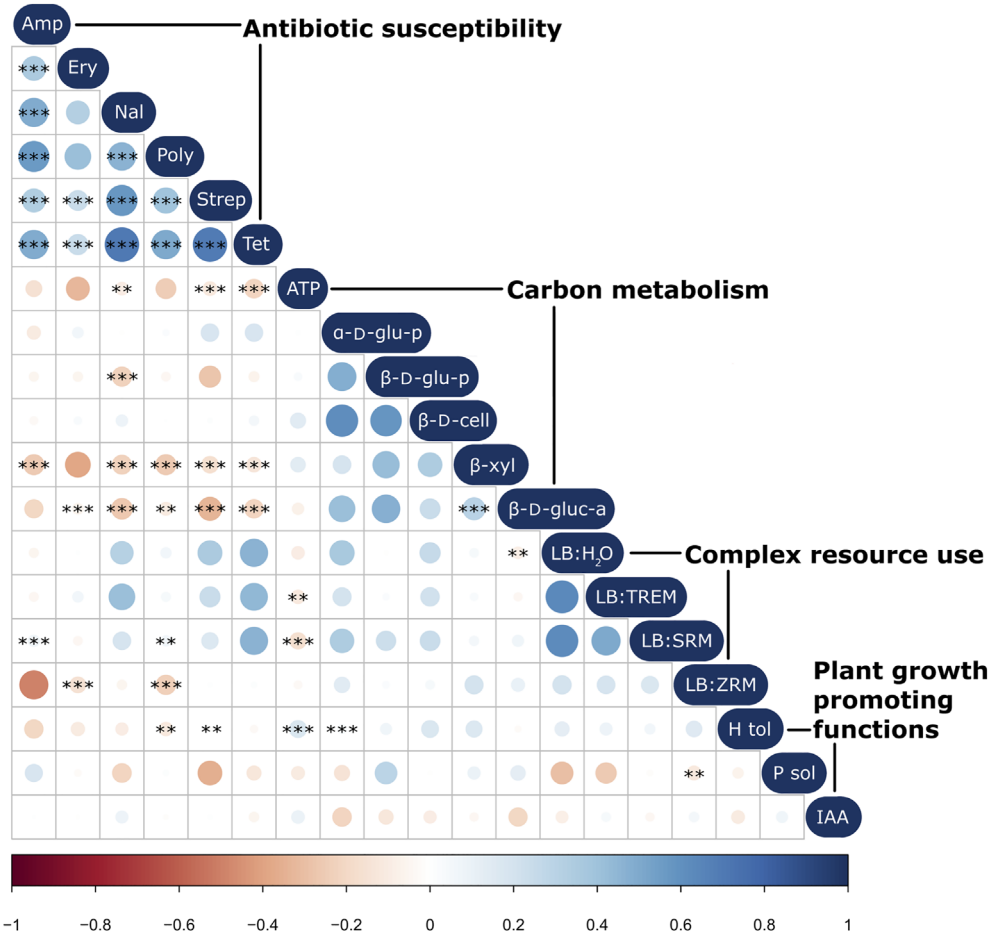

Fig 4. Isolate–trait interactions. The trait correlation matrix contrasts 19 traits listed down the autocorrelation line. Positive correlations are displayed in blue and negative correlations in red. Colour intensity and the size of circle are proportional to the correlation coefficients: strong OLS correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and −1 indicating perfect negative correlation (dark red) between two traits. Significance of correlation was assessed via PGLS tests that account for non-independence among related lineages ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

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values even when their phylogenetic composition differs. In this study *Pisum*, *Solanum* and *Zea* communities are relatively diverse phylogenetically but are functionally relatively conserved. Only one of the four communities (that on *Allium*) was phylogenetically conserved and also more variable in the functional trait values of community members (Fig. 2). Previous high-throughput 16S rRNA gene sequencing of this experiment has also shown that *Allium* communities were phylogenetically conserved and that *Zea* and *Pisum* communities were variable (Matthews *et al*., 2019) which confirms these broad findings. However, insights into the limits on functional variation in communities, which were independent of phylogenetic identity, would have been impossible without this complementary phenotypic study.

Differences in resource availability between plant species and competition between microbes are likely to be the dominant forces shaping rhizobacterial communities (Philippot *et al*., 2013; Baltrus, 2017). This study does not provide straightforward support for simple adaptation to species-specific resources. For instance, there was very little variation between communities in key enzymatic traits used to exploit complex root-associated carbon resources. This lack of variation may arise because these traits are essential for membership of rhizobacterial communities generally, but not important for species specificity. We might also expect community members to have more efficient growth on the complex media derived from their plant of origin. Instead, the overall pattern seen was that some communities tended to be more productive overall (and more copiotrophic) while other communities fared better in less nutrient-rich environments.

One explanation for this overall variation in growth is that there are trade-offs between different classes of functional traits and different phylogenetic groups tend to sit in different places in these trade-offs (Hibbing *et al*., 2010; Fierer, 2017; Ho *et al*., 2017). For instance, investment in antibiotic tolerance and production (secondary metabolism) may trade-off against metabolism. In this study, antibiotic susceptibility traits tended to be negatively correlated with basal metabolism. In our multivariate analyses of all trait values, the axis encompassing basal metabolism and antibiotic susceptibility was the largest principal component, indicating the importance of this trade-off in trait variation overall (Fig. S4). This variation was partly driven by phylogeny (Fig. S4) so some groups such as Actinomycetales and Rhizobiales have low metabolism, low antibiotic susceptibility and are more oligotrophic while the γ-Proteobacteria (Enterobacteriales, Xanthomonadales) tended to be at the other end of this spectrum (high metabolism, high antibiotic susceptibility, copiotrophy). Thus, communities dominated by γ-Proteobacteria (e.g. *Zea* and *Pisum* communities) do less well on complex media and in lower nutrient conditions, while communities with a higher proportion of Actinomycetales and Rhizobiales (*Solanum* and *Allium* communities) showed lower antibiotic susceptibility and more robust growth in low-nutrient conditions.

The likely importance of competitive interactions in community structuring was our motivation for measuring antibiotic susceptibility. Antibiotics can mediate antagonistic species interactions, benefiting producers in competitive, often nutrient-enriched, habitats (Williams and Vickers, 1986; Martinez *et al*., 2009). Antimicrobials have been shown to modulate transcription of genes that influence nutrient acquisition, virulence, motility, antibiotic production and biofilm formation (Ryan and Dow, 2008; Kinkel *et al*., 2014; Lareen *et al*., 2016) and thus may play a role in orchestrating microbe–microbe interactions in the rhizosphere, where complex multispecies communities form biofilms at the root surface (Schlatter and Kinkel, 2015; Yang *et al*., 2019). Thus antimicrobial production and tolerance may have complicated roles in community assembly, perhaps affecting community composition before host selection occurs (Romero *et al*., 2011; Cornforth and Foster, 2013). There are two lines of evidence suggesting that competitive interactions might have different importance in different plant communities in this study. First, *Solanum* and *Pisum* communities had distinct antibiotic susceptibility profiles relative to the other communities, with *Solanum* isolates having low antimicrobial susceptibility. Second, *Solanum* communities showed high phylogenetic evenness, a pattern associated with strong competitive interaction preventing the coexistence of closely related members (Vamosi *et al*., 2009).

Functional traits showed variation in phylogenetic signal. For instance, xylosidase activity, which metabolizes the xylose prevalent in hemicellulose, a major component of plant cell walls, had no detectable phylogenetic signal. Conversely, β-D-glucosaminidase, which is involved in the breakdown of chitin, a microbial cell wall component predominant in fungi, had a very strong phylogenetic signal. This pattern may reflect widespread horizontal gene transfer, and host coevolution with specific pathways, rather than specific lineages. The contrast between PGP traits and the use of complex resources is also interesting. All PGP traits showed phylogenetic signal, IAA (plant hormone) production and phosphate solubilization are notable given their use as markers for plant growth promotion (Rolli *et al*., 2017). At the family level, the Enterobacteriaceae and Pseudomonadaceae have the largest values of both these traits (Fig. S4). The consequence of using such traits to identify PGP bacteria is potentially to limit the taxonomic pool of isolates in any screening process. Given our results, this may have the effect of excluding the more oligotrophic taxa such as the Actinomycetales, even though these may be more

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competitive on some host species. In contrast to antibiotic and metabolic functional traits, where the host is the dominant driver of communities, differences in phosphate solubilization and IAA production appear to be linked to different soils, the implication being that these traits are not equally important in all habitats.

Culture-based studies inevitably impose biases in terms of what can be sampled and grown. Here we have the advantage of a high-throughput 16S rRNA gene sequencing analysis of the same experiment (Matthews *et al*., 2019). This means we can make some fruitful comparisons between this culture and functional based study and a culture-independent approach. Both studies identified that plants, rather than soils, shape rhizosphere communities. However, as might be expected, differences in bacterial community structure and composition between different host and soil groups are more pronounced in the high-throughput 16S rRNA gene sequencing analysis than in the culturable subset analysed here (Matthews *et al*., 2019). For example, culture-independent community structure analysis of OTUs indicates a higher β-diversity between hosts than soils. Our estimates of the least diverse rhizosphere community, Grassland *Allium*, via next-generation 16S rRNA gene sequencing, are predictably, much larger than culturable estimates (Mean observed OTUs $= 690 \pm 63$). However, culturable diversity is known to be a poor means of estimating alpha diversity as it introduces uneven sampling or phylogenetic bias (Donachie *et al*., 2007). Understanding bacterial root communities from a community ecology perspective requires interdisciplinary approaches as they are inherently complex: diverse, dynamic and spatially heterogeneous (Hinsinger *et al*., 2005; Thompson *et al*., 2017). A different and deeper understanding of community assembly emerges from having both high-throughput culture-independent data and functional data on phenotypes: functional traits are more significant than phylogeny alone. Given the prevalence of horizontal gene transfer in microbes, and our incomplete understanding of DNA sequences, there is still much value to be extracted from culture-based approaches.

Experimental procedures

Genotypic characterization

Isolates were genotyped by sequencing the variable region V1-V3 of the 16S rRNA gene. The following oligonucleotides were used for PCR: 27f 5′-AGAGTT TGATCMTGGCTCAG-3 and 519r: 5′-GWATTACC GCGGCK-GCTG-3′. PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by three loops each of 15 cycles of denaturation at 95°C, loop 1, 94°C, loop 2, 93°C, loop 3, for 30 s respectively followed by annealing for 1 min at 57°C and extension at 72°C, and a final extension at 72°C for 7 min. The PCR product was enzymatically cleaned by incubation for 15 min at 37°C followed by 15 min at 80°C with 1.8 μl ExoSAP mix containing 0.1 μl Exonuclease I and 0.1 μl Antarctic Phosphatase in 1.6 μl Exo1-Buffer (New England Biolabs). Sequencing reactions were conducted using BigDye terminator v3.1 cycle sequencing kit (Life Technologies, Paisley, UK) using the 27f primer only. The sequencing product was cleaned using ethanol and sodium acetate precipitation, and run on a 3130xl Genetic Analyser (Applied Biosciences, Foster City, CA). Electrophoregrams were quality checked and edited using Geneious (v5).

Antibiotic susceptibility

The first class of functional traits was growth in the presence of six antibiotics chosen as representatives of diverse antibiotic classes known in soil: ampicillin, erythromycin, nalidixic acid, polymyxin, streptomycin and tetracycline. Isolate growth kinetics were surveyed in 96-well plates containing 198 μl of media of one of four doses (0, 5, 10 and 20 $\mu g\ ml^{-1}$) of each of six antibiotics described in detail in Supplementary Methods Table SM1. Plates were inoculated with 2 μl of 1000× dilution of overnight culture and read on a BioTek Synergy 2 plate reader stacker (BioTek Instruments) recording optical density (OD) at wavelength of 600 nm every hour for 72 h. Growth curve data were used to calculate summary values for maximum growth rate, maximum density, total growth and doubling time.

Carbon metabolism: Each isolate was also assessed for overall metabolic rates and enzymatic activities against substrates likely to be present in the rhizosphere (Lynch and Whipps, 1990; Rudrappa *et al*., 2008; Bogino *et al*., 2013). Metabolic traits included: ATP production (overall metabolism), α-Glucosidases, β-Glucosidases, β-D-cellobiosidases, Xylosidases and N-acetyl-β-Glucosaminidases activity. For each isolate relative metabolic activity was surveyed using BacTiter-Glo™ Microbial Cell Viability Assay (Cat No. G8230, Promega, hereafter BacTiter-Glo). We determined relative ATP content within a sample, based on the luminous quantification of ATP. 25 μl BacTiter-Glo was automatically added using the plate reader's reagent dispenser to 100 μl of 1000× dilution of overnight culture in 0.85% (wt./vol.) NaCl. Emitted log luminescence was detected immediately and a total of five measurements were taken over 2 min using the BioTek Synergy 2 luminosity plate reader. The maximum value of a curve fitted to these data is our estimate of relative ATP content. The enzymatic activity assays for carbon sources used fluorescent moiety bound substrates described in Supplementary

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Methods Table SM2. This details substrates, target enzymes, their general catabolic action and experimental protocols.

Complex resource use

Growth on four complex media traits was quantified: two synthetic media LB and TREM, (Bertani, 1951; Meyer and Abdallah, 1978; Lugtenberg *et al*., 2002) and two naturally derived media, *Zea mays* and *Solanum lycopersicum* root medias. Isolate growth kinetics were measured using the 96-well plate method described for antibiotic dose–response assays above. Here, we aimed to characterize growth on species-specific media and to characterize isolates on a gradient of copiotrophy (efficient growth on high-nutrient conditions) and oligotrophy (efficient growth on low-nutrient conditions). We, therefore, measured how growth changed as LB was diluted and replaced with water or less nutritious root media, hypothesizing that oligotrophs would maintain robust growth as the high nutrient media (LB) was diluted. Data were summarized as slopes of change in area under the curve in response to changing concentration of media, details are included in Supplementary Methods SM4. Methods for preparation of media including root-derived media are provided in Supplementary Methods SM3.

Plant growth–promoting functions

The PGP traits scored in this scheme included heat tolerance, phosphate solubilization and IAA production (Kamilova *et al*., 2005; Mendes *et al*., 2013; Rolli *et al*., 2017). Pasteurization was used to screen for heat tolerance. Isolates were cultured in 96-well flat-bottomed plates in 180 μl of broth for 24 h at constant temperature 30°C and shaking at 150 rpm. Three media were tested: 2% LB (wt./vol.), DSM (Nicholson and Setlow, 1990) and HCO (Lecadet *et al*., 1980). Detailed media recipes are provided in Supplementary Methods SM5. An aliquot of 100 μl was transferred to PCR plates and incubated at 55°C for 20 min. Tolerant cells were recovered by plating on LBA plates and growth recorded after incubation for up to a week. Additionally, LB broth was returned to the pasteurization plate and the procedure was repeated after 24 h of further incubation. The ability to solubilize phosphate was ranked 0–5 by comparison of the zone of insoluble tri-calcium phosphate clearance when colonies were grown for 5 days on Pikovskaya Agar (Pikovskaya, 1948). IAA production was assayed by colour change in broth cultures grown with excess tryptophan and treated with FeH_2SO_4 0.5 M. Examples of spot test data are provided in Supplementary Methods SM6.

Phylogenetic analysis

We produced a phylogeny for the 593 bacterial isolates using maximum likelihood (ML) and the weighted neighbour-joining method of Bruno *et al*. (2000). The ML-WEIBOR tree was annotated with names via alignment to the Ribosomal database project (RDP) classifier (infernal v10 16S rRNA DB) and Phylip (Felsenstein, 1989; Cole *et al*., 2014). Additionally, we created a genus-level tree manually in Figtree v1.4.2 (Rambaut, 2012) and an accompanying dataset comprising trait means. Details are provided in Supplementary Methods SM7. Trait diversity was analysed at both the isolate and genus levels.

Community alpha diversity

We estimated phylogenetic relatedness and taxonomic richness in culturable bacterial communities defined by their origin in terms of crop species and soil. PD, the total branch length spanned by a community's sub-tree (Faith, 1992), and SR were calculated with the pd function in picante (Kembel *et al*., 2010).

Community composition

We measured patterns of phylogenetic relatedness among communities using the *comdist* and *comdistnt* functions to estimate respectively the mean pairwise distance (MPD) and mean nearest taxon distance (MNTD) (the mean distance separating each species in the community from its closest relative) between pairs of isolates drawn from different culturable communities. NMDS ordinations of community structure were computed from the resulting distance matrices and plotted in R using ggplot2 version 1.0.1 (Wickham, 2010). To visualize sample relationships as clusters, two factors, Soil type, Host genus, were plotted and tested for significant structuring effects on root-associated bacterial community diversity with ANOSIM and ADONIS. Correlation between phylogenetic and functional data was assessed using Mantel tests while comparisons between these data used permutation ANOVA tests on a thousand stochastic community matrices to contrast simulated mean dissimilarity to that associated with the observed community matrix. Tests were implemented in R via VEGAN version 2.0-10 (Oksanen *et al*., 2008).

We tested the similarity of communities in terms of traits by comparing the standardized effect sizes (SES) of trait diversity measured as dissimilarity among co-occurring isolates and compared this observed trait diversity to a null model in which tips were shuffled randomly 1000 times. Pearson correlation tests were used to test the correlation between phylogeny and traits. The function

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oecosimu was used to compare ADONIS models of trait and phylogeny-based communities.

Trait phylogenetic signal

We measured phylogenetic signal, which is the tendency of related organisms to resemble each other more than would be expected by chance, with two commonly used indices: Pagel's λ and Moran's *I* (Gittleman and Kot, 1990; Pagel, 1999). For each trait clustered across the 16S rRNA gene tree we calculated Pagel's λ in the package 'phylotools' with the function phylosig (Harmon *et al*., 2008). Tests were conducted in R v3.6.0 (R Development Core Team 2018). We tested the significance of λ with log-likelihood ratio tests to examine the significance of phylogenetic dependence by testing the null hypothesis that $\lambda = 0$, which indicates no phylogenetic signal in the trait data. The λ value varies between 0 and 1 but it can exceed 1 depending on the shape of the phylogeny (Freckleton *et al*., 2002). Details about the robustness of our analysis are presented in Supplementary Methods SM7.

Phylogenetic signal at different taxonomic ranks

We tested traits for variation in phylogenetic signal associated with taxonomic organization using Moran's *I* (Gittleman and Kot, 1990). Moran's *I* is an autocorrelation index and, unlike λ, is not based on evolutionary models and is thus able to make an independent estimate of phylogenetic structure.

Multivariate analysis of traits

We used PCA to explore covariance and the relative importance of functional traits. PCA was performed in R with *princomp* to reveal the main axis of variation in the traits based on Eigen analysis of a scaled correlation matrix using the VEGAN package in R 3.1.0 (Oksanen *et al*., 2008); the first two principal components were visualized in R with ggplot (Wickham, 2010).

Phylogenetic significance of interactions between traits and communities

We tested whether there is a significant relationship between traits using the PGLS approach (Orme *et al*., 2012). This method assumes that lineages may be similar based on recent shared phylogenetic history, and so are not independent. PGLS analysis accounts for the fact that residual errors will be affected by this shared evolutionary history in order to make unbiased consistent estimates of relationships. Ordinary least squares (OLS) coefficients were compared to those of PGLS in which the tree structure was expected to affect the covariance in trait values across taxa evolving by Brownian motion and thus the trait covariance between any pair of taxa was assumed to decrease linearly with the time (in branch length) since their divergence. The correlations were visualized as a coloured matrix using the package 'corrplot' (Wei *et al*., 2017). PGLS correlation significance was manually annotated in Gimp.

We tested whether a significant relationship between traits and communities was phylogenetically significant using a similar approach. PGLS models of traits were tested with phylogenetic error structure 'corBrownian' and the general formula trait x = Host $\times$ Soil. *Post hoc* Tukey comparisons of ecological factors in community trait means were calculated using the package 'lsmean' (Lenth and Lenth, 2018). The data were visualized as normalized trait means, calculated as (Community trait mean/Global trait mean).

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Data Availability Statement

The NCBI submission number for sequence data is SUB10019851. Experimental data are available from the University of Exeter's institutional repository via the permanent identifier http://hdl.handle.net/10871/126297.

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Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting information.

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