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Evolution of bacterial communities in the wheat crop rhizosphere

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Summary

The gap between current average global wheat yields and that achievable through best agronomic management and crop genetics is large. This is notable in intensive wheat rotations which are widely used. Expectations are that this gap can be reduced by manipulating soil processes, especially those that involve microbial ecology. Cross-year analysis of the soil microbiome in an intensive wheat cropping system revealed that rhizosphere bacteria changed much more than the bulk soil community. Dominant factors influencing populations included binding to roots, plant age, site and planting sequence. We demonstrated evolution of bacterial communities within the field rhizosphere. Early in the season, communities tightly bound to the root were simplest. These increased in diversity with plant age and senescence. Loosely bound communities also increased in diversity from vegetative to reproductive plant stages but were more stable than those tightly bound to roots. Planting sequence and, to a lesser extent, wheat genotype also significantly affected rhizosphere bacteria. Plasticity in the rhizosphere generated from crop root system management and genetics offers promise for manipulating the soil ecology of intense cereal systems. Analyses of soil microbiomes for the purpose of developing agronomic benefit should include roots as well as soil loosely adhered to the roots, and the bulk soil.

Introduction

Wheat, along with maize and rice, provides 50% of human calories and is a critical food source in regions with rapid population growth such as Asia, Africa and the Middle East. Productivity increases in wheat have slowed to 0.9% per year, yet need to lift to at least 1.5% per year by 2050 to avoid food price increases (Fischer and Edmeades, 2010). Intensive cereal systems, including wheat following wheat rotations, dominate many global food production systems and yields have been declining in these systems, often due to undiagnosed soil biological causes (Lobell *et al.*, 2009). Developing a predictive understanding of the links between soil biology, agronomy and crop performance would be a first step towards improving productivity of intensive cereals, and would have a major impact on global food production (Cassman, 1999).

Globally, expectations are high that soil biology can be manipulated through crop management and genetics to increase yields in agricultural production systems (Morrissey *et al.*, 2004; Welbaum *et al.*, 2004). Rhizobial associations with legumes for N_2-fixation, crop species rotation for pathogen control and mycorrhizal associations are obvious examples that have had clear productivity benefits; gains in other systems through manipulation of the soil biota have been much less predictable (Watt *et al.*, 2006a). Knowledge of root-associated bacterial populations largely comes from plants grown in pots, or simple laboratory conditions; knowledge of field soil communities frequently comes from sampling bulk soils, and the link to crop growth is unclear. Understanding these processes at spatio-temporal scales relevant to field-grown crops is a major challenge for soil microbial ecology (Hirsch and Mauchline, 2012). Here we describe soil bacterial populations associated with wheat plants grown in the field in a 2-year successive wheat sequence.

Rhizosphere bacteria were broadly defined by Hiltner in 1904 as those both within and on roots, and in soil associated with roots through substrates from roots (Hartmann *et al.*, 2008). We retain this definition of the rhizosphere including the roots, to capture all root-associated bacteria. We further divided rhizosphere bacteria into 'loosely bound' (LB, those that wash from roots and attached soil) and 'tightly bound' [TB, those that remained adhered to root surfaces (rhizoplane) after washing and root endophytes]. Recently communities associated with the plant model *Arabidopsis* were elucidated using sequencing in controlled soil conditions (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012), showing that bacterial endophytes were more soil and genotype-specific than bacteria in soil that can be shaken from the root.

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Table 1. ANOVA of populations cultured from wheat LB rhizosphere soil.

		Source	d.f.	*Pseudomonas*	*Actinobacteria*	Copiotrophs	Oligotrophs	C : O
Year 1		Time	3,48	20.0***	4.34*	4.43*	8.62***	7.4
		Site	1,18	5.04*	3.08	0.18	0.16	0.35
	Treatment	Fallow versus wheat	1,18	134.52***	52.52***	120.12***	144.98***	20.88***
		H45 versus Janz	1,18	0.23	0.72	1.07	1.78	0.12
	Interactions	Time × fallow/wheat	3,48	8.47***	10.96***	12.15***	5.13*	1.11
Year 2		Site	1,36	0.05	0.12	0.12	0.18	0.21
Vegetative	Treatment	Y1 fallow versus wheat	1,36	3.48	0.09	7.19*	2.64	1.45
		Y1 H45 versus Janz	1,36	0.14	1.12	0.8	0.05	1.13
		Y2 H45 versus Janz	1,36	8.21**	0.21	1.24	1.37	3.73
	Interactions	Site × Y2 wheat	1,36	4.14*				
Year 2		Site	1,36	5.59*	0.04	0.01	6.81*	10.84**
Reproductive	Treatment	Y1 fallow versus wheat	1,36	4	1.27	0.15	0.16	0.39
		Y1 H45 versus Janz	1,36	0.13	1.89	0.02	0.41	0.36
		Y2 H45 versus Janz	1,36	1.73	0.07	0.17	0.41	0.02
	Interactions	Site × Y2 wheat	1,36	6.77*				

Year 1 data were analysed by repeated measures ANOVA across four sampling times. Year 2 data were analysed within sampling times V2 and R2. F statistics are reported with significant results indicated by $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. All main factors and significant interactions are presented.
d.f., degrees of freedom, C : O, Copiotroph : Oligotroph ratio.

In addition to studying whole community diversity, we aimed to investigate the dynamics of populations known to play important roles in plant growth. *Pseudomonas* spp. are known to play varied roles in plant growth promotion but can also inhibit growth (Simpfendorfer *et al*., 2002) or in some cases are pathogenic. *Pseudomonas* are abundant on the surface of young roots (Watt *et al*., 2006b), although whether they persist in the rhizosphere of later plant stages is less well studied. *Actinobacteria* are also widely known for their growth promoting effects (Tarkka *et al*., 2008) though they have a markedly different ecology to pseudomonads, preferring organic substrates such as fungal mycelia or root fragments. The distinction between oligotrophs and copiotrophs may be studied to reveal resource use characteristics of the community (Fierer *et al*., 2007) with copiotrophs favouring high carbon environments and oligotrophs outcompeting copiotrophs in low resource environments. We used sequencing and culture-based approaches to characterize bacterial communities in wheat rhizospheres and in fallow soil. To better understand the dynamics, and therefore relevance of bacterial communities in the wheat rhizosphere, we sampled wheat root systems at multiple time points during two successive seasons in the field and analysed their associated rhizobacteria. Two widely grown wheat genotypes, cultivars Janz and H45, were used in year 1. In the second year, Janz and H45 were grown again in a fully crossed design (Supporting Information Fig. S1) to investigate the potential for the first year wheat cultivar to affect bacterial communities associated with the second year wheat crop. A fallow treatment included in the first year allowed comparison of communities associated with wheat following wheat and those of wheat following fallow. The experiment was replicated on two nearby sites with similar soils and environments.

Results

Major drivers of bacterial community composition

The major drivers of soil bacterial community structure were presence of a plant, crop growth stage and level of adhesion to the root. The plant's role as a driver of rhizosphere bacterial communities was reflected in culturing of selected populations (cultured from the LB fraction only), with populations of pseudomonads, *Actinobacteria*, copiotrophs and oligotrophs enriched 10-fold in the LB compared with bulk soil at the vegetative stage of crop development (V1) (Table 1, Supporting Information Fig. S2). Community structure was also driven by the presence of a plant (Fig. 1 rhizosphere versus bulk soil) and degree of adhesion to the root (TB versus LB). Changes in community structure over time were found in both the LB and TB rhizosphere soil but not in the bulk soil community (Fig. 1), implying temporal changes observed in the LB and TB bacteria were plant-driven rather than simply due to seasonal changes in the soil biota.

Secondary factors affecting community composition

Site significantly affected bacterial community structure as characterized by T-FRLP at all sampled stages (Table 2 and Supporting Information Fig. S3); however, among cultured populations, only abundance of *Pseudomonas* was different between sites in year 1 (site 1 > site 2). In year 2 at the reproductive stage (R2), abundance of *Pseudomonas* and oligotrophs was greater on site 2 (Supporting Information Fig. S2).

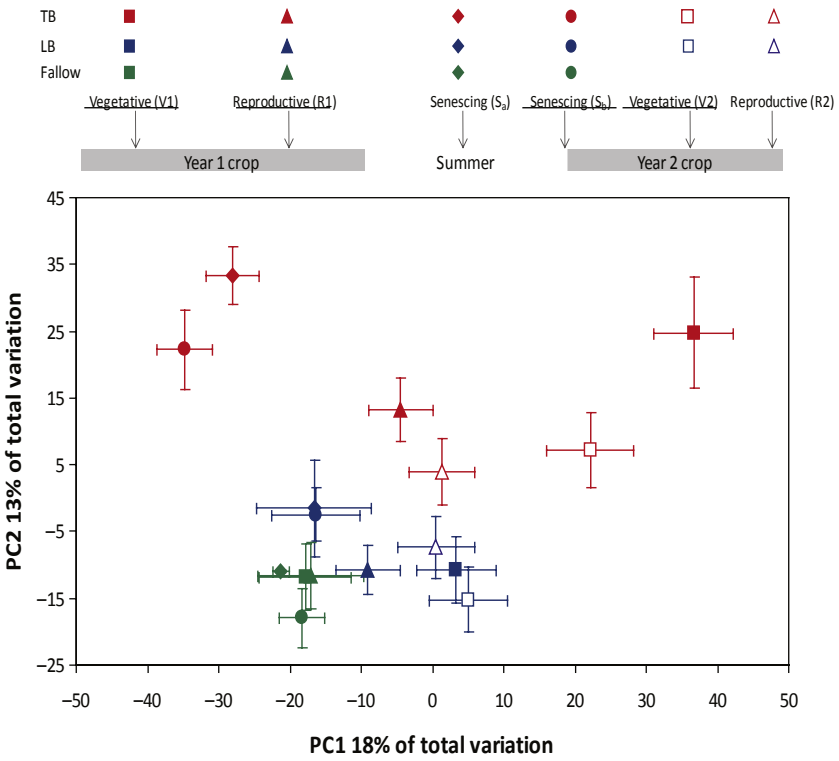

Fig. 1. Principal components plot of tightly bound (TB), loosely bound (LB) and fallow soil bacterial community analysed by 16S T-RFLP fingerprinting over 2 years of wheat cropping. Communities from underlined plant stages were subsequently sequenced. Mean PC scores for the first two components, explaining 31% of the total variation, are plotted ± 1 standard deviation (Year 1: TB and LB $n = 16$, fallow $n = 8$, Year 2: $n = 48$).

In year 2, community composition was significantly different on second year wheat than on wheat following fallow (Table 2, Supporting Information Fig. S3B and E), indicating evolution of soil bacteria due to planting wheat in year 1. At the vegetative stage in year 2 (V2), copiotrophs were enriched in the LB rhizosphere following wheat compared with wheat following fallow (Table 1).

In year 1, wheat genotype had no effect on the rhizosphere community (Tables 1 and 2). At the vegetative stage in the subsequent season (V2), community structure was, however, different on Janz and H45 (Table 2, Supporting Information Fig. S3). *Pseudomonas* were enriched in the Janz rhizosphere, at the V2 stage on site 1 and at the year 2 reproductive stage (R2) on site 2 only. At the V2 stage, copiotrophs were more abundant in the H45 than Janz rhizosphere on site 2 only (Table 1, Supporting Information Fig. S2).

In terms of wheat on wheat rotations, we were interested in whether the genotype planted in year 1 would have an effect on the rhizobacteria associated with the year 2 crop. At the vegetative stage in year 2, there was no significant effect of the first year wheat genotype on the bacterial composition associated with second year wheat

Table 2. PERMANOVA of T-RFLP profiles of wheat LB and TB soils.

Source of variation	Year 1		Year 2 vegetative stage		Year 2 reproductive stage	
Compartment (TB / LB)	478.48***	(15.98)	586.76***	(33.74)	218.48***	(14.97)
Site	53.52*	(1.79)	128.73***	(7.43)	120.17***	(8.23)
2010 Treatment	80.43**	(2.69)	37.42**	(2.15)	55.03***	(3.77)
Contrast 1: wheat versus fallow	190.06***	(6.34)	42.43**	(2.44)	68.42***	(4.69)
Contrast 2: H45 versus Janz	0		19.45	(1.12)	15.20	(1.04)
2011 Treatment (H45 versus Janz)			22.90*	(1.32)	8.03	(0.55)
Interactions:						
Site × compartment			125.28**	(7.2)	59.21**	(4.06)
Site × 2010					47.40**	(3.25)
site × 2010 Contrast 2: H45 versus Janz					63.20*	(4.33)
Compartment × 2010					30.43*	(2.09)
Residual variation	2156	(71.98)	643.81	(37.03)	630.93	(43.24)
Total variation	2995.12		1738.83		1459.3	

Estimates of components of variation are reported with significant results indicated by $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Estimated percent of total variation explained is in brackets. Results for all main factors are reported. Only significant interactions are reported.

Table 3. Mean Shannon–Weiner diversity index (H') of rhizosphere bacterial communities based on 454 sequencing OTU diversity.

Crop stage		Loosely bound	Tightly bound
Year 1	Vegetative (V1)	3.47 cd	2.79 a
	Reproductive (R1)	3.91 e	3.45 cd
	Senescing (S_b)	–	3.51 cd
Year 2	Vegetative (V2) wheat following wheat	3.80 d	3.35 bc
	Vegetative (V2) wheat following fallow	3.31 bc	3.19 b

Letters a-e indicate significant differences between sample groups (ANOVA $F_{6,37} = 13.03$ $P < 0.001$).

roots. However, analysis of the community structure on mature plants in the second season (R2) did reveal significant interactions between site and the genotype planted in the previous year (Table 2). These complex interactions explain a relatively small amount of the variation between bacterial communities; however, constrained analysis plots suggest a difference in communities following Janz and H45 that was evident in the TB soil compartment at site 1 (Supporting Information Fig. S4).

Composition of the wheat rhizosphere

To further define rhizosphere communities, we sequenced a subset of 44 samples from site 1 across four sampling times (Fig. 1). Samples from TB communities at V1, R1, S_b (senescing roots sampled immediately before year 2 sowing) were sequenced to study evolution of the community with plant stage, and at V2 to investigate the differences in communities between first and second year wheat. LB communities at V1, R1 and V2 stages were sequenced to compare with the TB communities on live roots. All sequences are submitted to the GenBank Sequence Read Archive under Bioproject PRJNA209386 and sample accession numbers SAMN02214119 to SAMN02214162.

Bacterial operational taxonomic unit (OTU) diversity, measured by the Shannon–Wiener index, was lowest in the year 1 vegetative stage (V1) in the TB fraction (Table 3) with the community composed of a subset of the OTUs present in LB soil (Supporting Information Fig. S5). At both vegetative and reproductive stages in year 1, diversity was significantly higher in the LB fraction than in the TB, and in both soil fractions, diversity increased between the vegetative and reproductive stage in the first

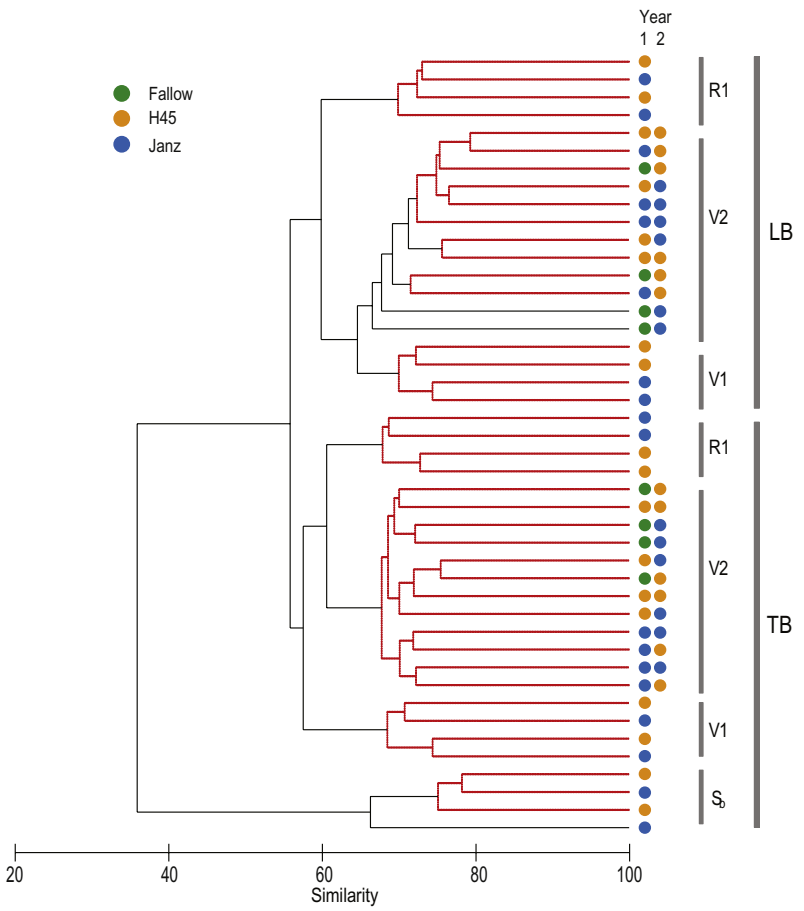

Fig. 2. Bray–Curtis clustering of 454 sequencing OTU data. Black lines show clusters significant at $P < 0.05$, clusters shown by red lines have no statistical support (SIMPROF, 999 simulations).

year (Table 3). Community diversity in the LB fraction was significantly greater following a wheat crop than on soil previously fallow (Table 3).

Clustering of the sequence data confirmed the terminal restriction fragment length polymorphism (T-RFLP) analysis with samples clustering by degree of rhizosphere binding and plant stage (Fig. 2, Supporting Information Fig. S6). The community tightly associated with senescing roots (S_b) was most distinct with less than 40% similarity to communities associated with live roots. There was no significant difference in diversity of communities associated with first or second year wheat and samples did not cluster by genotype.

At Phylum level, all soil samples were dominated by *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* (Fig. 3). An OTU unclassified at the Phylum level composed up to 20% of sequences, forming a greater part of the LB rhizosphere and the residue (S_b) TB rhizosphere community than the TB rhizosphere community associated with growing plants. TB soil was dominated by the *Proteobacteria*, particularly at the earliest sampling time, V1, when *Proteobacteria*, primarily β-*proteobacteria*, accounted for over 70% of the total community. At the same sampling time, *Proteobacteria* composed only 40% of the LB soil community, which composed greater proportions of *Acidobacteria*, *Firmicutes* and unclassified taxa. At later sampling stages, *Actinobacteria* were more prevalent in TB soil than in the LB soil.

Dynamic bacterial taxa in the rhizosphere

At genus level, distinct communities were associated with soil fractions and plant stages (Supporting Information Fig. S6). Communities associated with the vegetative plant stages in years 1 and 2 were more similar in the LB than TB soil. The reproductive stage communities were distinct from those at the vegetative stage and remained different between the tightly and LB soil fractions. The community associated with the senescing roots was well separated from the active plant stages, reflecting the distinct OTUs associated with this stage in Fig. 2.

For taxa composing greater than 0.5% of the community, pairwise comparisons were made between sampling times within the LB and TB communities, and between the LB and TB communities at each time point. At all three plant stages (V1, R1 and V2) sequenced for both fractions, *Streptomycetaceae* were more prevalent in the TB than LB fraction as were four families within the *Proteobacteria* (Fig. 4A). *Pseudomonadaceae* were most enriched in the TB fraction at the vegetative stage in year 1 (V1), whereas *Streptomycetaceae* were most enriched at a similar stage in year 2 (V2). A group belonging to the *Chloroflexi* which could not be classified beyond Phylum was significantly reduced in the TB compared with LB fraction, along with *Acidobacteria* Group 1 and *Nocardioidaceae*.

Among the TB bacteria, dominance of the *Proteobacteria* decreased with plant age (Fig. 3). In particular, members of the *Oxalobacteraceae* predominated on young roots, but were significantly reduced on older and senescing roots (Fig. 4B). Similarly, the *Pseudomonadaceae* composed significantly more of the community during early vegetative growth in the first year (V1) than at the reproductive stage (R1), and this was supported by culturing from both sites (Supporting Information Fig. S2). *Flavobacteriaceae* were the dominant family in *Bacteroidetes* and were significantly reduced on senescing roots (S_b). The bacterial community associated with S_b was distinct from that associated with growing plants (Figs 2 and 3) and, in addition to the unclassified group, was characterized by increases in *Micromonosporaceae* and *Pseudonocardiaceae* within the *Actinobacteria* (Fig. 4B).

Significant community shifts in LB soil were of smaller magnitude than in the TB fraction (Fig. 4B, Supporting Information Table S1). As in TB bacteria, *Proteobacteria* was the dominant Phylum and *Oxalobacteraceae* composed a greater proportion of the community around roots at vegetative growth stages (Figs 3 and 4B). As in the TB fraction, *Flavobacteria* were the dominant family from *Bacteroidetes* on vegetative stage roots (V1 and V2) but on older roots (R1) *Sphingobacteriaceae* composed a greater proportion of the LB community (Figs 3 and 4B).

Though wheat following wheat treatments harboured greater LB rhizosphere OTU diversity than wheat following fallow, pairwise comparisons at V2 showed no significant difference between first and second year wheat in individual genera.

Discussion

Selection for improved wheat varieties may result in changes to root architecture, development and exudates, which in turn affect the microbial community (Bertin *et al.*, 2003; de Graaff *et al.*, 2013). Consequent changes to the structure and function of the microbial community can be considered an extension of the plant's genotype and therefore something that can potentially be managed. This study showed that the largest changes in the soil bacteria in an intensive wheat rotation took place in the fraction TB to the roots (endophytes and outer surface-bound), providing field evidence the plant and its roots cause shifts in soil microbial communities over at least 2 years. Further, this study has methodological implications for soil biology research and diagnostics related to crop productivity: sampling for changes should include the roots and their bound microorganisms, as well as those from soil shaken from roots and the bulk soil.

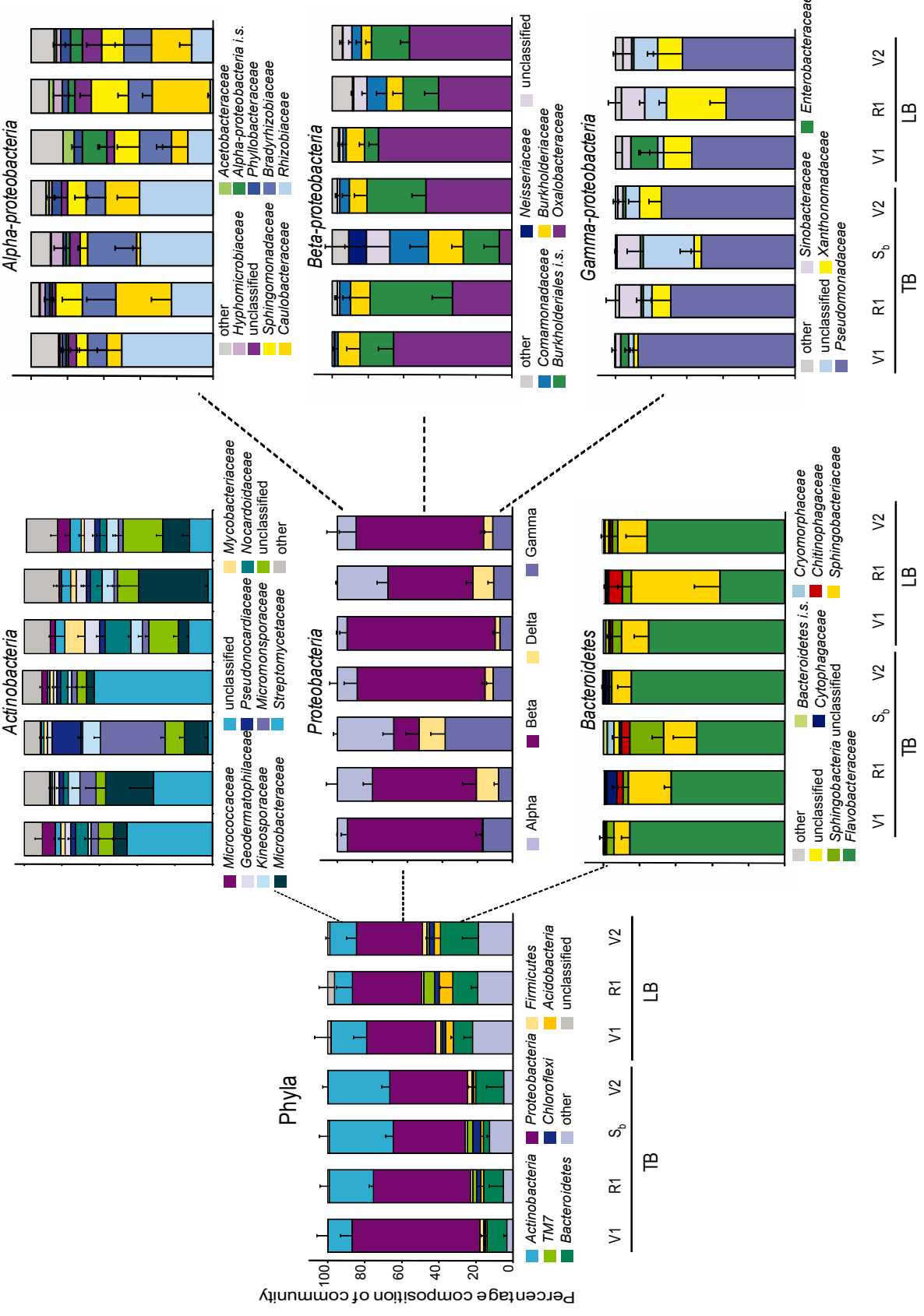

Fig. 3. Taxonomic assignment of sequence data. Composition of the three dominant Phyla: Actinobacteria, Bacteroidetes and Proteobacteria is given to family level (with uncertain placements indicated as *i.s. incertae sedis*). 'Other' includes low abundance taxa (< 5 % of the population). For taxa representing > 10 % of the population in any treatment, error bars show 1 standard deviation (year 1, $n = 4$; year 2, $n = 12$). Data for communities isolated from Janz and H45 are combined.

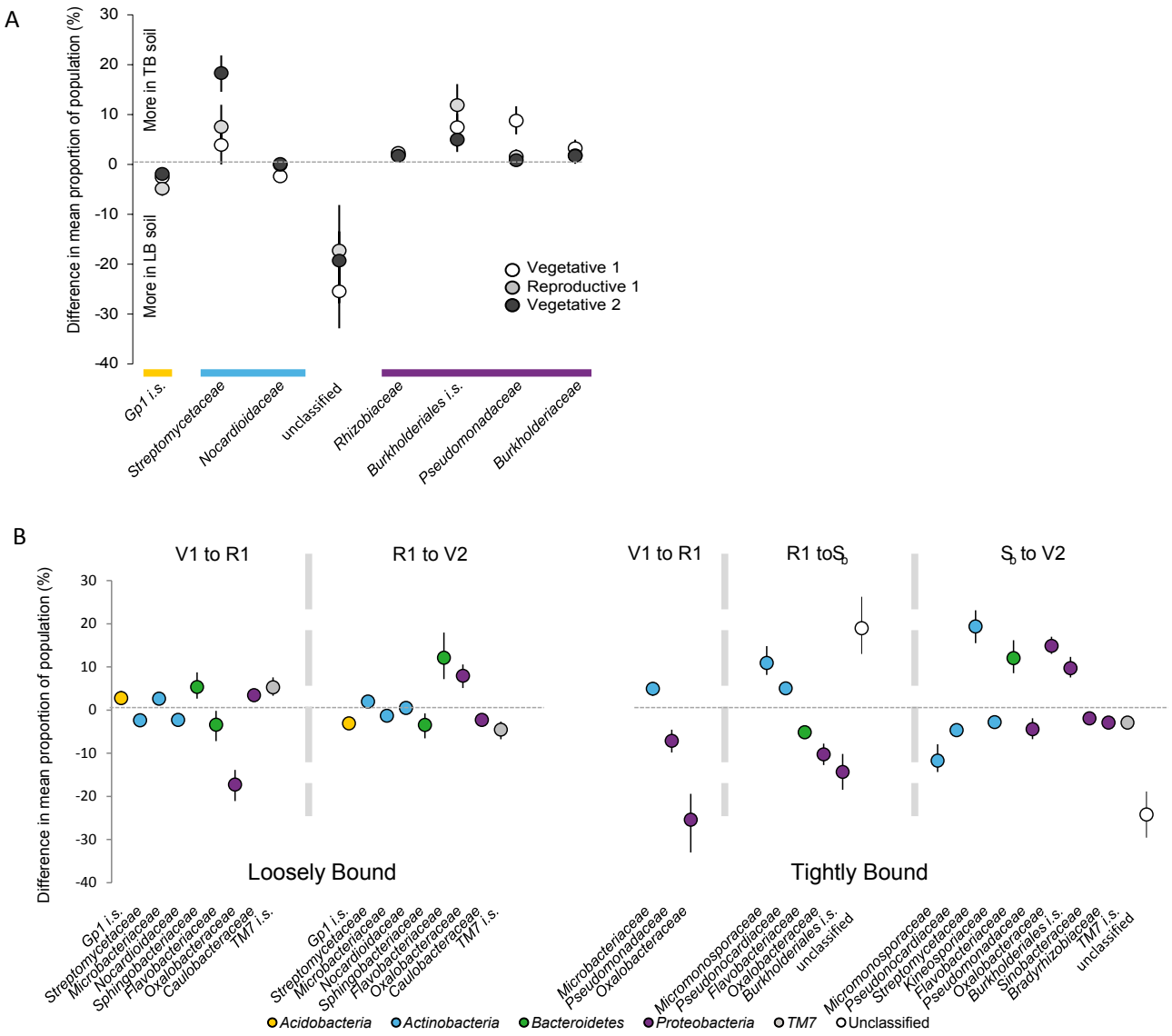

Fig. 4. Pairwise comparisons showing families changing significantly ($P < 0.05$) with time or soil fraction.
A. Between TB and LB communities. Families more abundant in TB have a positive value, in LB have a negative value.
B. Over time in communities LB and TB to roots and adhering soil. Families increasing in relative abundance between pairs of plant stages have a positive value and those decreasing have a negative value (*i.s. incertae sedis*). Pairwise comparisons were performed using White's non-parametric *t*-test; correction for multiple testing was done using the Benjamini–Hochberg false discovery rate. Coloured bars in A and data points in B indicate phyla *Acidobacteria* (orange), *Actinobacteria* (blue), *Bacteroidetes* (green) and *Proteobacteria* (purple).

In order to exploit the microbial community, we first need knowledge of its composition and dynamics. Bacteria TB to vegetative plant stages were dominated by families characterized by motile, aerobic or facultatively anaerobic taxa, such as the *Oxalobacteraceae* and *Burkholderiaceae* (*Betaproteobacteria*), *Pseudomonadaceae* (*Gammaproteobacteria*) and *Flavobacterium* (*Bacteroidetes*) as well as *Streptomycetaceae* (*Actinobacteria*). The key roles played by plant adhesive surfaces and plant growth stage suggest that TB communities were likely associated with roots in biofilms (Watt *et al.*, 2006b). Aside from characteristics favouring biofilm formation like motility and fast growth, the early TB community may have nutritional and signalling properties such as those identified in competent rhizosphere inoculants (Ghirardi *et al.*, 2012).

The community TB to the root surface was more dynamic than the LB community where amplitude of the changes in the mean proportions of constituent taxa were smaller (Fig. 4B). This reflects the more consistent state

of the surrounding bulk soil influencing this fraction of the rhizosphere. The TB community is more greatly influenced by plant growth stage and is therefore more dynamic through time.

Major components of the field-grown wheat rhizosphere community revealed here align with those found associated with *Arabidopsis thaliana* in controlled pot experiment conditions (Bulgarelli *et al*., 2012; Lundberg *et al*., 2012), yet we found the rhizosphere, in particular the TB community, to be more dynamic than previously suggested. This may be in part due to our different sampling strategy and represent a dynamic interface between the root and soil compared with the endophytic bacteria described for *Arabidopsis*, whose population was more stable over time. Moreover, our experiments were conducted over multiple time points for wheat plants grown under field conditions, as compared with controlled soil environments.

A distinct assemblage was associated with senescing roots compared with live roots, characterized by greater proportion of *Actinobacteria*, particularly *Micromonosporaceae* and *Pseudonocardiaceae*. We observed no differences in communities associated with different first year genotypes of decomposing roots, but by growing more diverse wheat genotypes than those used here, or other cereal species such as triticale, oats or barley, greater host-dependent shifts in community should be created enabling us to better examine the historical effects of cropping on the current crop rhizosphere populations. Bacteria utilizing recalcitrant root material are in competition with saprotrophic fungi and data presented here can be mined for organisms that may inhibit root fungal pathogens outside the cropping cycle and thus be used to develop natural suppression of soil-borne diseases.

We found that the soil bacterial community associated with field-grown wheat plants in a 2-year sequence evolved and that the strongest drivers of community structure were crop developmental stage and the adhesion of the community to the root. Therefore, identifying the response of the microbial community to different genotypes and management systems (e.g. rotations, soil factors) requires understanding the spatial and temporal dynamics of the rhizosphere, and sampling at relevant scales. TB rhizosphere microbes may be good targets for manipulation through plant genetics or for development as inoculants, as this was found to be the most dynamic soil fraction over different plant stages. In addition, sequencing showed that the LB communities of wheat following wheat had greater diversity than those of wheat following fallow, but that TB communities were not different. This indicates that the TB bacteria are at least partially buffered from changes in bulk soil diversity driven by management history.

Plant stage was a major driver of community structure; by comparison, there was little seasonal change in fallow soil communities observed in year 1. Population sizes of the selected rhizosphere bacteria decreased with maturing of the plant in year 1. This may reflect changes in the amount or nature of the resources in the rhizosphere, or the root and soil surfaces for adhesion (niches). Relocation of carbon to roots and its release through both respiration and rhizodeposition has been reported to decrease with plant stage, for example in wheat from 33% of pulse labelled ^{14}C at early tillering to 9% after anthesis (Gregory and Atwell, 1991). On the other hand, McCully and Canny (1985) found that young and older field-grown maize roots exuded similar total ^{14}C, but that the composition of the roots was different, older roots having more ethanol-soluble compounds (e.g. non-polar waxes and lignins). At the reproductive stage in year 2, enrichment of populations of Actinomycetes and copiotrophs was observed. It is unclear as to why copiotroph numbers were high, only that this complex group may be able to live on a variety of substrates. Older roots have been reported to have increased numbers of *Actinobacteria* (Thirup *et al*., 2001; Watt *et al*., 2006b). This is possibly related to their ability to degrade secondary cell walls with lignin and other complex molecules (Kirby, 2006) which are feature of senescing field-grown wheat roots (Watt *et al*., 2008). The higher *Actinobacteria* numbers in year 2-old roots may have arisen from inoculation by the year 1 root residues.

Community structure was consistently different between the two sites. In year 1, *Pseudomonas* were significantly more abundant on site 1. Selection of these sites was based on anecdotal evidence of declining yield with successive wheat crops at site 1 but not at site 2. In addition to different soil types (see Supporting Information Appendix S1), site 1 was sown to barley (*Hordeum vulgare*) and site 2 was sown to canola (*Brassica napus*) in the year before the experiment. In year 2, extra nitrogen was applied at 50 kg ha^{-1} to site 2 only to reduce N limitations on yield at this site. In addition, we found interactions between effects of genotype and site for abundance of *Pseudomonas* populations. Site, rotation history and nutrient management may all be important considerations when drawing any conclusions about the effect of a particular genotype on the microbial community.

We found shifts in the bacterial community between first and second year wheats, with greater bacterial diversity on second year wheat. Up to 50% of wheat roots come into contact with residues from previous crops (Watt *et al*., 2005) and so decomposing residues are a possible source of microbes for newly developing rhizospheres. We were unable to resolve the underlying taxonomic nature of the shift in community found on second year wheat, possibly due to the relatively low level of resolution

achieved by sequencing the 16S V5–7 region. T-RFLP revealed the effect of wheat following fallow or wheat following wheat was evident in the bacterial community at both the vegetative and reproductive growth stages in year 2, explaining more of the total variation in the community at the later growth stage (4.7% at reproductive stage versus 2.4% at vegetative stage).

Genotype Janz has previously been shown to perform poorly in unploughed soil, with lower root and shoot vigour and accumulation of inhibitory rhizosphere *Pseudomonas* (Simpfendorfer *et al*., 2002; Watt *et al*., 2003; 2005); while H45 is a vigorous and quicker maturing variety. The genus *Pseudomonas* is broad with many functional groups including plant growth promoting rhizobacteria, plant pathogens and non-pathogenic inhibitory bacteria. The lines between these groups is unclear and the role of a species may change depending on the circumstance. We found evidence of accumulation of pseudomonads on Janz in year 2 though this was dependent on site. *Pseudomonas* were more abundant on Janz at site 1 during vegetative growth in year 2 (V2) and on Janz at site 2 at the reproductive stage (R2). Whether this enrichment of pseudomonads on Janz is beneficial or detrimental to plant growth could not be resolved by this study; however, this work did reveal a genotypic effect on bacterial community. T-RFLP data revealed no effect of genotype on community composition in year 1, but in year 2, the community was significantly different at the vegetative stage only.

At the vegetative stage in year 2, no effect of the previous years crop genotype was evident. This result was confirmed by both T-RFLP and sequencing, covering two different 16S regions (V1-V3 and V5–7 respectively). At the reproductive stage in year 2, there were significant interactions of the year 1 genotype with site and soil compartment. These effects were small, dependent on site and warrant further study to confirm their robustness; however, they suggest that the previously planted genotype can affect the TB soil community at a late plant developmental stage.

Varietal and crop sequence effects accounted for relatively small amounts of the total variation in rhizosphere bacterial communities (Table 2), so to investigate these effects spatial, temporal and soil factors have to be carefully controlled. Our results suggest a model of bacterial community evolution in wheat crops, where the community associated with young plants is driven by the host plant (its presence and its genotype), and the later community is shaped by previous management, such as sequence and crop genotype in the preceding year. Timing and location of sampling depends on the question: for example host plant effects are best seen in the TB soil, and crop sequence effects in the LB rhizosphere soil. Competition with saprophytic fungi may be most evident on residues while direct pathogen plant interactions may be best observed when the plant is young, the roots most vulnerable to pathogen attack and the rhizosphere community is at its least complex.

We have demonstrated a cascade of factors that shape the rhizosphere microbiome. By sampling at appropriate time and spatial scales, we will have a better ability to predict how soil populations respond (e.g. rhizosphere competence and longevity) to root characteristics (e.g. physical structure, exudates). This study provides evidence that plant breeding and crop management can be used to shift soil communities in intensive cereal systems; the next challenge is to understand the functions and burdens of these root-associated bacterial groups, and which are likely to be beneficial to plant growth and yield.

Experimental procedures

Field experiment

Two comparable field experiments were established approximately 500 m apart within the same field at Gundibinyal, New South Wales, Australia (~ 34°28′S, 147°47′E) in May 2010. In year 1 (2010), three treatments were replicated four times in a randomized block design at both sites (Supporting Information Fig. S1) with individual plot size of 4.4×10 m. Treatments 1 and 2 were sown with bread wheat (*Triticum aestivum*) cultivars Janz and H45 and treatment 3 was left fallow but plots were subjected to the same disturbance and fertilizer as planted treatments. In year 2 (2011), each 2010 treatment plot was divided into four and duplicate plots of both Janz and H45 were sown directly onto the rows of the previous years' treatment. As a result of the treatment sequence, the treatments in year 2 were Janz on first year Janz residues (JJ), H45 on Janz (JH), Janz on H45 (HJ), H45 on H45 (HH), Janz on fallow (FJ) and H45 on fallow (FH) (Supporting Information Fig. S1).

A field plan and further details of the site history, soil types, wheat cultivars and management practices are detailed in the Supporting Information Appendix S1.

Sampling from the field and rhizosphere (TB and LB bacteria) and collection

Roots were sampled six times (Fig. 1, Supporting Information Fig. S1) and we assessed diversity and taxonomic affiliations of bacteria TB and LB to the rhizosphere, and in fallow soil. Bacteria of interest were also isolated and quantified from the LB fraction by culturing.

We harvested root systems and adhered soil from different stages of crop growth: vegetative (V, sampled at 3–4 leaf stage in year 1 and 4 to 5 leaf in year 2); reproductive (R, sampled at anthesis in year 1 and mid-grain filling in year 2) and senesced (S_a during summer fallow and S_b prior to sowing the second crop) plants (Fig. 1).

In the first year, there were 16 LB, 16 TB and 8 fallow soil samples per sampling time. In each of the second year samplings, there were 48 LB and 48 TB samples.

At each sampling time, a group of three to four plants was extracted by digging around the group to keep the root

systems as intact as possible. Plants were shaken by holding with the shoots to remove excess soil. Sampling was repeated in two additional locations in each plot to obtain a single composite sample. In the first year for fallow plots, an equivalent weight of soil was sampled at the same depth as root samples. Plots were machine harvested to leave the surface stubble and root residues in the field over summer as would occur in commercial wheat fields. After harvest, during the summer, fallow period root residues were sampled by collecting the remaining intact root systems attached to the crown. All samples were bagged in the field, maintained at a cool temperature and processed in the laboratory within 4 h of collection.

For each sample, roots were cut off below the crown, a 10–15 g subsample of roots and adhering soil was weighed and transferred to 100 ml 0.2 mM sterile $CaCl_2$ in a Schott bottle. The bottle was vortexed for 30 s three times to loosen adhering soil and microorganisms from the roots; this suspension was considered to contain the LB microorganisms. One millilitre suspension was taken for culturing, and 20 ml removed for DNA extraction. This 20 ml rhizosphere suspension was centrifuged at 5000 r.p.m. for 15 min at 5°C, the supernatant decanted and the pellet frozen in liquid nitrogen immediately. Samples were freeze-dried and stored at –20°C prior to DNA extraction.

To obtain the DNA of the microorganisms TB to the root, roots were recovered after shaking for rhizosphere collection, frozen in liquid nitrogen, freeze-dried and stored at –20°C until DNA extraction. Cultures were not obtained from the TB fraction.

Culturing

One millilitre LB soil suspension was serially diluted to 10^{-2}, 10^{-3} and 10^{-4}. One hundred microlitre of each dilution of each sample was cultured on four selective media: for pseudomonads *Pseudomonas* agar base was supplemented with $MgCl_2$ and cephalothin-fucidin-cetrimide; for *Actinobacteria* Actinomycete isolation agar with glucose; media for copiotrophs and oligotrophs were based on a stock solution following Semenov and colleagues (1999) (see Supporting Information for a detailed description of media). Plates were incubated at 15°C and colonies counted after the 3rd, 4th, 6th, 11th, 13th, 17th and 20th day of plating as there were no more new colony growth on *Pseudomonas* plates, and no significant colony growth on *Actinobacteria*, Oligotroph and Copiotroph plates after 20 days. Although this incubation time may have biased the *Actinobacteria* isolates recovered, we used culturing only to compare total numbers between treatments.

The number of colony forming units present in 100 ml of LB soil suspension was calculated from the plate dilutions and divided by the dry weight of soil washed from the root to give CFU g sdw^{-1}.

T-RFLP

DNA was extracted from 250 mg (± 0.5 mg) freeze-dried LB soil and microorganisms using a PowerSoil DNA isolation kit (Mo Bio, Carlsbad, CA, USA). Freeze-dried root was ground using a mortar and pestle and DNA was extracted from 100 mg (± 0.5 mg) root material with TB organisms, also using a PowerSoil DNA isolation kit. DNA was diluted 1 in 10 before further analysis to reduce inhibition from soil and root compounds.

For all samples collected from the field (352 in total, from both sites, at all plant stages and for TB, LB and fallow soil), bacterial diversity was assessed by T-RFLP. The 16S ribosomal gene was amplified using general bacterial primers FAM-27F (labelled with 6-carboxyfluorescein at the 5' end) and 519R (see Supporting Information for primer sequences and PCR conditions). Twenty nanograms purified polymerase chain reaction products were digested with five units *Hha*I (New England Biolabs, Ipswich, MA, USA), digest products were isopropanol precipitated and resuspended in 10 µl of Hi-di formamide with 0.1 µl LIZ-labelled Genescan 600 size standard (Applied Biosystems). FAM-labelled terminal restriction fragments (TRFs) were analysed on a 3130xl Genetic Analyser (Applied Biosystems).

T-RFLP data analysis

Raw data was retrieved from run files in Genemapper (Applied Biosystems) with a baseline threshold of two fluorescence units. Peaks at 136–140 bp and 472 bp were removed from further analyses as these correspond to fragment sizes occurring with digestion of wheat mitochondrial and chloroplast sequences respectively. Raw data were processed as described by Bissett and colleagues (2010) with data binned into peaks or TRFs over a two base pair window, resulting in a matrix of relative abundance of TRFs in each sample.

Statistical analyses

Cultured populations in the four sampling times for the first year crop were $\log_{10}$ transformed and analysed by two-way repeated measures analysis of variance (ANOVA) with site and treatment as main factors, including treatment contrasts between fallow and wheat crops, and between H45 and Janz. Replicate was included as a blocking factor. T-RFLP data for the first year were analysed using the PERMANOVA add-on in PRIMER v6 (Plymouth, UK) with plot nested within site and compartment to account for repeated sampling over time. The main factors were site, soil compartment and treatment, including contrasts as above.

Year 2 data were analysed within each sampling time. T-RFLP data were analysed by PERMANOVA with a model including main factors: site, compartment and rotation. Contrasts within rotation were performed to test whether there were differences between first and second year wheat (FJ, FH versus JJ JH HJ HH), differences between first year wheats (JJ JH versus HJ HH) and between second year wheats (FJ JJ HJ versus FH JH HH). Culturing data were analysed by ANOVA as above.

Unless described otherwise, analyses were carried out in GenStat version 13 (VSN International, Hemel Hempstead).

Sequencing

Pyrosequencing was performed for a subset of samples from site 1 only. For each treatment, DNA from replicate plots 1 and 2 and replicates 3 and 4 were combined to give duplicate

independent samples for sequencing. Duplicate samples from Janz and from H45 at year 1 vegetative (TB and LB, eight samples total), reproductive (TB and LB, eight samples total) and senescing (S_b) (TB only, four samples) and from FJ, JJ, HJ, FH, JH and HH at year 2 vegetative (TB and LB, 24 samples) were used for sequencing.

The V5 to V7 region of the 16S ribosomal gene was amplified and sequenced on a Roche GS FLX+ platform using primers 799f and 1193r (see Supporting Information Appendix S1) by Molecular Research LP (Shallowater, TX, USA). These primers were selected to exclude amplification of plant-derived sequences.

Sequences with Phred score > 25 were analysed in MOTHUR v123, following the method of Schloss and colleagues (2009). The barcodes and primers were stripped and sequences less than 200 bp in length, or with homopolymers longer than 8 bp were removed. Unique sequences were aligned with the SILVA database (release 111) and unaligned sequences removed. To reduce the chances of overestimating diversity due to pyrosequencing errors, pre-clustering was performed to merge any sequence groups differing by less than 2 bp from a more abundant sequence group, and chimeras were removed.

Sequences were clustered into OTUs at 97% similarity and randomly subsampled to standardize the number of sequences in each sample to 889. Shannon–Weiner diversity index was calculated from the raw data and compared by ANOVA. Log_2 transformed OTU data were plotted using the pheatmap package in R (Supporting Information Fig. S5). OTU data were $\log(x + 1)$ transformed and clustered using the group average linking of Bray–Curtis similarities in PRIMER-E v6 (Plymouth, UK), with significance testing over 999 simulations in SIMPROF.

Taxonomy was assigned to OTUs by comparing to the RDP database using Trainset9_032012. In order to identify the taxa changing significantly either with proximity to the root, or with plant stage, pairwise comparisons of sample groups were made at family level using STAMP (Parks and Beiko, 2010) with White's non-parametric *t*-test (White *et al*., 2009) and the Benjamini–Hochberg false discovery rate to correct for multiple testing. TB and LB fractions were compared within each plant stage where both fractions had been sequenced from (V1, R1 and V2). Within the TB fraction, the change in families was compared from V1 to R1, from R1 to S_b and from S_b to V2. Within the LB, fraction changes in families was compared from V1 to R1 and from R1 to V2.

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Supporting information

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

Fig. S1. Field plan and sampling schedule.
A. Field plan of experiment on site 1. First year treatments (Janz, H45, fallow soil) were replicated four times in a randomized block design, with composite samples taken from across the whole plot. First year treatments were oversown with Janz (J) and H45 (H) in the second year, and samples taken from subplots marked asterisk. The entire experiment was replicated in the first year on site 2. In the second year the two eastern blocks were unavailable for sampling on site 2 and all subplots in the western blocks were sampled in a pseudoreplicate structure (the total number of samples was the same but taken from only two blocks).
B. Sampling schedule. Above the time line are sampling dates with plant stage for samples collected for culturing and T-RFLP, communities from underlined plant stages were also sequenced.
Fig. S2. Abundance of cultured populations (log_{10} transformed) per gram dry weight of LB soil, over 2 years wheat cropping. Each point represents a mean of four samples.
Fig. S3. Year 2 vegetative (V2) stage T-RFLP data for A-C: LB and D-F: TB. A and D show principal coordinates analysis of Bray–Curtis similarities for communities labelled by site. Canonical analyses of principal coordinates for *a priori* groups defined by rotation and genotype are shown within site 1 (B and E) and site 2 (C and F). Colour highlights the genotype sown in the current year (Year 2).
Fig. S4. Year 2 reproductive (R2) stage T-RFLP data for A-C: LB and D-F: TB. A and D show principal coordinates analysis of Bray–Curtis similarities for communities labelled by site. Canonical analyses of principal coordinates for *a priori* groups defined by rotation and genotype are shown within site 1 (B and E) and site 2 (C and F). Note colour highlights the treatment (fallow or wheat genotype) of the previous year (Year 1).
Fig. S5. Evolution of bacterial diversity from 454 sequence data. Heatmap with rows showing operational taxonomic units (OTUs) represented by at least three sequences in three samples. Data are log_2 transformed. OTUs were defined by 97% sequence similarity. $V2^W$ indicates wheat grown after wheat, $V2^F$ indicates wheat grown after fallow.
Fig. S6. Sequence data allocated to genus level were log $(x + 1)$ transformed, clustered by Bray–Curtis similarity and displayed in multi-dimensional scaling plot. Points representing bacterial communities tightly bound to the root are coloured red and loosely bound communities blue. Plant stages are represented by symbols vegetative: squares, reproductive: triangles and senescing roots: circles.
Table S1. Fold changes in genera representing more than 0.5% of the population in any sample. Part A shows a summary of the number of genera changing in pairwise comparisons between i. plant stages within the TB fraction (year 1 vegetative, V1, to year 1 reproductive, R1, to senescing roots, S_b, to year 2 vegetative, V2); and from V1 to V2; ii. plant stages within the LB fraction; iii. TB and LB at each plant stage where both were sequenced from; iv. wheat following fallow, WOF, and wheat following wheat, WOW, within TB and LB soil fractions at V2. Part B lists the genera for each pairwise comparison in the summary table.
Appendix S1. Materials and methods.