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ORIGINAL ARTICLE

Contrasting patterns of seed and root colonization by bacteria from the genus *Chryseobacterium* and from the family Oxalobacteraceae

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Microbial colonization of plant seeds and roots is a highly complex process in which soil and plant type can influence the composition of the root-associated and rhizosphere microbial communities. Amendment of compost, a common agricultural technique, introduces exogenous nutrients and microorganisms to the soil–plant environment, and can further influence microbial community composition in the plant environment. Although compost amendments can strongly influence soil and rhizosphere microbial communities, there is evidence that with increasing proximity to the root, plant influences predominate over soil effects. We hypothesized that the 'rhizosphere effect' observed with proximity to plant surfaces does not act equally on all microorganisms. To explore this issue, we examined two bacterial taxa that reproducibly colonized seed and root surfaces in an experiment examining the influence of compost amendment on plant-associated bacterial communities. Population-specific analyses revealed striking differences in the ecology of bacteria from the genus *Chryseobacterium* and the family Oxalobacteraceae in potting mix and plant-associated environments. Seed- and root-colonizing Oxalobacteraceae populations were highly sensitive to plant effects, and phylogenetic analyses of root-colonizing Oxalobacteraceae revealed the presence of root-associated populations that were highly similar, regardless of treatment, and differed from the potting mix populations detected at the same sampling points. Conversely, *Chryseobacterium* community composition was found to be essentially invariant within treatments, but was strongly influenced by compost amendment. This persistence and stable nature of the *Chryseobacterium* community composition demonstrates that rhizosphere selection is not the exclusive factor involved in determining the composition of the cucumber spermosphere and rhizosphere communities.
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Introduction

Spermosphere and rhizosphere microbial populations are influenced by the type and quantity of exudates from plant seeds and roots, as well as plant development status and sampling location (Campbell and Greaves, 1990; Buyer *et al.*, 1999; Lugtenberg *et al.*, 1999; Andrews and Harris, 2000; Duineveld *et al.*, 2001; Whipps, 2001; Baudoin *et al.*, 2002; de Boer *et al.*, 2006). Although the plant can influence the abundance, diversity and composition of rhizosphere microbial communities, the role of soil microbial community composition and the soil organic matter cannot be neglected (Toal *et al.*, 2000; Marschner *et al.*, 2001; De Ridder-Duine *et al.*, 2005).

Compost amendment to soil is a common agricultural technique and, by introducing large amounts of organic matter as well as high numbers and a large diversity of microorganisms, it can substantially modify soil chemistry and structure and can significantly influence the composition of plant-associated microbial communities (Dick and McCoy, 1993; Beffa *et al.*, 1996; Tiquia *et al.*, 2002;

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Ryckeboer *et al*., 2003; Inbar *et al*., 2005; Green *et al*., 2006). Soil organic matter can be a significant source of carbon for some rhizosphere organisms (Toal *et al*., 2000), and Boehm *et al*. (1997) have suggested that the addition of compost to soil can support microorganisms that are not sustained by root exudates. This capacity for compost-derived organic matter to sustain some microorganisms suggests that the 'rhizosphere effect' does not act equally on all microbial populations.

To explore this issue, we examined bacterial communities associated with seeds and roots during the early development of cucumber in soils and potting mixes amended with cow manure composts. Previously, using domain- (Bacteria), phylum- (*Bacteroidetes*) and family- (Streptomycetes) level molecular analyses, we demonstrated shifts in community composition as a function of compost amendment and as a function of plant development and competing influences of root and compost on microbial communities associated with plants. With increasing proximity to the root, we observed a strong rhizosphere effect, and that the plant influence on bacteria from the genus *Streptomyces* predominated over soil and compost effects (Inbar *et al*., 2005). In a more general analysis of bacterial communities associated with plant seeds and roots, we observed that while seed surfaces were largely colonized by dominant potting mix populations, root microbial communities differed significantly from the source potting mix communities (Green *et al*., 2006). However, despite a general divergence between the community composition of seed and root bacteria, two distinct taxonomic groups were consistently detected in seed and root communities. These taxa included a clade of organisms closely related to the genus *Telluria*, within the family Oxalobacteraceae (*Betaproteobacteria*) and bacteria from the genus *Chryseobacterium* (*Bacteroidetes*) (Green *et al*., 2006). Both are common to soil environments, and are aerobic, heterotrophic organisms (Kwok *et al*., 1987; Spiegel *et al*., 1991; Bowman *et al*., 1993; Bernardet *et al*., 2005). However, isolated members of the genus *Telluria* are flagellated and have chitinolytic, proteolytic and collagenolytic activity (Spiegel *et al*., 1991; Bowman *et al*., 1993), while *Chryseobacterium* are non-motile, heterotrophic organisms, frequently found in composted materials and other organic-rich environments (Kwok *et al*., 1987; Bernardet *et al*., 2005; Green *et al*., 2004, 2006). In this study, we investigated these taxa more closely to characterize the interaction of the rhizosphere effect and the influence of compost amendment as it varied between these seed- and root-colonizing microorganisms.

Materials and methods

Plant growth sampling and DNA extraction

Cucumber seeds (*Cucumis sativus* L. 'straight eight') were sown in three peat-based potting mixes amended with composts, as previously described (Green *et al*., 2006). The two composts employed were sawdust-amended ('sawdust compost') and straw-amended ('straw compost') cow manure composts, as previously described (Changa *et al*., 2003; Green *et al*., 2004). Genomic DNA was extracted from potting mix and plant material from three separate pots at each of the three stages of plant development: seed germination (1 day post sowing), seedlings with fully extended cotyledons (7 days post sowing) and seedlings with four true leaves (21 days post sowing), using the UltraClean soil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA). Before DNA extraction, seeds and roots were removed from each pot, shaken to remove loosely adhering potting mix and washed twice with distilled water. Roots were homogenized using sterile razors and comprised rhizoplane, endosphere and any tightly adhering rhizospheric potting mix. Full experimental design details have been published previously (Green *et al*., 2004, 2006).

Primer design and PCR amplification

Portions of bacterial 16S ribosomal RNA (rRNA) genes were amplified from extracted genomic DNA samples using a nested PCR methodology (Green *et al*., 2006). Initially, DNA samples were separately amplified with two primer sets: CH45F (5′-GGCCTA ACACATGCAAGC-3′)/CH1112R (5′-GCAACTAGTG ACAGGGG-3′) ('*Chryseobacterium*') and OX225F (5′-TGGAGCGGCCGATATCTG-3′)/OX1249R (5′-GGG TTGGCGGCCCTCTG-3′) ('Oxalobacteraceae'). Primers were designed using the 'probe design' feature of the phylogenetic analysis program package ARB (Ludwig *et al*., 2004), and checked for specificity by basic local alignment search tool (BLAST) analyses, sequence analyses of recovered PCR products and comparison to short sequences previously recovered (Altschul *et al*., 1997; Green *et al*., 2006). The *Chryseobacterium* primers target 16S rRNA genes from bacteria of the genera *Chryseobacterium* and some closely related organisms from the order *Flavobacteriales*. The Oxalobacteraceae primers target 16S rRNA genes from bacteria of the genera *Telluria*, *Massilia*, *Duganella*, *Janthinobacterium*, as well as others from the β-proteobacterial family Oxalobacteraceae.

DNA samples were amplified with *Chryseobacterium* and Oxalobacteraceae primer sets in PCR mixes containing 1.5 U (per 50 μl) of *Taq* polymerase (Red Taq, Sigma Chemical Co., St Louis, MO, USA), and the following reagents: 1 × Sigma PCR buffer, 0.2 mM PCR nucleotide mix (Promega, Madison, WI, USA), 6.25 μg (per 50 μl) bovine serum albumin (BSA; Roche Diagnostics, Mannheim, Germany) and 25 pmol of each primer. Final PCR mix magnesium concentrations were adjusted to 2 mM (Oxalobacteraceae) or 4 mM (*Chryseobacterium*) with a 25 mM solution of $MgCl_2$. These samples were initially denatured for 3 min at 95°C, and then cycled 35

times through three steps: denaturation (94°C; 30 s), annealing (64°C and 65°C for *Chryseobacterium* and Oxalobacteraceae respectively; 30 s) and elongation (72°C; 60 s). These PCR products were then PCR amplified with a general bacterial primer set suitable for denaturing gradient gel electrophoresis (DGGE) analysis (nested PCR), using the primers 341F and 907R, as described previously (Muyzer *et al.*, 1993, 1998; Green *et al.*, 2006).

Community composition analysis
DGGE analyses were performed on the nested PCR products with a D-Gene system (Bio-Rad, Hercules, CA, USA), as previously described (Green *et al.*, 2004). Cloning reactions were performed on the population-specific PCR products (that is those generated with Oxalobacteraceae or *Chryseobacterium* primer sets). The resulting clones were individually screened by PCR amplification with the general bacterial DGGE primer set and DGGE analysis. The PCR products amplified from selected clones were compared, via DGGE analysis, to nested PCR products generated from environmental samples. For several bands that could not be recovered via screening of clones, bands were individually excised from DGGE gels and sequenced as described previously (Green *et al.*, 2004). These sequence data have been submitted to the GenBank database under accession numbers AY621813-AY621834 (*Chryseobacterium*) and AY624608-AY624650 (Oxalobacteraceae).

Phylogenetic analyses
Recovered sequences were aligned to known bacterial sequences using the 'greengenes' 16S rRNA gene database and alignment tool (DeSantis *et al.*, 2006). Aligned sequences were submitted to the Bellerophon chimera checking tool, via the greengenes website (Huber *et al.*, 2004). Aligned sequences and close relatives were imported and alignments were manually refined by visual inspection in the Mega software package version 3.1 (Kumar *et al.*, 2004). Neighbor-joining phylogenetic trees were constructed on aligned 16S rRNA gene sequences using the Kimura 2-parameter substitution model with pairwise deletion of gapped positions. The robustness of inferred tree topologies was evaluated by 1000 bootstrap resamplings of the data. Additionally, Bayesian analyses were performed on the aligned sequence data (MrBayes version 3.1; Ronquist and Huelsenbeck, 2003) by running five simultaneous chains (four heated, one cold) for ten million generations, sampling every 1000 generations. The selected model was the general time reversible (GTR) using empirical base frequencies and estimating the shape of the gamma distribution and proportion of invariant sites from the data. A resulting 50% majority-rule consensus tree (after discarding the burn-in of 25% of the generations) was determined to calculate the posterior probabilities for each node.

Results

The community composition of bacteria from the family Oxalobacteraceae and the genus *Chryseobacterium* in peat and compost samples, as well as in potting mix, seed and root samples from cucumber plants grown in three different potting mix treatments ('peat-only', 'sawdust compost + peat' and 'straw compost + peat'), was analyzed. At each time point (start of experiment, 24 h, 7 days and 21 days post sowing), three pots from each treatment were destructively sampled. Genomic DNA was extracted from each of the triplicate samples for each treatment and time point and subjected to PCR, DGGE and sequence analyses. Replicate samples were examined via Oxalobacteraceae- and *Chryseobacterium*-specific PCR-denaturing gradient gel electrophoresis (PCR-DGGE) analyses, and for each primer set, community composition profiles were found to be highly reproducible. As a result, single representative samples from each treatment and time point were taken for more extensive analysis.

Oxalobacteraceae community composition
PCR-DGGE analysis of the Oxalobacteraceae amplicons revealed that seed and potting mix community profiles at 1 day were highly similar in all treatments (Figure 1). However, profiles of potting mix and root samples at 1 and 3 weeks differed substantially. In all treatments, including the peat-only and two different compost-amended treatments, three main band positions were detected. These included an upper position ('A'), detected in nearly all samples with the exception of 3 weeks root samples, represented in the peat-only treatment by bands P4, P7, P12 and P18, in the sawdust compost + peat treatment by bands S3, S6, S8, S11, S14 and S20 and in the straw compost + peat treatment by bands T2, T7, T14 and T20. Bands migrating to position 'B' were detected only in root samples, and included bands P11, P16, S12, S17, T13 and T18. Bands migrating to position 'C' were detected in potting mixes, and included bands P1, P8, P13, P20, S9, S16, S22, T15 and T21. In addition to the dominant band positions above, several less dominant bands were detected and sequenced (bands P2, P10, P19, S2, S5, S15 and T1). Sequence analyses were conducted on these bands, and in total, 43 clones representing 36 bands were sequenced and submitted to the GenBank database.

The most striking feature of the sequence analyses was the clustering of Oxalobacteraceae clones recovered from root samples regardless of treatment and sampling time (Figure 2). The root clones clustered with *Telluria mixta*, *T chitinolytica*, an environmental clone from a tree root (AJ863419), and several potting mix organisms. The grouping of root Oxalobacteraceae together, and with the genus *Telluria*, was indicated by neighbor-joining and Bayesian analyses. While high (that is >70%)

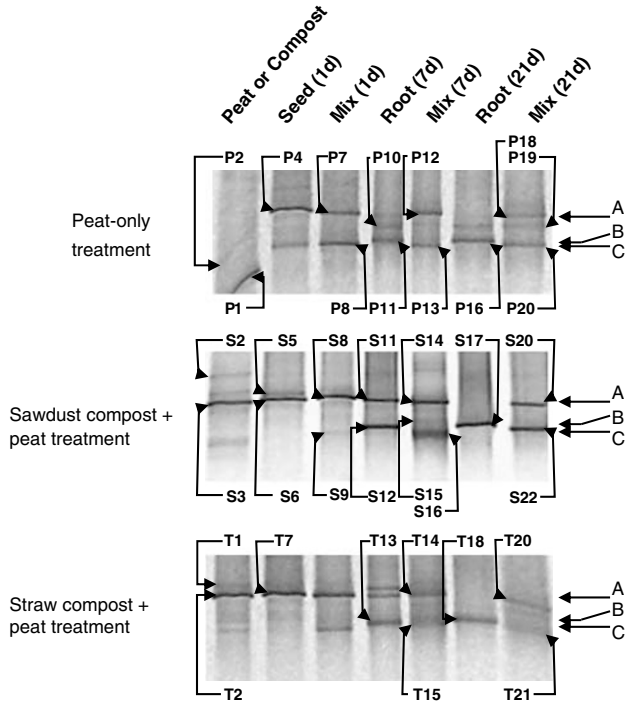

Figure 1 Composite figure of two DGGE analyses of partial 16S rRNA gene fragments amplified initially with the Oxalobacteraceae primer set and then subject to nested PCR with the general bacterial DGGE primer set, as described in the text. PCR-DGGE analyses were performed on peat and compost samples, as well as potting mix and plant samples (seed or root) from 1, 7 and 21 days post sowing. Sequenced bands are marked. Three dominant band positions, indicated by arrows and labels A–C, were present in each treatment, and are discussed in the text. Bands B and C have very similar electrophoretic mobility; however, band B migrates to a position slightly above (lower denaturant concentration) that of band C and is found only in root samples. DGGE, denaturing gradient gel electrophoresis; PCR-DGGE, PCR-denaturing gradient gel electrophoresis; rRNA, ribosomal RNA.

bootstrap values were not observed, this node was strongly supported by a Bayesian posterior probability of greater than 70% (Figure 2).

Chryseobacterium *community composition*

Genomic DNA was PCR amplified with the *Chryseobacterium* primer set, nested with the general bacterial DGGE primer set and the resultant PCR products were analyzed by DGGE (Figure 3). These analyses revealed the presence of three bands within each treatment (Figure 3; bands D–F for peat treatment; bands G–I for the two compost treatments). Within each treatment, regardless of sampling time or location, DGGE profiles were nearly identical. DGGE profiles of samples from the two compost-amended treatments were identical to each other, but shared no bands in common with samples from the peat-only treatment. Multiple sequences from the dominant bands (D–I) were recovered from different samples within each treatment (Figure 3). In total, 22 clones were sequenced, and 20 of these sequences belonged to six clusters (highlighted in gray; Figure 4). These clusters, supported by high bootstrap values and Bayesian posterior probabilities, consist of sequences recovered from different samples but identical band positions (Figure 4). Two of the recovered sequences did not group with the six defined clusters. These sequences included the sequences ChsSC and ChsP1, recovered from the sawdust compost before incorporation into the potting mix and from the seed surface of the peat treatment, respectively. Sequence ChsSC formed a clade with sequences recovered from band 'I', but was more divergent than the other recovered sequences, and was most closely related to a bacterial isolate from an industrial wastewater treatment system. Sequence ChsP1, closely related to *Chryseobacterium scophthalmum* (AJ271009), did not cluster with the other sequences recovered from band 'D'.

Discussion

Studies of the interaction between soil amendments, such as compost, and plant effects have shown that while such amendments can have a dramatic influence on soil and rhizosphere bacterial communities, this influence is mitigated with increasing proximity to the plant surface (Inbar *et al*., 2005). Nonetheless, the influence of compost amendments at seed and root surfaces is not negligible, particularly at elevated compost levels. Since composts may serve as a food source for microorganisms that are not sustained by root exudates (Boehm *et al*., 1997), we hypothesized that the interaction between exogenous soil amendments and the influence of the plant or rhizosphere effect does not impact all microorganisms equally.

To explore this issue, the community composition of two distinct taxa, one closely related to the genus *Telluria* and the second from the genus *Chryseobacterium*, both consistently detected in seed and root communities, was characterized by the application of population-specific analyses. These analyses revealed that the two groups of seed- and root-colonizing taxa responded to compost and root effects in dramatically different manners.

Spermosphere and rhizosphere communities tend to be dominated by fast-growing, motile, versatile, Gram-negative bacteria, such as *Pseudomonas* spp, competing for root exudates (Weller *et al*., 2002). The response of Oxalobacteraceae populations to plant growth stage and proximity to roots was consistent with the response of saprophytic bacteria largely influenced by plant exudates, resulting in a strong rhizosphere effect. The Oxalobacteraceae community composition changed dramatically during plant development, as seed communities resembled the initial potting mix while the 21-day root communities were comprised of a single dominant population, which differed from those detected on the seeds.

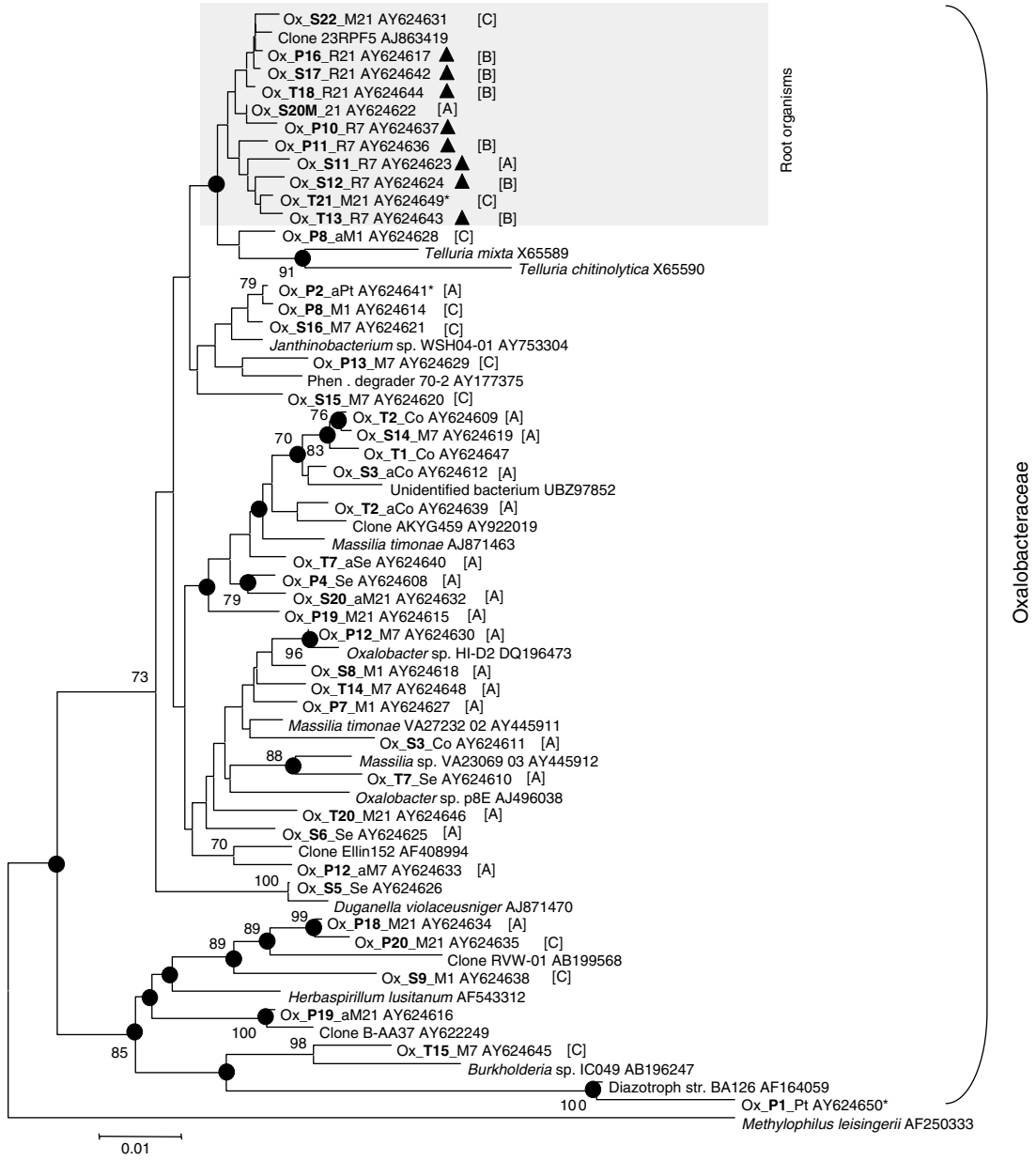

Figure 2 Bootstrapped neighbor-joining tree of Oxalobacteraceae 16S rRNA gene sequences. Short sequences (less than 1000–1005 bases) are indicated by asterisks. Nodes supported by bootstrap values greater than 70% are indicated by numeric values, and nodes supported by Bayesian analysis, with posterior probability values greater than 70%, are indicated with black circles. Each sequence is labeled by clone name, with OX representing Oxalobacteraceae; DGGE band identification (in bold; according to Figure 1); a sample indicator: Pt (Peat), Co (Compost), Se (seed), M (Potting Mix) and R (Root); and date indicator: 7 (7-day sampling) or 21 (21-day sampling). Sequences labeled with an 'a' after the DGGE band identification represent a second sequenced clone from the same band. Sequences recovered from root samples are indicated by triangles, and the cluster containing the root sequences is highlighted in gray. The DGGE band position associated with each sequence (positions A–C, Figure 1) is indicated within brackets. The scale bar represents 0.01 substitution per nucleotide. DGGE, denaturing gradient gel electrophoresis; rRNA, ribosomal RNA.

Furthermore, all root Oxalobacteraceae sequences were at least 98.3% similar to each other, and clustered together with a poplar tree root bacterium (Graff and Conrad, 2005). The shift in Oxalobacteraceae population composition from seed to root was consistent with our prior observation that the seed-colonizing bacterial communities differed substantially from the root microbial communities (Green *et al*., 2006). However, the observed community shift occurred within the narrow phylogenetic context of Oxalobacteraceae closely related to the genus *Telluria*.

The close phylogenetic relationship of the root-associated Oxalobacteraceae with the genus *Telluria* is intriguing. At least one study has noted the strong rhizosphere capabilities of *Telluria* species; *T chitinolytica* was found to colonize tomato roots, particularly elongation zones and roots tips

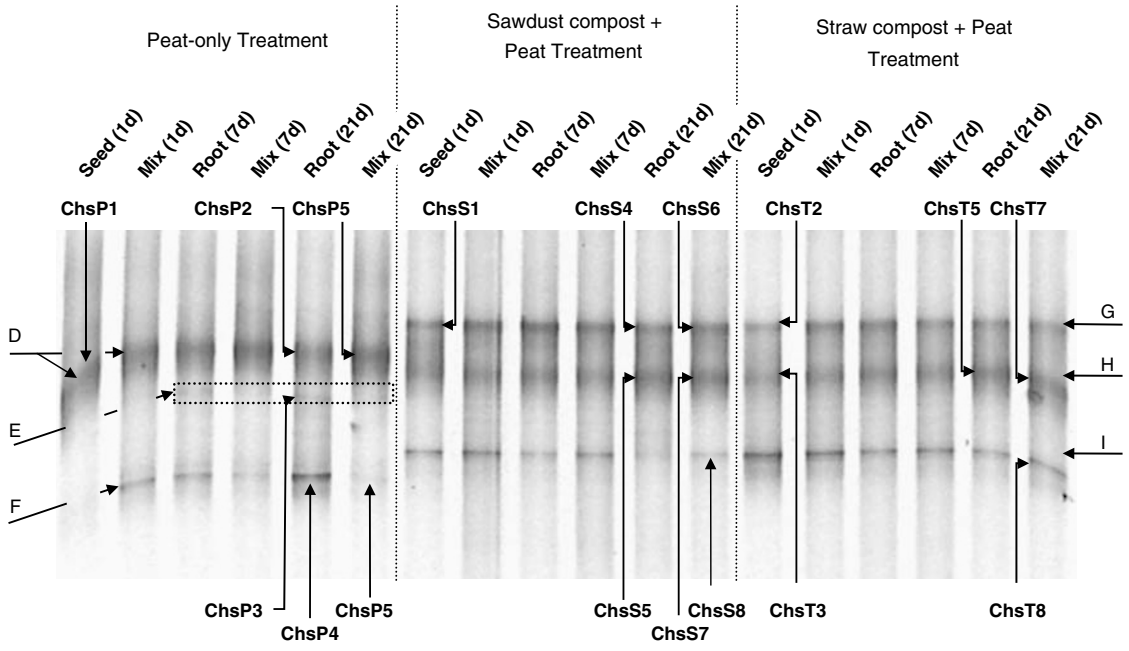

Figure 3 DGGE analysis of partial 16S rRNA genes amplified initially with the *Chryseobacterium* primer set and then subject to nested PCR with the general bacterial DGGE primer set, as described in the text. PCR-DGGE analyses were performed on potting mix and plant samples (seed or root) from 1, 7 and 21 days post sowing. Six dominant band positions, labeled D–F (peat-only treatment) and G–I (compost treatments), are discussed in the text. Sequenced bands are marked. Bands migrating to position E were only faintly detected in samples from 7 and 21 days post sowing, and are marked by a rectangle. DGGE, denaturing gradient gel electrophoresis; PCR-DGGE, PCR-denaturing gradient gel electrophoresis; rRNA, ribosomal RNA.

(Spiegel *et al.*, 1991). Physiological analyses of *Telluria* reveal many features that are consistent with traditional copiotrophic rhizosphere bacteria (Spiegel *et al.*, 1991; Bowman *et al.*, 1993; Anzai *et al.*, 2000). Bacteria from the family Oxalobacteraceae, previously detected in root environments (Hallmann *et al.*, 1997; Mahaffee and Kloepper, 1997a, b; Olsson *et al.*, 1999; McSpadden-Gardner and Weller, 2001; Johansen and Binnerup, 2002; Schmalenberger and Tebbe, 2002; Graff and Conrad, 2005), have increasingly been seen as important components of rhizosphere communities and have been specifically targeted in rhizosphere studies (Dohrmann and Tebbe, 2005).

Unlike the Oxalobacteraceae, the community composition of compost-derived *Chryseobacterium* was not sensitive to the seed or root environment. This ecological behavior is more consistent with organisms that are influenced by bulk soil (for example soil organic matter). Soil organic matter can be a significant source of carbon for rhizosphere organisms (Toal *et al.*, 2000), and high levels of organic matter and nutrients, such as those that are supplied via compost amendment, can influence the strength of the observed rhizosphere effect (Semenov *et al.*, 1999). Although *Chryseobacterium* spp are found frequently in organic-rich environments such as composted materials (Ryckeboer *et al.*, 2003), these organisms are not particularly well known as rhizosphere organisms, even though they have been previously detected in or isolated from rhizosphere environments (McSpadden Gardener and Weller, 2001; Young *et al.*, 2005; Park *et al.*, 2006).

The consistent presence of *Chryseobacterium* spp on seed and root surfaces may be a result of their persistence in the compost-amended potting mixes. The ability of bacteria to survive in large numbers in soil can be a major determinant of their ability to subsequently colonize rhizosphere environments (Jjemba and Alexander, 1999; De Ridder-Duine *et al.*, 2005), and compost amendments have been previously shown to sustain *Chryseobacterium* in peat-based potting mixes for longer than in unamended potting mixes (Krause *et al.*, 2001). This is consistent with our prior observation that in the peat-only treatment, *Chryseobacterium* spp were not detectable members of the total bacterial community on the roots at 21 days, while in both compost-amended treatments they were detectable (Green *et al.*, 2006).

Furthermore, the *Chryseobacterium* detected in the compost-amended treatments were derived from the composts (as opposed to having been enriched from the peat). This was demonstrated by PCR-DGGE analyses with general bacterial-, *Bacteroidetes*-specific and *Chryseobacterium*-specific primer sets (data not shown), and by sequence analysis of *Chryseobacterium* from compost materials, from potting mix and plant samples from treatments with and without compost. There was no overlap between the *Chryseobacterium* community composition in the peat-only treatment and either of the compost-amended mixes, and the *Chryseobacterium* sp pre-

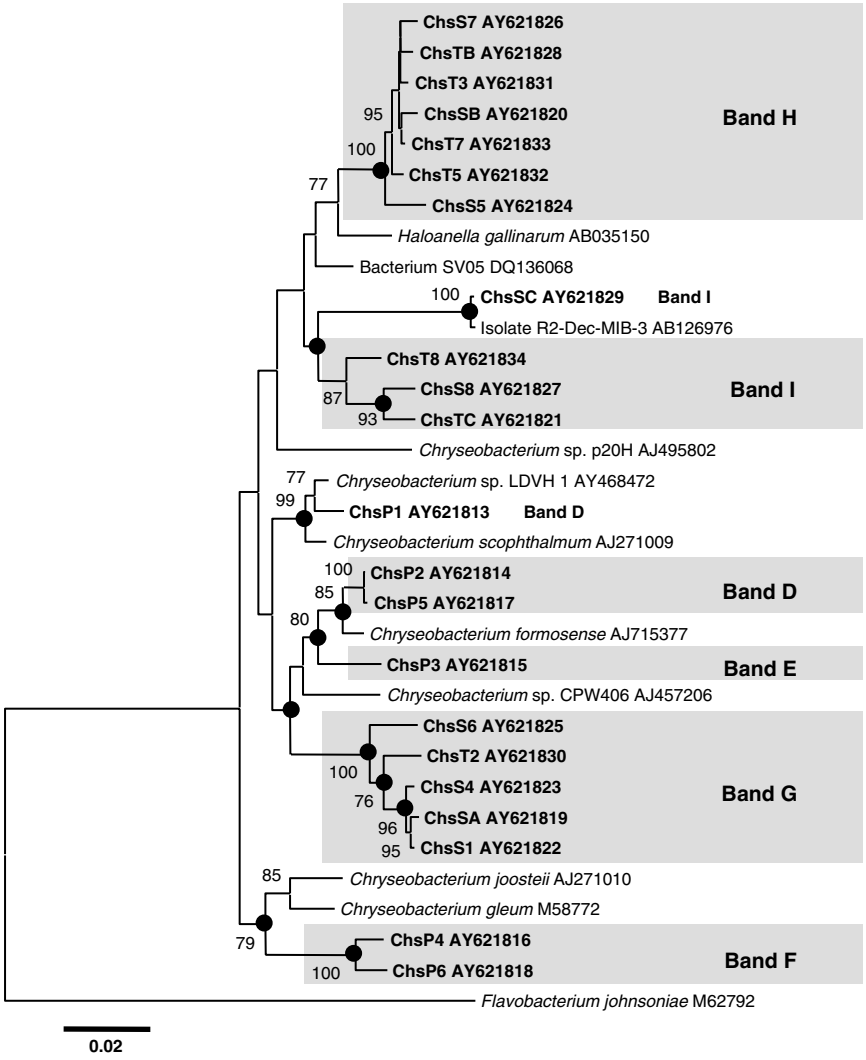

Figure 4 Bootstrapped neighbor-joining tree of *Chryseobacterium* 16S rRNA gene sequences (1031–1035 bases) recovered from DGGE analyses (Figure 3). Nodes supported by bootstrap values greater than 70% are indicated by numeric values, and nodes supported by Bayesian analysis, with posterior probability values greater than 70%, are indicated with black circles. Six monophyletic clades of recovered *Chryseobacterium* sequences, labeled D–I (Figure 3), are highlighted in gray. The scale bar represents 0.02 substitution per nucleotide. DGGE, denaturing gradient gel electrophoresis; rRNA, ribosomal RNA.

viously detected in the peat-only treatment was not detected in root samples (Green *et al*., 2006). The essentially invariant community composition of the *Chryseobacterium* in all sampling points and locations, combined with the persistence of *Chryseobacterium* in seed and root as significant components of the entire bacterial community (Green *et al*., 2006), suggests that *Chryseobacterium* populations in the compost treatments are primarily sustained by the compost itself.

Thus, although root-associated bacterial communities can be heavily influenced by the plant, our work has shown that some microorganisms can be sustained in root environments with relative insensitivity to the rhizosphere effect. The persistence of the same *Chryseobacterium* populations in potting mixes and on seed and root surfaces demonstrates that the cucumber rhizosphere selection is not the exclusive factor involved in determining the composition of the spermosphere and rhizosphere communities. Although it seems unlikely that this is a plant-specific phenomenon, further research is required to characterize the multiple factors that can mitigate the rhizosphere effect, and allow the persistence of 'non-traditional' rhizosphere microorganisms in close proximity to plant seed and root surfaces.

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