Seed-Colonizing Bacterial Communities Associated with the Suppression of *Pythium* Seedling Disease in a Municipal Biosolids Compost

Mei-Hsing Chen, Allison L. H. Jack, I. Cristina McGuire, and Eric B. Nelson

Cornell University, Department of Plant Pathology and Plant-Microbe Biology, 334 Plant Science Building, Ithaca, NY 14853.

Current address of M.-H. Chen: Taiwan Agricultural Research Institute, Zhongzheng Rd., Number 189, Wufeng Dist., Taichung City 413, Taiwan.

Current address of I. C. McGuire: Rheonix Inc., 22 Thornwood Drive, Ithaca, NY 14850.

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ABSTRACT

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This study was designed to characterize seed-colonizing microbial communities that were previously shown to be involved in the suppression of seedling disease caused by *Pythium ultimum* in a municipal biosolids compost. Selective microbial inhibitors were employed to inactivate portions of the microbial community associated with seed germinated in a compost medium to evaluate their impact on disease suppression. After initial screenings for toxicity to both cucumber and *P. ultimum*, six selective inhibitors were eventually used to assess the impact of seed treatment on the reduction of bacterial and fungal populations and on disease suppression. Rifampicin was the most effective inhibitor for inactivating disease suppression. Bacterial communities that colonized cucumber seed sown in compost medium for 8 h and seed sown in compost medium for 8 h followed by a 3-h treatment of either rifampicin at 500 ppm or water were dislodged from seed surfaces and subjected to RNA extraction and reverse transcription to cDNA. Differences in the composition of seed-colonizing bacterial communities were assessed using terminal restriction fragment length polymorphisms (T-RFLP) of polymerase chain reaction-amplified 16S rDNA genes. T-RFLP profiles revealed a diversity of distinct bacterial taxa, a number of which dominate seed surfaces within 8 h of sowing. Analysis of similarity (ANOSIM) using terminal restriction fragment (T-RF) presence or absence showed

that community profiles of nontreated and water-treated seed were quite similar whereas community profiles from rifampicin-treated seed were distinct. Differences in community profiles based on T-RF abundance (peak height and peak area) indicated that all treatments were unique (ANOSIM, all pairwise comparisons $P < 0.05$). Peaks heights and areas of relatively few T-RFs were reduced to zero following rifampicin treatment and 34 T-RFs explained 85% of the observed difference between treatments. Tentative taxon assignments for each of the T-RFs that contributed to the treatment differences revealed a preponderance of sequences with affinities to the α -, β -, and γ -Proteobacteria and Firmicutes. Limited sequencing of clones associated with water-treated and rifampicin-treated seed revealed the presence of similar taxa dominated by members of the γ -Proteobacteria. Many species within these taxa (such as *Pseudomonas* spp., *Enterobacter* spp., and *Bacillus* spp.) are known to be suppressive to *Pythium* diseases. Results of our study have confirmed that *Pythium* disease suppression in a municipal biosolids compost is mediated by compost-associated bacteria that colonize seed within hours after sowing. By focusing on actively growing microbes in the infection court during important stages of pathogen infection, we believe we can more efficiently determine the mechanisms of disease suppression and the microbes involved. Although specific to this pathosystem and compost, our results have a much broader scope of inference and illustrate the utility of such a targeted approach in identifying a relatively small subset of microbial taxa from complex communities likely to be involved in disease suppression.

Disease-suppressive soils represent useful models for understanding the possible mechanisms by which complex microbial communities interact with plants and plant pathogens, resulting in natural disease control. Suppressive soils have been described from many parts of the world (40,45), often occurring naturally or intentionally manipulated to induce suppressiveness, either through continuous monoculture (61) or through the addition of organic amendments such as composts (2,52). Overwhelming evidence now links disease-suppressive properties of natural or compost-induced suppressive soils with the activities of microbial consortia (8,72). However, the specific microorganisms and processes that lead to reduced disease development in these soils are just beginning to be revealed (4,50,61).

Identifying the microbes that contribute to disease suppression has been challenging, largely because of the massive populations

and diversity of prokaryotic (60,73) and eukaryotic soil microbes (3,33), many of which carry out important ecosystem functions that do not contribute to disease suppression. This level of complexity makes it difficult to find those microbes and microbial activities that contribute to disease suppression, in part because of the sheer numbers of microbial taxa but also because of both direct and possible indirect interactions that may lead to disease suppression. Because it would be quite challenging to identify indirect interactions among microbes that could potentially contribute to disease suppression, a common approach for identifying disease-suppressive microbes has been to focus directly on comparisons of microbial communities between suppressive and non-suppressive soils to find differences in microbial taxa that correlate with disease suppression (9,20,36,39,70). Often, such a strategy does not offer a clear explanation of the microbes involved in disease suppression (7,56), again because many taxa may differ in the presence or absence or relative abundance between suppressive and nonsuppressive soils but have nothing to do with disease suppression.

Corresponding author: E. B. Nelson; E-mail address: ebn1@cornell.edu

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A more directed strategy for identifying the microbial species and processes involved in disease suppression is to focus on microbes whose populations change in the pathogen infection court with corresponding changes in disease suppression (8). This strategy has proven effective in revealing the role of 2,4-diacetylphlorogucinol-producing *Pseudomonas* spp. in the suppression of take-all disease of wheat (72), root colonizing actinobacteria and Proteobacteria in the suppression of Rhizoctonia root rot of apple (44,46), nonpathogenic *Fusarium* spp. in the suppression of Fusarium wilt diseases (26), and cyst-associated fungi in the suppression of *Heterodera schachtii* (76,77). Such a strategy focuses on spatial and temporal scales that are ecologically relevant to the pathosystem and it also facilitates the characterization of disease-suppressive microbes by filtering out much of the soil microbial community that does not contribute to disease suppression. It also focuses on actively growing microbes, which are most likely to express disease-suppressive traits, in part because their activity and responses to the plant are synchronized with the time and site of infection.

Seed-colonizing microbial consortia have been shown previously to be associated with the suppression of Pythium diseases, especially in compost-amended soils (5,6,22). Previous work with Pythium seedling diseases of cotton (47), cucumber, wheat, and pea (11) demonstrated that active bacterial communities developing on seed within the first few hours of seed germination could fully explain the suppression of *Pythium ultimum*-induced seedling diseases. However, little is currently known about the microbial taxa associated with seed, especially those communities that develop soon after sowing (51) that may also contribute to Pythium disease suppression. In some studies of seed-colonizing microbes, species of *Xanthobacter*, *Enterobacter*, *Microbacterium*, *Paracoccus*, *Curtobacterium*, *Micrococcus*, *Agrobacterium*, *Paenibacillus*, and other actinobacteria were identified from cottonseed surfaces germinated for as little as 4 h in a leaf compost (47). No fungal species were detected within this time frame. Other studies have identified species of *Bacillus*, *Oxalobacter*, *Stenotrophomonas*, *Sphingobacterium*, and *Rhizobium*, as well as strains of Bacteroidetes and unclassified β -Proteobacteria colonizing cucumber seed within 24 h of sowing in a cow manure compost (30). Yet, although *Enterobacter*, *Paenibacillus*, *Bacillus*, and *Stenotrophomonas* spp. are known to suppress diseases caused by *P. ultimum* (24,48,74), their roles in disease-suppressive soils or composts are unknown, and even less is known of the Pythium disease-suppressive properties of many of the other genera described from seed.

The purpose of the present work was to determine which microbial taxa present on seed surfaces likely explain the suppression of *P. ultimum*-incited seedling disease in a municipal biosolids compost. Using a terminal restriction fragment length polymorphism (T-RFLP) analysis (42), we focused on microbial communities that develop in the *P. ultimum* infection court (i.e., seed surface and spermosphere) soon after sowing because these are the communities previously shown to be associated with suppressiveness (11,47). An aim of our work was to utilize selective inhibitors to reduce or eliminate suppressiveness while, at the same time, providing a means of detecting species whose abundances are correspondingly altered as a result of the inhibitor treatment. We reasoned that, if we could reduce or eliminate disease suppression and also reduce or eliminate certain seed-colonizing microbial populations, these reduced or eliminated taxa would likely be among those involved in Pythium seedling disease suppression. We also focused our microbial community analyses on RNA extractions from seed surfaces as opposed to DNA extractions, reasoning that this approach would enrich actively metabolizing bacterial cells that respond directly to the germinating seed, and also avoid bacterial DNA from cells potentially lysed as a result of the inhibitor treatments. Furthermore, by focusing on interactions in the infection court, we are more

likely to find the microbes that interact directly with the pathogen during a critical stage of plant infection.

MATERIALS AND METHODS

Compost and plant material. A municipal biosolids compost was collected from a composting facility in the village of Endicott, NY in 2005 and used throughout this study. The general properties of biosolids composts produced from this facility have been described previously (16) and the suppressiveness of these composts to Pythium seedling diseases has been previously documented (11). Composts used in this study were sieved through a 2-mm screen, placed in large aliquots, and stored at -20°C until use. Although we recognize that any storage method may alter compost microbial communities, freezing has been shown to be a reliable means of preserving composts for subsequent microbial analyses (71). For all laboratory experiments, each compost was mixed with sterile, washed, oven-dried quartz sand sieved to a particle size of 0.5 to 1.0 mm at a rate of 100 mg (dry weight) of compost per cubic centimeter of mix (total volume), which is referred to as "compost medium".

Nontreated cucumber seed (*Cucumis sativus* L. 'Marketmore 76') were used in all experiments. Seed were inspected and those with cracks or other visible deformations were discarded. For use in experiments, seed were surface disinfested for 5 min in 0.5% (vol/vol) sodium hypochlorite containing 1 or 2 drops of Tween 20 (polyoxyethylene [21] sorbitan monolaurate) Sigma-Aldrich, St. Louis) as a wetting agent. Seed were rinsed three times with sterile MilliQ-purified water (MQW), blotted, and air dried before use in experiments.

Production and germination of *P. ultimum* sporangia. Sporangia of *P. ultimum* were used as inoculum in all seedling bioassays. *P. ultimum* isolate P4 was maintained on solid SM+L medium as described previously (69). To induce the formation of sporangia, mycelial disks (5 mm in diameter) were excised from cultures after 5 days of growth at 27°C and placed in sterile petri dishes (9 cm in diameter). Disks were leached for two consecutive 10-min periods in a buffer (pH 5.8) containing 10 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 4 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 5 mM KNO_3 . This was followed by a final 3-h leaching period in darkness. Buffer was replaced after each leaching period. After the final leaching period, the leachate was removed and disks were rinsed twice with MQW and incubated at 24°C in darkness. Abundant sporangia were formed after 2 days. Sporangia of this isolate only germinate directly by forming germ tubes; zoospores have never been observed.

For seedling bioassays in which microbial inhibitors were used, the entire SM+L culture was used to produce sporangia. The culture medium was leached as described for disks above. After incubation for 2 days in the dark at 24°C , sporangia were scraped from the surface of the SM+L culture by placing 2 ml of MQW on the culture surface and gently scraping the surface with an inoculation loop. The 2 ml containing the dislodged sporangia was removed from the dish, vortexed for 5 min, and then filtered through four layers of cheesecloth to remove hyphal fragments. The filtrate was then centrifuged at 8,000 rpm for 5 min and the sporangial pellet was resuspended in MQW. Sporangia were intact, viable, and free of hyphal contamination. The concentration of sporangia was adjusted to 250 per 10 μl and used immediately in experiments.

Cucumber seed exudate (CSE), prepared as described previously (74), was used to germinate sporangia of *P. ultimum* isolate P4 for phytotoxicity assays. Based on preliminary sporangium germination experiments with different concentrations of CSE, 5 mg/ml was chosen as the stimulatory concentration in all subsequent experiments.

Microbial inhibitors. A number of selective microbial inhibitors were employed as seed treatments as a means of altering

microbial communities on seed surfaces so that the impact on disease suppression could be evaluated. The following inhibitors were evaluated: benomyl (methyl [1-[(butylamino)carbonyl]-1H-benzimidazol-2-yl]carbamate), pentachloronitrobenzene (PCNB), streptomycin sulfate, neomycin sulfate, rifampicin, vancomycin, pimaricin, penicillin G, nystatin, chlorophenicol, chlorotetracycline, and cycloheximide. For these inhibitors to be acceptable for our experiments, we needed to first ensure that they were not inhibitory to cucumber seedlings or to *P. ultimum* sporangia.

Phytotoxicity tests. Toxicity of microbial inhibitors to cucumber seedlings was evaluated by soaking 10 surface-disinfested cucumber seed in the inhibitor at 0, 10, 100, and 1,000 ppm for 30 min and assessing their subsequent germination. Germination in the presence of the inhibitor was compared with germination of nontreated seed and seed soaked for 30 min in water. In some experiments with rifampicin, seed were also soaked in a 500-ppm concentration for 1, 2, and 3 h. Seed were then transferred to a premoistened double layer of sterile Whatman number 1 filter paper in 9-cm petri dishes and incubated in the dark at 24°C. After 3 days, seed were assessed for germination whereas shoot length and root length were assessed after 7 days. There were 10 replicates for each treatment and each experiment was repeated at least once.

Toxicity of microbial inhibitors to *P. ultimum* sporangia. Inhibitors previously shown to be nonphytotoxic were chosen and used in experiments to assess their toxicity to *P. ultimum* sporangia. Inhibitors were added to CSE at a final concentration of 1,000 ppm. CSE (5 mg/ml) was used as a positive control because of its known stimulatory activity to *P. ultimum* sporangia (51). Water served as a negative control. Each solution (10 µl) was placed directly on three replicate agar disks containing sporangia. After 3 h at 24°C, disks were examined for germinated sporangia by staining with 0.03% acid fuchsin in 85% lactic acid and counting under the microscope at ×250 magnification.

Inhibition of seed-colonizing bacterial and fungal populations. The inhibition of culturable seed-colonizing bacterial and fungal population development from the surface of cucumber seed was determined by dilution plating. Ten cucumber seeds were incubated in compost medium at 24°C and at a water potential of -2 kPa. After 8 h, seed were removed and either not treated or soaked in water or in solutions of either benomyl, PCNB, penicillin G, neomycin sulfate, rifampicin, vancomycin, or pimaricin at a concentration of 1,000 ppm. In most experiments, seed were removed from solutions after 1 h. However, in some experiments with rifampicin, seed were also soaked in a 500-ppm solution for 3 h. After inhibitor treatment, seed were rinsed with sterile water; then, 10 seeds were vortexed in 10 ml of MQW for 10 min and a dilution series was prepared. Appropriate dilutions were then plated on 10% tryptic soy agar for enumerating bacterial populations and on 1/10-strength potato dextrose agar (PDA) containing streptomycin sulfate and chlorotetracycline, each at 100 ppm, for enumerating fungal populations (10). Colony counts were expressed as CFU per seed. There were three replicates for each treatment.

Effects of microbial inhibitors on *Pythium* seedling disease suppression. Seedling bioassay experiments were modified from those described earlier (11). Bioassays were conducted in 2.5-cm-diameter glass cylinders placed on a moistened blotter paper supported on a plexiglass sheet. Glass cylinders were partially filled with 30 cm³ of sterile sand. MQW (5 ml) was added to each cylinder followed by five filter paper disks (5 mm in diameter) containing 250 *P. ultimum* sporangia. Seed that had germinated in either compost medium or sand for 8 h were removed and either not treated further, soaked in water, or soaked in inhibitor solutions as described above. Seed were then placed directly onto each filter paper disk. In some experiments, cucumber seed removed after 8 h from compost medium or sterile sand were soaked in rifampicin solutions at 500 or 1,000 ppm for 1 h.

Because rifampicin blocks the initiation of RNA synthesis by binding to the β-subunit of DNA-dependent RNA polymerase (23), time may be required for the inhibitor to enter the cell. Therefore, we extended the incubation time to 3 h to ensure that RNA synthesis was more completely blocked. Cylinders were then filled with a final 45 cm³ of sterile sand followed by the addition of another 7 ml of MQW. MQW and noninoculated sand served as controls. There were three replicate cylinders per treatment. The entire assembly was placed in a clear plastic box and incubated at 24°C with a 16-h photoperiod. For our disease assessments, we chose to use shoot lengths as our measure of disease severity in all of our experiments. Based on a previous comparative analysis of disease assessment metrics (11), shoot heights not only reflected observed differences in stand establishment but also served as a measure of the stunting due to seed and seedling infections by *P. ultimum*. Shoot lengths of each seedling were determined after 7 days of incubation. The experiment was repeated twice.

RNA extraction and reverse-transcription polymerase chain reaction. Fifty cucumber seeds were sown in compost medium as described above for 8 h, removed, lightly rinsed, and either (i) not treated, (ii) soaked in water (water treated), or (iii) soaked in a 500-ppm rifampicin solution for 3 h (rifampicin treated). Seed were then removed from solutions, lightly rinsed, and vortexed for two consecutive 5-min periods. The dislodged cell suspension was filtered through four layers of cheesecloth and centrifuged at 8,000 rpm for 10 min. The pellet was resuspended in 100 µl of Tris-EDTA buffer. Total microbial RNA was extracted using an RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. There were three replicate extractions for each treatment. Contaminating DNA was removed from RNA extracts using a Turbo DNA-free Kit (Life Technologies Corp., Grand Island, NY) and RNase-Free DNase Set (Qiagen, Inc.). cDNA was prepared by reverse-transcription polymerase chain reaction (RT-PCR) of RNA with the aid of a QuantiTect Reverse Transcription Kit (Qiagen, Inc.).

PCR amplification. T-RFLP microbial community fingerprinting was used to characterize the bacterial communities in the cucumber spermosphere (42). 16S rRNA genes were amplified from cDNA using carboxyfluorescein (FAM)-labeled bacterial universal forward primer 27f (5'-FAM/AGA GTT TGA TCM TGG CTC AG-3') (29) and reverse primer 1392r (5'-ACG GGC GGT GTG TRC-3') (1). Each PCR reaction mixture contained 1 µg of cDNA template, 3.0 mM MgCl₂, 2.5 units of Taq polymerase (Sigma-Aldrich), 1× PCR buffer, 0.2 mM PCR nucleotide mix (Sigma-Aldrich), 0.2 µM DNA primers, and bovine serum albumin (Life Technologies Corp.) at 5.0 µg µl⁻¹ in a final volume of 50 µl. The following PCR protocol was used: an initial denaturation step of 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 45 s, primer annealing at 55°C for 1 min, and extension at 72°C for 90 s. A final extension at 72°C for 7 min was performed after the cycling was complete in a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA). To obtain sufficient DNA for T-RFLP analysis and to minimize PCR bias, amplicons from six PCR runs for each sample were pooled and run on 1% agarose gel and the band at ≈1,400 bp was excised, then purified using a PCR purification kit (Wizard PCR Preps DNA Purification System; Promega Corporation, Madison, WI).

T-RFLP analysis. Purified PCR products (450 ng) were digested with 20 U of *Msp*I (CC/GG) (New England Biolabs Inc., Ipswich, MA) in a volume of 50 µl overnight at 37°C, then inactivated at 65°C for 20 min. Of the various restriction enzymes tested, this was the only enzyme where the water and rifampicin treatments could be resolved. Finally, excess salts were removed from digestions with Micro Bio-Spin 30 Chromatography Columns (Bio-Rad Laboratories) according to the manufacturer's instructions. All samples were dried, then dissolved in Liz

standard 500 (0.1 µl) and Hi-Di Formamide (9.9 µl). All samples were then run in an ABI 3730 DNA Analyzer system (Life Technologies Corp.) and the products were analyzed using GeneMapper software (version 3.7; Life Technologies Corp.).

Peak data from the GeneMapper software were uploaded to the online T-RFLP analysis software T-REX (17). This software package was used to prepare raw data for analysis, identify and align terminal restriction fragment (T-RF) peaks among the three samples, and construct data matrices for subsequent statistical analyses. T-RF peaks were identified by rounding to the nearest integer and aligned, and peak height and peak areas were calculated. T-RFs were considered to be different if they differed by >1 bp (17). T-RFs that were present in all three replicate extractions were then subjected to searches within the Ribosomal Database Project (RDP) database (R10, Update 26 of over 1.5 million bacterial 16S rDNA sequences) as implemented in the online tool MiCA 3 (64). This analysis allowed us to obtain putative species matches. Recognizing that our database hits would identify matches to multiple taxa, our goal was to identify the most likely phylum associated with each T-RF and the relative abundance of hits to known as opposed to unknown genera as a rough assessment of the microbial taxa present. Thus, any database hits to unidentified or uncultured taxa were removed from taxonomic assignments if they were associated with the same phyla as known taxa.

Cloning, sequencing, and operational taxonomic unit (OTU) sharing between water-treated and rifampicin-treated seed. Purified PCR products were also cloned using INVαF' competent cells and the pCR2.1 vector from the TA Cloning Kit (Life Technologies Corp.) according to the manufacturer's directions. Transformants were transferred to Luria-Bertani (LB) broth containing kanamycin (50 µg/ml) in 96-well tissue culture plates, and then incubated for 16 h at 37°C at 225 rpm. Transformants were PCR screened using M13f (5'-GTAAAACGACGGCCAG-3') and M13r (5'-CAGGAAACAGCTATGAC-3') primers to check for the presence of an insert. Proper-sized transformants were purified with exonuclease 1 and antarctic phosphatase (New England Biolabs, Inc.) for 45 min at 37°C then at 95°C for 15 min prior to sequencing. Purified plasmid DNA was mixed with the M13f primer, then submitted to the Cornell University Life Sciences Core Laboratories Center. Sequencing was performed on an Applied Biosystems Automated 3730 DNA Analyzer using Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase. Sequences were compiled and edited in Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI) to remove vector sequences and eliminate poorly resolved regions.

Edited and trimmed sequences were imported into the software MEGA 5.0 (68) and aligned using the Clustal W algorithm (13) under default settings. After initial alignments, sequences were manually edited using MEGA 5.0 to correct misaligned sequences and ambiguous base designations. During this final editing, all alignments were further trimmed to a fixed length of 582 bp (gaps included). Jukes-Cantor-corrected distance matrices were then constructed from combined water treatment and rifampicin treatment alignments using the DNAdist utility within the Phylip v3.68 program. Distance matrices were then used to estimate OTUs using the program DOTUR (62). The relatedness of each of our calculated OTUs to known taxa was determined based on BLAST searches of the National Center for Biotechnology Information GenBank database. The level of OTU overlap between water-treated communities and rifampicin-treated communities was estimated using the SONS software package (63). This analysis allowed us to determine which OTUs were present on seed treated with water but absent on seed treated with rifampicin.

Statistical analyses. Seed germination percentages and seedling lengths from the phytotoxicity tests were compared with water controls using the Dunnett test (SAS Institute, Cary, NC). All other experiments with microbial inhibitors and fungal and

bacterial populations were analyzed using analysis of variance using the general linear model. All possible pairwise means comparisons among nontreated seed, water-treated seed, and rifampicin-treated seed were made using Tukey's test at an α level of 0.05.

The magnitude and significance of qualitative and quantitative differences in T-RF abundance between seed-colonizing microbial communities from nontreated seed, water-treated seed, and rifampicin-treated seed were determined by comparing T-RF presence or absence and relative abundance as estimated by peak height and peak area, respectively. Data matrices were constructed and analyzed with the additive main effects and multiplicative interaction model (AMMI) in T-REX (17) to assess whether T-RF community structures differed among the three treatments (nontreated, water-treated, and rifampicin-treated). Analysis of variance was then used in the AMMI model to partition the variation into main effects and interaction effects. Principle components analysis was then applied to the interactions to create principal component axes (IPCA) from which the data were ordinated and visualized (18). The two-dimensional data matrix constructed in T-REX for the AMMI and IPCA analysis was also used in an analysis of similarity (ANOSIM) as implemented in the software program PAST v. 2.10 (32). The Bonferroni correction was used to adjust *P* values for simultaneous pairwise comparisons. ANOSIM was carried out with Bray-Curtis distance measures for T-RF presence-or-absence data, as well as the quantitative measures of peak height and peak area. To determine which T-RFs contributed most significantly to the observed differences between water-treated and rifampicin-treated communities, quantitative data (peak heights and peak areas) were subjected to similarity percentage (SIMPER) analysis (14,59), again using Bray-Curtis distance measure as implemented in PAST.

RESULTS

Toxicity of microbial inhibitors to cucumber seed germination and *P. ultimum* sporangium germination. Fungal and bacterial inhibitors varied in their phytotoxicity to cucumber seedlings (data not shown). Toxicity was expressed as reductions in shoot development, root development, or both. However, none of the inhibitors reduced the germination percentage of cucumber seed. Cycloheximide was phytotoxic at all concentrations tested (10 to 1,000 ppm) whereas chlortetracycline was phytotoxic only at 100 and 1,000 ppm. Among the other inhibitors tested, nystatin, streptomycin sulfate, and chloramphenicol were phytotoxic only at 1,000 ppm. The remainder of the inhibitors tested (benomyl, neomycin sulfate, PCNB, penicillin G, pimaricin, rifampicin, and vancomycin) were nonphytotoxic and subsequently chosen for use in tests to evaluate toxicity to sporangium germination.

Sporangium germination was generally unaffected by most of the inhibitors tested (data not shown). Only pimaricin significantly ($P < 0.05$) reduced sporangium germination relative to germination in the cotton seed exudate control. The remaining six nontoxic inhibitors were chosen for subsequent experiments to determine their impacts on seed-colonizing fungal and bacterial populations.

Inhibition of seed-colonizing bacterial and fungal populations. Log populations of bacteria on the surface of cucumber seed removed from compost medium after 8 h were an order of magnitude higher (6.6) than surface populations of seed removed from compost medium that had been lightly rinsed (5.7). Among the six inhibitors tested at a concentration of 1,000 ppm (benomyl, PCNB, penicillin G, neomycin sulfate, rifampicin, and vancomycin), only rifampicin and neomycin sulfate significantly ($P < 0.05$) reduced log bacterial populations to 5.3 and 4.6, respectively (Fig. 1A).

Mean fungal populations were extremely low and variable on the surface of cucumber seed that had germinated in compost medium for 8 h. Because of the wide variation in colony counts

(29 to 259 CFU/seed), none of the inhibitors significantly ($P < 0.05$) reduced fungal populations compared with the water control (Fig. 1B).

Impact of microbial inhibitors on *Pythium* seedling disease suppression. Differential effects of various microbial inhibitors

Fig. 1. Inhibition of **A**, bacterial and **B**, fungal populations on the surface of cucumber seed in the presence of various microbial inhibitors. Seed were germinated in compost for 8 h. Populations of bacteria and fungi were determined by plating on 10% tryptic soy agar and on 10% potato dextrose agar containing streptomycin sulfate at 100 ppm and chlorotetracycline at 100 ppm, respectively. Columns followed by an asterisk (*) represent means that are significantly different ($P < 0.05$) from the water control.

on *Pythium* seedling disease suppression were observed. Seedling disease severity was high from seed germinated in sand for 8 h, then transplanted to sand and inoculated with *P. ultimum* (Fig. 2). Unexpectedly, seed treated with neomycin sulfate and inoculated with *P. ultimum* gave rise to significantly greater seedling lengths compared with the noninoculated control. This response was not observed in the absence of *P. ultimum* inoculations. Because we felt that this would complicate and possibly confound our microbial analysis, we removed this inhibitor from further experimentation. Of the five other inhibitors tested, none significantly altered seedling disease incidence or severity in sand-to-sand transplants. However, cucumber seed germinated in compost medium for 8 h followed by transplant to sand, and challenge by *P. ultimum* resulted in low levels of seedling disease severity. Although treatment of seed with benomyl, PCNB, vancomycin, or rifampicin at 1,000 ppm gave rise to seedlings with a significantly shorter shoot length than seedlings from nontreated seed, only rifampicin-treated seed (either 500 or 1,000 ppm) resulted in a complete loss of disease suppression (Fig. 2).

T-RFLP community profiling. Because rifampicin was the only inhibitor effective in eliminating *Pythium* seedling disease suppression without affecting *P. ultimum* pathogenesis or cucumber seedling development, we employed a T-RFLP analysis of 16S rRNA genes to compare seed-colonizing microbial communities that had been treated with rifampicin at 500 ppm for 3 h with those that had only been treated with water or those that were not treated. Microbial communities from seed germinated in sand were not analyzed.

Comparative T-RFLP peak profiles of 16S rRNA genes from suppressive and nonsuppressive seed-colonizing microbial communities (data not shown) revealed a wide diversity of distinct bacterial taxa, a number of which dominated seed surfaces within 8 h of sowing. There was a significant treatment effect as determined by our AMMI analysis (Table 1), and T-RF peak height and area explained more of the variation in the dataset than T-RF presence or absence (Fig. 3). Community profiles of non-

Fig. 2. Suppression of *Pythium* seedling disease on cucumber seedlings by seed-colonizing microbial communities in the presence of various microbial inhibitors. Cucumber seedlings grown in compost medium (gray bars) or in sterile sand (black bars) for 8 h. Seed were removed, treated with inhibitors for 1 h, then planted in sterile sand and inoculated with *Pythium ultimum*. Shoot heights were assessed 7 days later. Bars with the same letter are not significantly different according to Tukey's HSD test ($P < 0.05$).

treated and water-treated seed were similar, based on principle components analysis, whereas the community profile from rifampicin-treated seed was distinct (Fig. 3). Results from the ANOSIM analysis of T-RF presence or absence indicated that only the nontreated and rifampicin-treated communities differed significantly ($P = 0.0129$) (Table 2). However, the ANOSIM R value of 0.1228 for T-RF presence or absence indicated that the treatments are barely separable (58). In contrast, comparing communities using T-RF peak heights and peak areas with ANOSIM revealed that the seed-colonizing communities from all three treatments were distinct, with good overall separation (R values > 0.5 , all pairwise comparisons $P < 0.05$) (Table 2).

To arrive at the bacterial T-RFs representing taxa reduced or eliminated by the rifampicin treatment and likely to be involved in *Pythium* seedling disease suppression, we focused on comparisons between water-treated seed and rifampicin-treated seed. SIMPER analysis revealed those T-RFs that contributed the most to the observed differences between water-treated and rifampicin-treated communities. These communities had an overall community dissimilarity of 67.83 and 66.32 for peak height and peak area, respectively. This allowed us to focus the analysis only on T-RFs whose abundance in the community was most significantly impacted by treatment with rifampicin. Here, we report the top 34 T-RFs out of 367 total T-RFs in the dataset that explained $\approx 85\%$ of the treatment differences and whose abundance was either decreased or increased in the rifampicin treatment relative to the water treatment (Fig. 4). Similar results, with slight differences in T-RF rankings, were obtained with both peak height and peak area measures. Of those 34 T-RFs that changed the most in response to rifampicin treatment, 22 decreased in abundance whereas 12 were increased.

Taxon assignments based on the frequency of RDP database hits for each of the 34 T-RFs are shown in Table 3. Although the in silico analyses using MiCA gave rise to multiple taxa for each T-RF, each with distinct phylogenetic affiliations, many T-RFs aligned with the α -, β -, and γ -Proteobacteria whereas others had affinities to the Firmicutes, Bacteroidetes, Actinobacteria, Cyanobacteria, Chlorobi, Tenericutes, and Planctobacteria.

Bacterial clones from seed surfaces. A small sampling of 43 sequences from water-treated and rifampicin-treated seed revealed a similar assemblage of taxa as that determined by T-RFLP analysis. This level of sampling yielded a total of 21 OTUs, some of which were shared between the water treatment and rifampicin treatment. Many of the clones were singletons present predominantly in the water-treated samples (Fig. 5). As with the T-RFLP analysis, those clones present in the water treatment that were also eliminated by the rifampicin treatment were represented by taxa largely in the γ -Proteobacteria, with affinities to the genera *Enterobacter* and *Pseudomonas*.

TABLE 1. Additive main effects and multiplicative interaction model (AMMI) results from T-REX showing the percent variation and relative contributions of terminal restriction fragments (T-RFs) to variation in the data set^a

Effects	Variation (%)		
	T-RF presence or absence	T-RF peak height	T-RF peak area
Main effects			
T-RFs	74.00	69.34	68.35
Treatments	0.63	0.32	0.22
Interaction effects ^b			
Pattern	18.54	24.67	24.93
Noise	6.84	5.68	6.50

^a Analysis of variance was used to partition the variation into main effects and interactions. The AMMI model was then implemented in the T-REX software.

^b Higher percentages of interaction pattern reflect greater similarity among replicates within the same treatment.

DISCUSSION

A particular goal of this study was to provide a more direct means of understanding which microbes present in disease-suppressive composts could potentially contribute to disease

Fig. 3. Interaction principle components analysis (IPCA) of the additive main effects and multiplicative interaction (AMMI) analysis data matrix of bacterial terminal restriction fragments (T-RFs) from nontreated (squares), water-treated (circles), and rifampicin-treated (triangles) seed. A, T-RF presence or absence; B, T-RF peak height; and C, T-RF peak area.

suppression. Our overarching hypothesis was that the organisms most likely to contribute to compost-induced disease suppression would be those active in the infection court at a time when the pathogen is actively infecting the host. Of the diverse assemblages of microbes in composts (55), the vast majority should have little to do with disease suppression. Therefore, of the many microbial differences that potentially exist between bulk suppressive and nonsuppressive composts, relatively few would likely be related to disease-suppressive properties, making the detection of suppression-related differences quite difficult. An advantage of the *Pythium* spp.–cucumber pathosystem utilized in this study is that the infection court (i.e., the germinating seed) is well defined and the critical temporal availability of the infection court to pathogen infection (i.e., within 8 h of sowing) has been well established (11,47,74).

We demonstrated previously that compost microbes that colonize seed within 4 to 8 h after sowing can explain much of the suppressiveness of a municipal biosolids compost to *Pythium* seedling disease (11,47). Removal of the microbial consortia from seed surfaces through centrifugation eliminates disease suppression whereas treating seed with the microbes that were previously removed can restore disease suppression (11). Therefore, in this system, it is likely that interactions taking place at some distance from the plant have little significance to disease suppression. Instead, the microbial interactions and processes that contribute the greatest to *Pythium* seedling disease suppression occur on the seed surface and in the spermosphere and would also be expected to occur synchronously with plant infection by *P. ultimum*.

As a seed-infecting pathogen, *P. ultimum* responds rapidly to seed exudates. Sporangia are primarily responsive to long-chain unsaturated fatty acids released from imbibing seed where they germinate and initiate infections within a few hours after sowing (74). Other soil microbes also actively respond to the rapid efflux of exudate molecules from germinating seed and utilize many of the simple organic compounds that are released into the spermosphere within hours of sowing (74,75). These communities of fast-growing microbes have the greatest potential for interfering with pathogenesis because they not only must compete with *P. ultimum* for many of the same exudate resources (69) but also would need to be in an active growth phase for pathogen-suppressive processes such as antibiotic biosynthesis (57). Furthermore, fast-growing microbes present in composts have been shown previously to be more effective than slower-growing microbes in suppressing *P. ultimum* on cucumber root tips (5,67).

TABLE 2. Analysis of similarity (ANOSIM) among terminal restriction fragment (T-RF) presence or absence, peak height, and peak area in seed-colonizing communities from not-treated (NT), water-treated (W), and rifampicin-treated (R) seed^a

Variables	Mean rank of all distances		
	Presence or absence	Peak height	Peak area
Within treatments	161.1	107.9	111.4
Between treatments	182.6	206.3	204.7
R value	0.1228	0.5607	0.5316
P value ^b	0.0314	0.0001	0.0001

Treatments	Bonferroni corrected P values					
	Presence or absence		Peak height		Peak area	
	NT	R	NT	R	NT	R
R	0.0129	...	0.0117	...	0.0126	...
W	0.6975	0.9093	0.0003	0.0003	0.0003	0.0003

^a The ANOSIM function was used in the software package PAST v. 2.10 using Bray-Curtis distance measures. T-RF presence or absence, peak height, and peak area were identified using T-REX.
^b Based on 10,000 permutations of community membership.

The apparent necessity of rapid microbial growth to achieve *Pythium* disease suppression may also explain why the low fungal populations on seed during the early stages of seed germination are unlikely to contribute to disease suppression because the spores of many fungi require relatively long germination times to achieve only low germination rates in soils (66).

Our perturbation experiments with microbial inhibitors were an attempt to follow on from our previous strategy to better under-

Fig. 4. Differential abundance of terminal restriction fragments (T-RFs) comprising bacterial communities from seed treated with water (W) or rifampicin (R) ranked from left to right by the percent contribution to overall treatment differences. **A**, Differential abundances based on peak height. The 33 T-RFs collectively explained 85.23% of the treatment differences between water and rifampicin as determined by similarity percentage (SIMPER) analysis; 22 T-RFs were reduced (positive values) whereas 11 were enhanced (negative values) by the rifampicin treatment. **B**, Differential abundance based on peak area. The 24 T-RFs collectively explained 85.44% of the treatment differences between water and rifampicin as determined by SIMPER analysis; 16 T-RFs were reduced (positive values) whereas 8 were enhanced (negative values) by the rifampicin treatment.

stand which microbial taxa within the consortium of seed-colonizing microbes could be responsible for the observed disease suppression. Our previous findings that the seed-colonizing microbial community was directly related to *Pythium* disease suppression (11,47) prompted us to ask whether the selective removal of some members of the seed-colonizing microbial community using specific microbial inhibitors might also reduce or eliminate *Pythium* seedling disease suppression. If so, this should provide a more direct means of detecting those seed-colonizing microbes most likely to contribute to disease suppression, especially when the successional stage at which they are assessed corresponds with stages of pathogenic development and host infection by *P. ultimum*.

Our choice of rifampicin as a means of further targeting the specific subset of seed-colonizing microbes responsible for *Pythium* seedling disease suppression was based not only on its ability to reduce bacterial populations on seed and eliminate disease suppression but also on its lack of toxicity to cucumber seedlings and to sporangia of *P. ultimum*. Although inhibitors such as neomycin sulfate were especially effective at reducing seed-colonizing populations of bacteria, this inhibitor did not reduce or eliminate disease suppression, indicating that the bacterial taxa inhibited by neomycin were not involved in *Pythium* seedling disease suppression. On the other hand, rifampicin dramatically reduced *Pythium* disease suppression, even though the reduction in populations of seed-colonizing bacteria was not

TABLE 3. Primary phylogenetic affiliations of 34 terminal restriction fragments (T-RFs) that collectively contribute up to 85% of the differences between water-treated and rifampicin-treated seed-colonizing bacterial communities^a

T-RF (bp) ^b	Taxon name ^c	Phylum	Change in abundance in response to rifampicin ^d
79	<i>Tistrella</i> sp. (4) <i>Rhodobacter</i> sp. (1)	α-Proteobacteria α-Proteobacteria	Decreased
86	<i>Ralstonia solanacearum</i> (30) <i>Parabacteroides distasonis</i> (5)	β-Proteobacteria Bacteroidetes	Decreased
124	<i>Geodermatophilus</i> sp. (8) <i>Bordetella</i> sp. (2)	Actinobacteria β-Proteobacteria	Decreased
126	<i>Halomonas</i> sp. (65) <i>Spongiobacter</i> sp. (11) <i>Salinicola salarius</i> (5)	γ -Proteobacteria γ -Proteobacteria γ -Proteobacteria	Increased
148	<i>Bacillus</i> sp. (401) <i>Staphylococcus</i> sp. (52) <i>Listeria</i> sp. (47)	Firmicutes Firmicutes Firmicutes	Decreased
201	<i>Chryseobacterium</i> sp. (3) <i>Pantoea</i> sp. (1)	Bacteroidetes γ-Proteobacteria	Decreased
396	<i>Rhizobium leguminosarum</i> (1)	α-Proteobacteria	Decreased
399	<i>Rhizobium</i> sp. (1) <i>Ochrobactrum</i> sp. (1)	α-Proteobacteria α-Proteobacteria	Decreased
438	<i>Devosia</i> sp. (2) <i>Rhodospirillum rubrum</i> (1)	α -Proteobacteria α -Proteobacteria	Increased
439	<i>Ruegeria</i> sp. (22) <i>Rhodopseudomonas</i> sp. (18) <i>Magnetospirillum</i> sp. (17) <i>Paracoccus</i> sp. (16) <i>Rhodobacter</i> sp. (14)	α-Proteobacteria α-Proteobacteria α-Proteobacteria α-Proteobacteria α-Proteobacteria	Decreased
440	<i>Sulfitobacter</i> sp. (26) <i>Paracoccus</i> sp. (18) <i>Rhodobacter</i> sp. (15)	α-Proteobacteria α-Proteobacteria α-Proteobacteria	Decreased
453	<i>Stenotrophomonas</i> sp. (31) <i>Xanthomonas</i> sp. (9)	γ-Proteobacteria γ-Proteobacteria	Decreased
454	<i>Stenotrophomonas</i> sp. (15) <i>Rhodobacter</i> sp. (3)	γ -Proteobacteria α -Proteobacteria	Increased
482	<i>Francisella</i> sp. (10) <i>Pseudomonas</i> sp. (2)	γ-Proteobacteria γ-Proteobacteria	Decreased
483	<i>Phytoplasma</i> sp. (19) <i>Francisella</i> sp. (7)	Firmicutes γ-Proteobacteria	Decreased
484	<i>Francisella</i> sp. (42) <i>Alkaliphilus</i> sp. (20)	γ -Proteobacteria Firmicutes	Increased
485	<i>Elizabethkingia</i> sp. (12) <i>Riemerella</i> sp. (9) <i>Chryseobacterium</i> sp. (9) <i>Teredinibacter</i> sp. (8)	Bacteroidetes Bacteroidetes Bacteroidetes γ -Proteobacteria	Increased

(continued on next page)

^a T-RFs whose peak heights or peak areas were significantly reduced in the presence of rifampicin at 500 ppm (conditions that eliminate disease suppression) as compared with a water control. Taxa in bold represent those likely involved in *Pythium* seedling disease suppression.

^b T-RFs from *MspI* digests of cDNA from compost-derived seed-colonizing microbial communities.

^c Numbers in parentheses following each taxon name represent the number of hits to that taxon in the Ribosomal Database Project database (R10, Update 26) of over 1.5 million bacterial 16S rDNA sequences. Hits to uncultured or unidentified taxa were removed from the taxonomic assignments.

^d Increase = T-RF was more abundant in communities from seed treated with rifampicin at 500 ppm than in communities from seed treated with water; decrease = T-RF was less abundant in communities from seed treated with rifampicin at 500 ppm than in communities from seed treated with water.

as great as with neomycin. This further suggests that the suppression of *Pythium* seedling diseases in this municipal solid waste compost is induced by very specific assemblages of bacteria and does not result from a general suppression phenomenon (37), commonly associated with *Pythium* disease suppression. Our results with rifampicin treatments also provide more evidence that the suppression of *Pythium* seedling disease is expressed at the time and site where *P. ultimum* infection occurs. Similar studies to understand how suppressive soils reduce *Rhizoctonia solani* infections of sugar beet seedlings demon-

strated clearly through the combined use of microbial inhibitors and transplant experiments that, unlike with *Pythium* spp., disease suppression did not occur in the spermosphere but in the laimosphere (i.e., hypocotyl), which is the infection site for *R. solani* (27).

From the perturbation of seed-colonizing bacterial communities with rifampicin, we predicted three possible microbial responses that would influence suppressiveness: (i) particular taxa or groups of taxa would be eliminated or reduced in relative abundance, which would directly translate into a reduction or elimination of

TABLE 3. (continued from preceding page)

T-RF (bp) ^b	Taxon name ^c	Phylum	Change in abundance in response to rifampicin ^d
486	<i>Cellvibrio</i> sp. (2) <i>Glaciecola</i> sp. (3) <i>Crenothrix</i> sp. (1)	γ-Proteobacteria γ-Proteobacteria Bacteroidetes	Decreased
487	<i>Crenothrix</i> sp. (7) <i>Cellvibrio</i> sp. (7) <i>Comamonas</i> sp. (5) <i>Pseudomonas</i> sp. (4)	Bacteroidetes γ-Proteobacteria β-Proteobacteria γ-Proteobacteria	Increased
488	<i>Thiomonas</i> sp. (158) <i>Pseudoalteromonas</i> sp. (140) <i>Acidovorax</i> sp. (56) <i>Acidithiobacillus</i> sp. (31) <i>Delftia</i> sp. (30)	β-Proteobacteria γ-Proteobacteria β-Proteobacteria γ-Proteobacteria β-Proteobacteria	Decreased
489	<i>Pseudomonas</i> sp. (19) <i>Pseudoalteromonas</i> sp. (9)	γ-Proteobacteria γ-Proteobacteria	Decreased
490	<i>Pseudomonas</i> sp. (865) <i>Pseudoalteromonas</i> sp. (370) <i>Achromobacter</i> sp. (49) <i>Variovorax</i> sp. (35)	γ-Proteobacteria γ-Proteobacteria γ-Proteobacteria γ-Proteobacteria	Increased
491	<i>Acinetobacter</i> sp. (158) <i>Pseudomonas</i> sp. (32) <i>Synechococcus</i> sp. (34)	γ-Proteobacteria γ-Proteobacteria Cyanobacteria	Increased
492	<i>Pseudomonas</i> sp. (111) <i>Herbaspirillum</i> sp. (21) <i>Acinetobacter</i> sp. (18)	γ-Proteobacteria γ-Proteobacteria γ-Proteobacteria	Increased
493	<i>Pseudomonas</i> sp. (84) <i>Klebsiella</i> sp. (55) <i>Marinomonas</i> sp. (47) <i>Enterobacter</i> sp. (39) <i>Pantoea</i> sp. (32)	γ-Proteobacteria γ-Proteobacteria γ-Proteobacteria γ-Proteobacteria γ-Proteobacteria	Decreased
494	<i>Klebsiella</i> sp. (55) <i>Marinomonas</i> sp. (45) <i>Enterobacter</i> sp. (43) <i>Pantoea</i> sp. (32) <i>Halomonas</i> sp. (25)	γ-Proteobacteria γ-Proteobacteria γ-Proteobacteria γ-Proteobacteria γ-Proteobacteria	Decreased
495	<i>Haemophilus</i> sp. (42) <i>Salmonella</i> sp. (15) <i>Shigella</i> sp. (10) <i>Klebsiella</i> sp. (7)	γ-Proteobacteria γ-Proteobacteria γ-Proteobacteria γ-Proteobacteria	Increased
534	<i>Chlorobaculum</i> sp. (6)	Chlorobi	Increased
535	<i>Chlorobium limicola</i> (1) <i>Mycoplasma wenyonii</i> (1) <i>Kordiimonas</i> sp. (1)	Bacteroidetes Tenericutes α-Proteobacteria	Increased
537	<i>Spiroplasma</i> sp. (2) <i>Pseudomonas</i> sp. (1) <i>Clostridium</i> sp. (1)	Tenericutes γ-Proteobacteria γ-Proteobacteria	Decreased
538	<i>Spiroplasma</i> sp. (29) <i>Gelidibacter</i> sp. (4)	Tenericutes Bacteroidetes	Decreased
539	<i>Spiroplasma</i> (18) <i>Gelidibacter</i> sp. (2)	Tenericutes Bacteroidetes	Decreased
599	<i>Planctomyces</i> sp. (3) <i>Pseudomonas</i> sp. (1)	Planctobacteria γ-Proteobacteria	Decreased
600	<i>Pseudomonas</i> (2) <i>Methylomonas</i> (1) <i>Nitrospira</i> (1)	γ-Proteobacteria γ-Proteobacteria γ-Proteobacteria	Decreased

Pythium seedling disease suppression; (ii) even though disease suppression was eliminated, particular taxa or groups of taxa would increase following rifampicin treatment because of their resistance to rifampicin; or (iii) if disease suppression results from bacteria–bacteria interactions that lead only to indirect interactions with *P. ultimum*, the elimination or reduction of one or a few of these cooperative bacterial taxa by rifampicin may not necessarily alter disease suppression, making it very difficult to determine the specific taxa that contribute to disease suppression. Although studies with combinations of individual microbes have demonstrated the possibility of this scenario (19,21,35), we have no evidence for such interactions based on comparisons of the relative abundance of T-RFs between water and rifampicin treatments in this study.

Following our rifampicin treatment, T-RFs were either reduced or enhanced in frequency as predicted. Again, the factors that contributed to the enhancement of some taxa are not entirely clear but may simply represent rifampicin-resistant strains that were able to proliferate in the presence of the rifampicin treatment because of increased niche availability upon the inhibition of rifampicin-sensitive bacteria. It is unlikely that this enhancement represents strains that proliferated once the rifampicin selection was removed because there was insufficient time for significant growth following the removal of the inhibitor. If the T-RFs that were increased represent rifampicin-resistant strains, it is unlikely that these taxa are involved directly in Pythium seedling disease suppression because, in the presence of rifampicin, suppression was eliminated. This does not rule out the possibility of their indirect associations with disease suppression but, again, this would be very difficult to resolve and our study was not designed to address these indirect interactions adequately.

Therefore, those T-RFs believed to be most likely involved in Pythium seedling disease suppression are represented by the 22 T-RFs reduced by the rifampicin treatment. Of those 22 T-RFs, none were present on seed treated with rifampicin that were not also present on seed treated with water. Based on the relative contributions of the different T-RFs to the rifampicin effects (from our SIMPER analysis), we would conclude that T-RFs 482 (γ -Proteobacteria), 483 (Firmicutes), 486 (γ -Proteobacteria), 489 (γ -Proteobacteria), and 539 (Tenericutes) would play the greatest role in disease suppression. Many of the remaining T-RFs have affinities to α -, and β -Proteobacteria. Although the predominance of α -, β -, γ -Proteobacteria, and Firmicutes would be predicted based on other observations of spermosphere bacteria (47,54,65), it is likely that other compost sources give rise to different seed-colonizing consortia. For example, Green et al. (30) found that microbial communities associated with cucumber seed that were germinated in manure-based composts were dominated by the bacterial phyla Bacteroidetes, with fewer β -Proteobacteria, and only a low frequency of α - and γ -Proteobacteria. This suggests that seed-associated communities are dictated, in part, by the composition of the compost communities but also by plant-specific effects (31) that may ultimately influence the level and spectrum of disease suppression. The importance of the plant in recruiting disease-suppressive microbes from the soil (28) lends further support to our strategy of focusing on the infection court as a target site for observing disease-suppressive microbial interactions and, therefore, the site for community analyses to determine which microbes are involved in disease suppression in soils or composts.

Of the major phyla of rifampicin-sensitive bacteria in the cucumber infection court, the γ -Proteobacteria and Firmicutes are composed of many bacterial genera that are known to suppress *Pythium* spp., including *Pseudomonas* (γ -Proteobacteria) (49), *Enterobacter* (γ -Proteobacteria) (43), *Stenotrophomonas* (γ -Proteobacteria) (24), and *Bacillus* spp. (Firmicutes) (48). Few species within the α -Proteobacteria have been described as being suppressive to plant pathogens, yet they are well-known plant

mutualists (34). Much of our current understanding of the mechanisms by which plant-associated microbes suppress infections of plants by pathogens has come largely from studies of the γ -Proteobacteria and Firmicutes (15,41). Elucidating the processes involved in the suppression of Pythium seedling disease by these groups of seed-colonizing bacteria requires further work. However, communities of these taxa may carry out many of the same processes that have already been demonstrated to be important to the suppression of *Pythium* spp. infections of seed, such as antibiotic biosynthesis (57), the metabolism of exudates molecules required for *P. ultimum* pathogenic development (74), or competition for carbon or other resources in the spermosphere (25,38). We recently observed that these same seed-colonizing microbial consortia, in addition to preventing very early stages of plant infection, may also compete with *P. ultimum* for general carbon compounds, resulting in the abortion of sporangium germ tubes subsequent to initial plant responses and, thus, suppressing further infection (12).

In summary, the results of our study have confirmed that Pythium disease suppression in a municipal biosolids compost is mediated by compost-associated bacteria that colonize seed within hours after sowing. By focusing on actively growing microbes in the infection court during important stages of pathogen infection, we believe we can more efficiently determine the mechanisms of diseases suppression and the microbes involved. This is especially important given that seed microbial communities are often quite different than those associated with roots or bulk soils (30,53,54) and, often, the compost-inhabiting taxa that colonize seed and roots do so in a very host-specific and compost-specific manner (31). Therefore, no single microbial consortium dominated by a fixed set of taxa will be found that can explain disease suppression. Rather, each consortium is likely to be very context (host, pathogen, soil, or substrate) dependent. Our results, although specific to this pathosystem and compost, have a much broader scope of inference and illustrate the utility of such a targeted approach in identifying a relatively small subset of microbial taxa from complex communities likely to be involved in disease suppression. A better understanding of the interactions of

Fig. 5. Distribution of operational taxonomic units (OTUs) between water-treated seed and rifampicin-treated seed. Taxon designations represent the best BLAST hits from the National Center for Biotechnology Information GenBank database. OTUs were determined using the software packages DOTUR and SONS. OTUs marked by an asterisk (*) represent those eliminated from seed-colonizing consortia by the rifampicin treatment.

these microbes with the pathogen, the host, and other microbes will greatly facilitate our understanding of the mechanisms underlying disease-suppression processes.

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