Applied and Environmental Microbiology

Streptomycin Application Has No Detectable Effect on Bacterial Community Structure in Apple Orchard Soil

Ashley Shade, Amy K. Klimowicz, Russell N. Spear, Matthew Linske, Justin J. Donato, Clifford S. Hogan, Patricia S. McManus and Jo Handelsman

Appl. Environ. Microbiol. **2013, 79(21):6617. DOI: 10.1128/AEM.02017-13.**
Published Ahead of Print 23 August 2013.

AEM
Journals.ASM.org

Streptomycin Application Has No Detectable Effect on Bacterial Community Structure in Apple Orchard Soil

Ashley Shade,[a] Amy K. Klimowicz,[b] Russell N. Spear,[b] Matthew Linske,[c] Justin J. Donato,[c,d] Clifford S. Hogan,[c] Patricia S. McManus,[b] Jo Handelsman[a]

Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut, USA[a]; Department of Plant Pathology, University of Wisconsin—Madison, Madison, Wisconsin, USA[b]; Department of Bacteriology, University of Wisconsin—Madison, Madison, Wisconsin, USA[c]; Department of Chemistry, University of St. Thomas, St. Paul, Minnesota, USA[d]

Streptomycin is commonly used to control fire blight disease on apple trees. Although the practice has incited controversy, little is known about its nontarget effects in the environment. We investigated the impact of aerial application of streptomycin on nontarget bacterial communities in soil beneath streptomycin-treated and untreated trees in a commercial apple orchard. Soil samples were collected in two consecutive years at 4 or 10 days before spraying streptomycin and 8 or 9 days after the final spray. Three sources of microbial DNA were profiled using tag-pyrosequencing of 16S rRNA genes: uncultured bacteria from the soil (culture independent) and bacteria cultured on unamended or streptomycin-amended (15 μg/ml) media. Multivariate tests for differences in community structure, Shannon diversity, and Pielou's evenness test results showed no evidence of community response to streptomycin. The results indicate that use of streptomycin for disease management has minimal, if any, immediate effect on apple orchard soil bacterial communities. This study contributes to the profile of an agroecosystem in which antibiotic use for disease prevention appears to have minimal consequences for nontarget bacteria.

Antibiotic use on crops is controversial, although it represents less than 0.5% of the total antibiotic use in the United States (1). The controversy stems in large part from the lack of knowledge about the impact of antibiotics used in plant agriculture on development of resistance among human pathogens (1, 2). Resistant bacteria and resistance genes appear to flow between animals and the humans who care for them (3–7), and there are various routes by which antibiotics used on plants could influence resistance in bacteria that inhabit or infect humans. For example, antibiotics applied to plants could change the antibiotic resistance gene profile in soil, which has long been regarded as a reservoir of antibiotic resistance genes (8). Furthermore, antibiotics could alter nontarget microbial communities by killing some sensitive taxa, thereby selecting for resistant taxa, which could ultimately alter the microbial community diversity or structure. Growing evidence that indicates the critical role of native microbiota in the health of hosts and of the planet (see, e.g., references 9 and 10) calls for assessment of the impact of antibiotics on nontarget microorganisms in cropping systems.

Application of antibiotics is a standard practice to prevent fire blight, the most important bacterial disease of apple, pear, and related ornamental plants in the Rosaceae family. The disease is caused by the Gram-negative, enteric bacterium *Erwinia amylovora*, which overwinters in stem cankers, multiplies at the canker margins in the spring, and is disseminated to flowers by rain splash (11, 12) and pollinating insects (13). *E. amylovora* first colonizes the stigma of flowers and then invades the primary site of infection, the nectary (14, 15). The pathogen then invades shoots and roots, which in turn leads to the loss of large branches and entire trees. Nearly all pear varieties and many apple varieties and rootstocks with desirable horticultural characteristics are susceptible to fire blight. Therefore, to prevent outbreaks, many orchardists apply streptomycin (StrR) (and, to a lesser extent, oxytetracycline) to trees with newly opened flowers one to five times per season (1). A 2002 paper estimated that 20% of apple acres and 30% to 40% of pear acres were treated with streptomycin (1). A U.S. Department of Agriculture report from 2007 stated that 12% of bearing acres of apple are sprayed in the major apple-producing states (including California, Michigan, New York, North Carolina, Oregon, Pennsylvania, and Washington; 16). Currently, streptomycin remains the most effective chemical treatment available for fire blight prevention (1).

To apply streptomycin, antibiotic salts are dissolved in water and then blown into tree canopies using an air blast sprayer. As the mist settles, nontarget plants, animals, and soil are exposed to antibiotics (17), although the extent or impact of this exposure is not known. Therefore, the apple orchard ecosystem provides an opportunity to determine what impact antibiotic application on trees has on the nearby soil microbial community.

Streptomycin application reportedly has little or no effect on the proportion of streptomycin-resistant bacteria in soils and other environmental samples (18, 19) and a minimal effect on the proportion of streptomycin-resistant soil streptomycetes (20). In these studies, however, effects of streptomycin on bacterial community membership and diversity were not assessed. Studies of other types of antibiotic resistance in soil have reported increases (21), decreases (22), or no change (23), suggesting that the spread of antibiotic resistance in soils is complex and dependent on multiple biological and environmental factors (24). In the few studies that investigated the impact of antibiotics on soil bacterial com-

Received 20 June 2013 **Accepted** 15 August 2013
Published ahead of print 23 August 2013
Address correspondence to Jo Handelsman, jo.handelsman@yale.edu.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02017-13.

doi:10.1128/AEM.02017-13

munity structure, molecular fingerprinting tools were used that did not have the resolution to determine the taxonomic affiliation of community members (see, e.g., references 21, 25, 26, 27, and 28). Most of these fingerprinting studies found that, though the membership of the community did not always change after exposure to antibiotics, the intensity of the signals (for instance, denaturing gradient gel electrophoresis [DGGE] bands or terminal restriction fragment length polymorphism [T-RFLP] peaks, representing bacterial operational taxonomic units) often changed (24). Furthermore, there is evidence that antibiotics alter the functional profile of soil microbial communities, which may be linked to community membership and structure (29). Therefore, the effects of agricultural antibiotics on nontarget, environmental microbial communities remain unclear. The effects of antibiotics on soil microbial community membership and structure, including the taxonomic affiliations of community members, is a particularly understudied area.

We posed the hypothesis that streptomycin applied to apple tree canopies for fire blight management changes microbial community structure by reducing diversity and increases the proportion of streptomycin-resistant bacteria in the soil bacterial community. To test this hypothesis, we directly compared bacterial communities in soils beneath four streptomycin-treated and four untreated apple trees (*Malus domestica*) over two growing seasons in a commercial apple orchard. From each of these trees, DNA profiling was accomplished with DNA extracted directly from the soil (culture-independent sample) or from microorganisms cultured on rhizosphere isolation medium (RIM; 30) or from microorganisms cultured on RIM amended with streptomycin. There was no evidence that spraying trees with streptomycin changed the composition or structure of soil bacterial communities in the orchard soil sampled within 9 days of streptomycin application.

MATERIALS AND METHODS

Study site, experimental design, and sample collection. The study was conducted in 2008 and 2009 at a diversified fruit and vegetable farm in southern Wisconsin (42°58′N, 89°28′W) containing an 8-hectare apple orchard. Fire blight had been an ongoing problem, and the grower had applied streptomycin two to three times in spring since 1997.

Four trees were selected from each of two rows of different apple cultivars. One row was planted with the fire blight-susceptible cultivar "Gala" grafted to M.9 rootstock and was directly sprayed with streptomycin (designated "treated"). A row 74 m uphill from the first contained the relatively resistant cultivar "Delicious" grafted to M.9 rootstock and was not sprayed with streptomycin (designated "untreated"). According to the orchard owner and manager, these untreated trees had never been sprayed with streptomycin in past growing seasons. Prior to streptomycin application, on 8 May 2008 and 1 May 2009, soil was collected from beneath the eight trees for soil chemistry and texture analyses, which were performed by the University of Wisconsin—Madison Soil and Plant Analysis Laboratory according to standard protocols (http://uwlab.soils.wisc.edu/madison/). One soil core 10 cm in diameter and 5 cm deep was collected under the drip-line at the edge of the canopy on each of the north and south sides of trees, totaling two cores per tree. Soil was homogenized in the collection bags by hand mixing. Samples from four treated trees were paired with samples from four untreated trees that had similar soil textures (percent sand, silt, and clay). These samples were designated "prespray." Immediately after the first apple flowers opened, the grower applied plant-grade streptomycin formulated as a powder containing 21% streptomycin sulfate (Agri-mycin17; Nufarm Americas, Inc., Burr Ridge, IL) (163 ppm in 2008 and 245 ppm in 2009) dissolved in water and delivered to the trees at a pressure of 2 MPa (250 to 300 lb/in^2). The trees were sprayed twice during flower bloom on 12 and 18 May 2008 and on 11 and 14 May 2009. Approximately 2 weeks after the first spray, on 27 May 2008 and 22 May 2009, postspray soil samples were collected.

To visualize the amount of spray that reached the orchard floor, water-sensitive cards (Spraying Systems Co., Wheaton, IL) that changed color when wet were placed below trees prior to spraying in spring 2007, when the grower sprayed in a manner similar to that described above. Visual inspection of the cards indicated that some of the streptomycin sprayed on trees reached the soil and that streptomycin drift from treated trees to untreated trees was minimal (see Fig. S1 in the supplemental material).

Culturing of bacteria. A 5-g subsample of soil was removed from each homogenized soil core, suspended in 25 ml sterile MilliQ water, shaken for 1 min, and sonicated in a bath sonicator (Model 2210; Branson, Danbury, CT) as described previously (31). Duplicate serial dilutions (10^{-3}, 10^{-4}, and 10^{-5}) were cultured on RIM (30) with or without streptomycin (15 mg/ml). RIM is a dilute medium containing 15 amino acids selected to inhibit the growth of *Bacillus mycoides*, a common soil inhabitant that spreads rapidly across agar surfaces, thereby interfering with culturing other members of the community (30). We modified RIM by replacing the nystatin with 100 μg/ml cycloheximide to inhibit fungal growth. Plates were incubated at ambient temperature for 6 days before enumeration of CFU. For each tree, colonies from the north and south dilution plates were scraped, pooled by soil core (a total of 8, 10, or 12 plates per pool, omitting plates that were overgrown), and then homogenized by mixing with a spatula and sonicating. The samples were stored in 10% glycerol at −80°C. Bacteria cultured on RIM with and without streptomycin are referred to as "cultured" and "StrR cultured," respectively. The frequency of streptomycin resistance was determined by dividing the total number of CFU per gram of soil on RIM plates amended with streptomycin by the total number of CFU per gram of soil on RIM plates that did not contain streptomycin.

DNA extraction and barcoded pyrosequencing. To obtain a culture-independent profile of the bacterial communities, metagenomic DNA was isolated directly from the soil within 6 h of collection. Extraction was accomplished with a Power Soil kit (MoBio, Carlsbad, CA) with published modifications (32), and DNA was purified by phenol-chloroform/isoamyl alcohol extraction followed by ethanol precipitation. Profiles of metagenomic DNA extracted directly from the soil are referred to as "culture independent." To obtain a profile of the cultured bacterial communities, genomic DNA was isolated from 75 to 100 μl of the frozen cell suspension (described above) using the GenElute bacterial genomic DNA kit (Sigma, St. Louis, MO) protocol for Gram-positive bacteria. Prior to addition of lysozyme solution, the cells were vortexed for 4 min with 50 μl of 0.1 mm zirconia silica beads to aid in cell lysis.

Barcoded pyrosequencing was performed on 96 samples (4 trees × 2 spray treatments × 2 sampling times × 3 sample types × 2 years) on a 454 Life Sciences FLX Genome Sequencer (454 Life Sciences Roche, Branford, CT) using the prokaryotic 16S rRNA gene primers 515 and 806 to target the V3 and V4 variable regions. Sequence quality control was performed using the QIIME (version 1.2.0) default parameters, which included a minimum quality score of 25, a minimum sequence length of 200, and a maximum length of 1,000, and no ambiguous or no primer mismatches were permitted (33). Operational taxonomic units (OTUs) were assigned based on 97% sequence identity using uclust (34), and the most abundant sequence within each OTU was used as the representative for alignment and taxonomic assignment. Alignments were performed using PyNAST (35), and taxonomy was assigned using the RDP Classifier with 0.8 minimum confidence and E value minima of 0.001 (36). To be conservative and avoid potential pyrosequencing biases that lead to an overestimate of richness (see, e.g., reference 37), taxa that were observed only once across the entire data set (singletons) were removed before community analyses were performed (38), and all samples were relativized prior to multivariate analyses. The quality-controlled data set (QIIME's split-library output) is available through MG-RAST (metagenomics rapid annotation using subsystem technology; project identification [ID] no. mgp2603).

Multivariate analyses. After singleton removal, species accumulation curves were calculated in PRIMER v6 software (39) with 999 permutations, and rarefied richness was calculated using the QIIME alpha_rarefaction.py script with 10 samplings of the data set for every 20 steps from 50 to the minimum number of OTUs observed in any community for each of the culture-independent, cultured, and StrR-cultured groups.

Differences in untreated and treated soil characteristics (phosphorus and potassium content, percent organic matter, and texture) were identified using analysis of similarity on Euclidean distances of normalized environmental data in PRIMER. Welch's *t* test was used *post hoc* to determine exactly which chemistry measurements were different between untreated and treated soils. Variation in the bacterial communities was related to soil chemistry using a Mantel test, with Euclidean distance used for the soil analysis and Bray-Curtis for the bacterial communities. These tests were performed in the R environment for statistical computing (40).

Nonmetric multidimensional scaling (NMDS) analysis was performed with PRIMER, using Bray-Curtis similarity. Additionally, Hellinger distances based on relative abundances and Sørenson distances based on presence or absence were used for NMDS analysis; these analyses resulted in the same overall ordination patterns. Therefore, only Bray-Curtis results are shown for NMDS. Pielou's evenness and Shannon diversity were calculated using PRIMER.

The vegan package (41) in R was used to test for differences in bacterial community structure across treatments. Four resemblance metrics were chosen to test for differences in community structure: Bray-Curtis, modified Gower index with a log base 10 transformation (42), Sørenson's, and Morisita-Horn. Some of the properties of these metrics are discussed in reference 43 and are discussed in more detail in reference 44. These four metrics were chosen because they emphasize complementary aspects of the community. The Bray-Curtis emphasizes changes in prevalent members (those OTUs in high relative abundance) and has been favored in microbial ecology because it is robust in analyses of data sets from species-rich communities in which many of the members are rare and therefore absent from many samples. Sørenson's is analogous to Bray-Curtis but considers only the presence or absence of taxa; thus, prevalent and rare taxa are indistinguishable in the analysis. The modified Gower emphasizes fold changes in taxon abundances between communities. For example, the modified Gower index log base 10 would weight a rare OTU that experiences a log 10-fold difference between communities similarly to a prevalent OTU that has the same fold difference. The Morisita-Horn metric of overlap emphasizes differences in the most prevalent taxa (45). The utility of the Morisita-Horn index for microbial ecology remains unclear; some find that it performs well (see, e.g., reference 46), but others find that it does not consistently reveal clustered or gradient community patterns (47). We include the Morisita-Horn index because it is a common resemblance metric and it was important that our study could be directly compared with other studies that have used this metric.

Four hypothesis tests were used to determine differences in various aspects of community structure, including the centroid (mean) and dispersion around the centroid (spread). In these tests, all pairwise group comparisons are performed, resulting in a global test statistic. When the global test statistic is significant, there is a difference between the results from at least two of the groups, and then the pairwise tests reveal precisely which groups exhibit differences. All tests were performed using the vegan package in the R environment, and each test was permuted 999 times. The analysis of similarity (ANOSIM; 39), multiple-response permutation procedure (MRPP; 48), and permuted analysis of variance (PERMANOVA; 49) test for differences in either the centroid or the spread among communities. ANOSIM is based on rank-order distances and effectively uncovers apparent differences in clusters. ANOSIM returns an R statistic that indicates the degree of difference between groups, where a value of 1 indicates that no members are shared (i.e., communities are completely different), and values closer to 0 indicate that communities are identical. MRPP uses original resemblances and thus is more sensitive to subtle differences across experimental groups. MRPP returns a delta value representative of the observed versus permuted within-group distances. PERMANOVA is an analysis-of-variance analog for multivariate data and returns an R^2 with a Monte Carlo-permuted P value. PERMANOVA was performed using the adonis function in the vegan package. Finally, permutated analysis of dispersion (PERMDISP; 50) was used to test whether there were differences in spread (dispersion) among communities. PERMDISP was performed using the betadisper function in the vegan package. If significant differences among groups were detected with any global ANOSIM, MRPP, PERMANOVA, or PERMDISP test, pairwise *post hoc* tests were performed to determine specifically which experimental groups were distinguishable.

To investigate the influence of the less abundant OTUs that may have been masked by the more prevalent OTUs, data sets that included only the 65% least-abundant OTUs (as suggested in reference 51 and informed by rank abundance curves) were created. Summative relative abundances (across all samples) were used for ranking OTUs, and then the most abundant 35% were removed.

RESULTS

Pyrosequencing summary. Ninety of 96 soil samples yielded 16S-tag sequences that met our quality control standards. The data set included 141,884 recovered good-quality tag sequences, with an average length of 370 bp (minimum of 200, maximum of 512). An average of 1,493 OTUs (97% sequence identity level) were recovered per observation (minimum of 407, maximum of 3,813). Rarefied richness and species accumulation curves revealed that our field-sampling efforts (number of soil samples collected) were exhaustive for all samples and that the sequencing effort (number of sequences obtained) was not exhaustive for the culture-independent samples (see Fig. S2 in the supplemental material). A total of 14,624 OTUs were observed over the data set; of these, 8,372 (~57%) OTUs were singletons, and 6,252 OTUs remained after omitting singletons.

Community variation across location and years. To reveal trends that were unrelated to streptomycin that could influence interpretation of results, we paired samples with similar texture and chemistry characteristics from treated and untreated soil and found few differences in soil traits (see Table S1 in the supplemental material; ANOSIM $P = 0.11$). However, there were differences in phosphorus and potassium levels between soils from untreated and treated trees ($P = 0.02$ and 0.01, respectively), but there was no relationship between the chemistry and the bacterial communities observed in the same soil (Mantel $P = 0.56$). These analyses affirmed that there were no differences in measured soil chemistry that were important in explaining differences in community structure. There also were no differences in bacterial community structure between the 2008 and 2009 growing seasons (ANOSIM $P = 0.23$).

Community differences across culture-based and culture-independent methods. Our observations were based on three types of samples: metagenomic DNA from the soil (culture independent; $n = 26$); DNA from isolates cultured on RIM (cultured; $n = 32$), and DNA from isolates cultured on RIM amended with streptomycin (streptomycin resistant, StrR cultured; $n = 32$). The most striking differences detected were between groups of samples as follows: for the culture-independent group versus the cultured group, ANOSIM $R = 1$; for the culture-independent group versus the StrR-cultured group, ANOSIM $R = 1$; and for the cultured group versus the StrR-cultured group, ANOSIM $R = 0.81$ (all $P < 0.001$) (Fig. 1A). Though the expectation was that the culture-based samples would represent subsets of the culture-indepen-

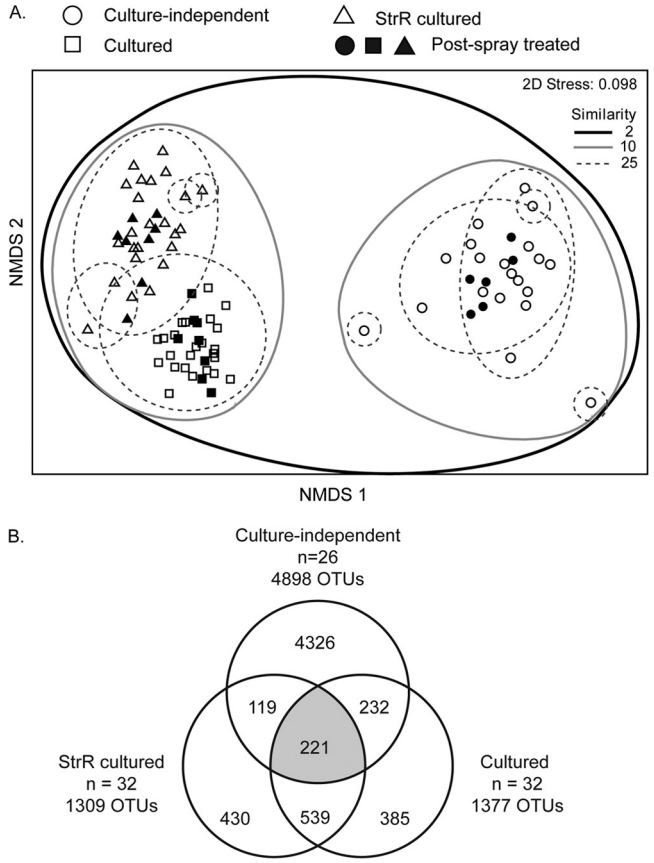

FIG 1 Differences between culture-independent, cultured, and StrR-cultured community samples. (A) Nonmetric multidimensional scaling analysis of orchard soil bacterial communities, based on ranked Bray-Curtis similarities. The large circles indicate the minimum shared similarity within the surrounded communities, as referenced in the "Similarity" key. All communities were within 2% Bray-Curtis similarity. Filled symbols represent the postspray treated samples, which cluster with the postspray untreated and all prespray samples, indicating no effect of streptomycin on community structure. (B) Venn diagram of overlapping memberships among culture (with streptomycin [StrR cultured] or without streptomycin [Cultured])-dependent and culture-independent groups. The shading indicates that 221 OTUs were shared among all three groups. 2D, two dimensional.

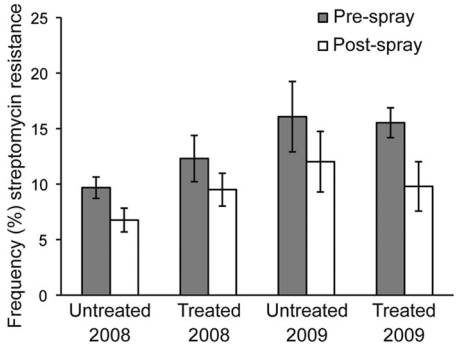

FIG 2 Frequency of streptomycin-resistant (15 mg/liter) isolates from soil collected below untreated and treated trees before (prespray) and after (postspray) streptomycin application. The frequency of resistance was assessed by dividing the total number of CFU per gram of soil on RIM plates amended with streptomycin by the total number of CFU per gram of soil on RIM plates that were not amended with streptomycin. Error bars represent standard deviations around the means of the results obtained with eight independent soil samples (one north and one south sample collected beneath each of four trees). There were no differences between prespray and postspray communities for either the untreated or treated soils (all $P > 0.05$).

dent group, each instead contained a larger proportion of unique OTUs than shared OTUs (Fig. 1B), affirming the results of a previous comparison of the culture-based and culture-independent OTUs (38). Also, the StrR-cultured community was not a complete subset of the cultured community, although 58% and 55% of the OTUs in the StrR-cultured and cultured communities, respectively, were shared between the two groups (Fig. 1B). Because of these large differences between culture-independent, cultured, and StrR-cultured samples, all subsequent tests for differences between prespray and postspray communities were performed independently for each group of samples.

Effect of streptomycin on soil community diversity. There were no differences in the frequency of streptomycin-resistant isolates between treated and untreated soils (Fig. 2). There was a general trend of a lower frequency of resistance after streptomycin spraying. Similarly, we found no differences in Pielou's evenness (equitability of taxa) or Shannon diversity before and after spraying (Fig. 3). One exception was in the culture-dependent samples, in which communities of soil under treated trees sampled after spraying were slightly less even in their diversity than before spraying ($P = 0.03$).

We performed four hypothesis tests to assess whether there were global differences in community structure between groups (prespray untreated, prespray treated, postspray untreated, and postspray treated soil communities; Table 1). We performed these hypothesis tests on each of four community resemblance metrics. These resemblance metrics were chosen because each emphasized a different aspect of community structure (see Materials and Methods for details). The analyses of culture-based samples revealed more differences between communities in the prespray and postspray samples than the culture-independent analyses (Table 1). However, the Morisita-Horn metric and the PERMDISP test each revealed no differences for any group of samples. The remaining three tests, MRPP, ANOSIM, and PERMANOVA, generally were consistent with each other in detecting differences. Because these three tests detect differences in both the centroid and the spread, while PERMDISP detects only differences in the spread, we concluded that experimental groups were distinct because they had different centroids rather than different dispersions.

In cases in which the global test revealed significant differences among experimental groups, *post hoc* tests were performed to determine specifically which communities were distinct from one another. Four representative *post hoc* tests for the MRPP test are given in Table 2. All other results are available (see Tables S2 to S6 in the supplemental material).

The differences among communities could not be attributed to use of streptomycin. If there were an effect of streptomycin, the postspray treated community should have differed from all other communities. However, this was not the case (Table 2; see also Tables S2 to S6 in the supplemental material). Consider, for example, the tests using the modifier Gower metric for the culture-dependent communities (Table 2): both the prespray untreated and the prespray treated samples differed from the postspray treated samples. However, the postspray untreated and postspray

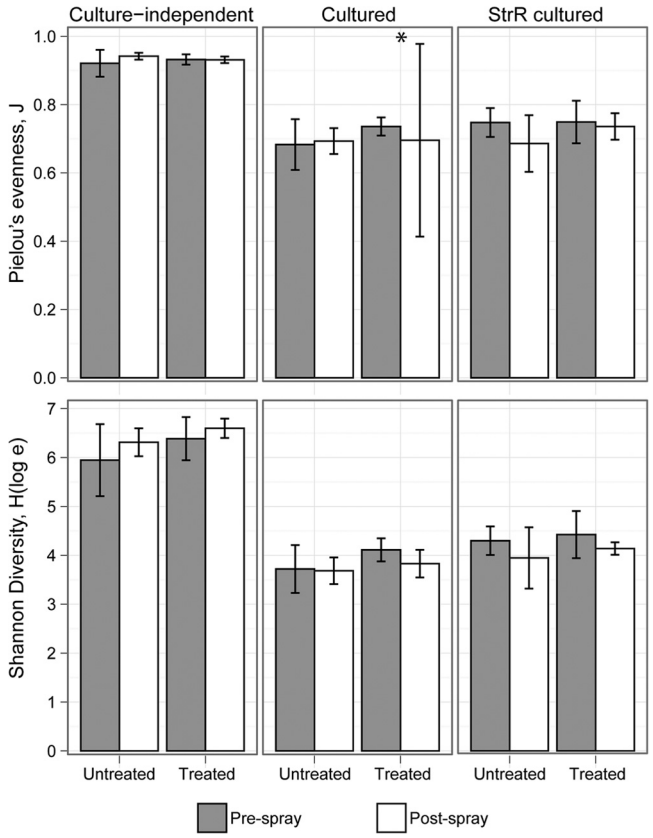

FIG 3 Pielou's evenness and Shannon diversity before and after streptomycin application, compared across culture-independent, cultured, and StrR-cultured community sample groups. Error bars represent standard deviations. All prespray versus postspray communities were not different in evenness or Shannon diversity (all $P > 0.05$), with one exception (indicated by the asterisk): the postspray evenness was slightly lower than the prespray evenness ($P = 0.03$) in the treated cultured communities.

treated communities were not distinct ($P = 0.206$ for StrR cultured, $P = 0.212$ for cultured; Table 2). Thus, these results do not support the hypothesis that streptomycin affected the culture-dependent communities.

Furthermore, the results do not consistently support the idea of an influence of time, as prespray versus postspray communities were not always different from one another. In aggregate, 12 of 19 comparisons of untreated communities detected no difference between prespray and postspray time points, while all treated comparisons showed differences between prespray and postspray time points. Though not conclusive (and removed from the comparative context of the individual untreated controls for each test), these results might mean that untreated communities were less likely than treated ones to change over the course of the experiment. The postspray treated community did not differ from any other communities, and the prespray or postspray untreated community was not different from all others with the exception of the postspray treated community, and thus we cannot decisively conclude that streptomycin-treated communities changed in time and untreated communities did not.

We detected no differences among communities in culture-independent samples, except a marginal difference ($P = 0.054$) when the Sørenson metric was used with the PERMANOVA test (Table 1). *Post hoc* tests revealed that the differences were between prespray untreated and postspray treated communities, as well as between postspray untreated and postspray treated communities. However, there were no differences detected between prespray and postspray communities (see Table S6 in the supplemental material). Thus, as was true for culture-based communities, the differences among culture-independent communities could not be attributed to use of streptomycin.

To better evaluate responses of rare taxa to streptomycin, we reduced the data set to include only the 65% least abundant OTUs (51) and performed NMDS analysis (see Fig. S3 in the supplemental material). As with the whole community, there were no detectable differences in the subset of rare taxa.

Although there was no effect of streptomycin at the community level, and although the compositions of pre- and postspray treated communities substantially overlapped each other and those of the untreated communities, we found taxa that were uniquely detected after streptomycin application. We report the identities of these taxa as information possibly useful for future monitoring. They include 308, 100, and 148 taxa, respectively, for the culture-independent, StrR-cultured, and cultured samples (Fig. 4A). These taxa were very rare, contributing on average 0.0001, 0.0002, and 0.0001 relative abundances to the postspray treated communities in the culture-independent, StrR-cultured, and cultured samples, respectively. Many of these taxa were affiliated with the *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* (Fig. 4B). One taxon, OTU 14542, a *Flavobacterium* member of the *Bacteroidetes*, was detected by all three detection methods at the postspray time point. Because these taxa were rare and because sequencing efforts were not exhaustive, it is difficult to determine whether their occurrence patterns in the postspray treated samples are due to streptomycin application to the trees or random variation around the limits of detection.

Spatial effects. Small but significant differences in community structure were not related to experimental treatment or time (comparison of the pre- and postspray untreated samples provided no evidence for change over time in the absence of treatment), so we tested for evidence of spatial structuring. If there were a spatial influence, soils in close proximity to each other would have harbored bacterial communities more similar in structure than soils farther apart. Two tests were used to assess spatial structuring: (i) a Mantel test between a matrix of the distances in meters separating sampling locations in the orchard and a Bray-Curtis matrix of bacterial community similarities and (ii) the RELATE test in PRIMER-e for evidence that a similarity between communities can be explained by their relationship to one another along a gradient (e.g., representing a spatial series of non-independent communities, in which communities in close proximity are more similar than communities that are distant). No test showed significant differences (all $P > 0.10$), indicating that there was no evidence of spatial effects on the bacterial communities we tested in the orchard soil.

Taxonomic composition of communities. We examined the taxonomic affiliations of the recovered tag sequences (Fig. 5). At the phylum level, *Proteobacteria* dominated both cultured and culture-independent samples. Despite quality control and singleton removal efforts, there remained a large proportion of unidentified bacteria in the culture-independent samples. With or without streptomycin, cultured samples contained *Bacteriodetes* as the second most abundant phylum. The third most abundant phylum

TABLE 1 Four hypothesis tests for differences in community structure (mean or variation) among prespray and postspray untreated and treated soil microbial communities, assessed using each of four resemblance metrics[a]

Sample group	Metric	Differences in mean or variation in community structure			Differences in variation in community structure (PERMDISP)
		PERMANOVA	MRPP	ANOSIM	
Culture independent	Bray-Curtis	n.s. ($P = 0.070$)	n.s. ($P = 0.058$)	n.s. ($P = 0.166$)	n.s. ($P = 0.434$)
	Modified Gower log10	n.s. ($P = 0.082$)	n.s. ($P = 0.087$)	n.s. ($P = 0.176$)	n.s. ($P = 0.127$)
	Morisita-Horn	n.s. ($P = 0.233$)	n.s. ($P = 0.177$)	n.s. (0.438)	n.s. ($P = 0.388$)
	Sørenson	**$R^2 = 0.131$, $P = 0.054$**	n.s. ($P = 0.079$)	n.s. ($P = 0.136$)	n.s. ($P = 0.535$)
StrR cultured	Bray-Curtis	**$R^2 = 0.14$, $P = 0.004$**	**deltaA = 0.6371, $P = 0.008$**	**$R = 0.12$, $P = 0.011$**	n.s. ($P = 0.284$)
	Modified Gower log10	**$R^2 = 0.12$, $P = 0.001$**	**deltaA = 1.15, $P = 0.001$**	**$R = 0.10$, $P = 0.002$**	n.s. ($P = 0.144$)
	Morisita-Horn	n.s. ($P = 0.096$)	n.s. ($P = 0.105$)	n.s. ($P = 0.094$)	n.s. ($P = 0.057$)
	Sørenson	**$R^2 = 0.13$, $P = 0.001$**	**deltaA = 0.6625, $P = 0.001$**	**$R = 0.15$, $P = 0.002$**	n.s. ($P = 0.155$)
Cultured	Bray-Curtis	**$R^2 = 0.13$, $P = 0.024$**	**deltaA = 0.55, $P = 0.02$**	**$R = 0.102$, $P = 0.016$**	n.s. ($P = 0.276$)
	Modified Gower log10	**$R^2 = 0.12$, $P = 0.001$**	**deltaA = 1.162, $P = 0.001$**	**$R = 0.12$, $P = 0.001$**	n.s. ($P = 0.766$)
	Morisita-Horn	n.s. ($P = 0.257$)	n.s. ($P = 0.164$)	n.s. ($P = 0.236$)	n.s. ($P = 0.367$)
	Sørenson	**$R^2 = 0.12$, $P = 0.001$**	**deltaA = 0.670, $P = 0.001$**	**$R = 0.186$, $P = 0.001$**	n.s. ($P = 0.617$)

[a] Significant test results are shown in bold. n.s., not significant ($P > 0.05$); PERMANOVA, permuted analysis of variance; MRPP, multiple-response permutation procedure; ANOSIM, analysis of similarity; PERMDISP, permuted analysis of multivariate dispersion.

was *Actinobacteria*, members of which were proportionally less abundant in the StrR-cultured samples. *Deltaproteobacteria* and *Acidobacteria* were not detected by culturing but were detected by the culture-independent analysis.

DISCUSSION

This study investigated the impact of streptomycin application to apple trees for fire blight prevention on nontarget bacterial communities in soil under the trees. We predicted that streptomycin would reduce diversity but found no evidence that streptomycin affects the overall diversity, evenness, or community structure of orchard soil bacterial communities, as assessed with a suite of multivariate analyses of 16S rRNA gene sequences. Although there were some differences among experimental groups, these were not explained by experiment design, time, soil chemistry, or spatial proximity of soil samples to one another. There could have been an influence of some unmeasured environmental parameter, or these differences might be attributable to the inherent variability and complexity of soil communities.

It is possible that there were experimental effects that were not detected. For example, perhaps the temporal scale of sampling (before spraying and then 20 days after the first sample) was too coarse and a more immediate sampling was necessary to detect community response. Regardless, if communities were altered in the short term by streptomycin application, the effects were transient. Alternatively, there might be changes in the frequency of certain traits without an accompanying change in community membership. Tag-pyrosequencing of the 16S rRNA genes would not detect such an effect, which would require metagenomic or functional analyses.

Though our data suggest some tolerance to streptomycin among orchard soil bacteria, evidenced by growth of soil isolates on streptomycin (15 μg/ml), the resistance detected could represent a typical baseline for microorganisms in the orchard soil (see, e.g., reference 8). Long-term monitoring of antibiotic resistances in multiple agricultural settings, including settings with no streptomycin exposure, would be necessary to address these questions. In our analyses, we have highlighted some taxa that were detected in postspray treated communities and could provide an interesting springboard for monitoring. As previously discussed, because

TABLE 2 MRPP to test for differences in community structure between prespray and postspray untreated and treated soil samples[a]

Sample group	Treatment category	Time point	Test results			
			Untreated		Treated	
			Prespray	Postspray	Prespray	Postspray
Cultured	Untreated	Prespray		n.s. ($P = 0.161$)	n.s. ($P = 0.295$)	n.s. ($P = 0.151$)
		Postspray	**delta = 1.03, $P = 0.027$**		**delta = 0.54, $P = 0.007$**	n.s. ($P = 0.452$)
	Treated	Prespray	n.s. ($P = 0.224$)	**delta = 1.02, $P = 0.001$**		**delta = 0.54, $P = 0.014$**
		Postspray	**delta = 1.01, $P = 0.026$**	n.s. ($P = 0.212$)	**delta = 1.00, $P = 0.002$**	
StrR cultured	Untreated	Prespray		n.s. ($P = 0.063$)	n.s. ($P = 0.856$)	**delta = 0.63, $P = 0.006$**
		Postspray	n.s. ($P = 0.066$)		**delta = 0.64, $P = 0.017$**	n.s. ($P = 0.325$)
	Treated	Prespray	n.s. ($P = 0.078$)	**delta = 1.04, $P = 0.004$**		**delta = 0.060, $P = 0.009$**
		Postspray	**delta = 1.05, $P = 0.001$**	n.s. ($P = 0.206$)	**delta = 1.04, $P = 0.001$**	

[a] For each sample group of cultured and StrR-cultured communities, the upper diagonal (gray shading) shows results determined using the Bray-Curtis metric for community dissimilarity, and the lower diagonal (no shading) shows results determined using the alternative Gower metric. *Post hoc* MRPP results are not reported for culture-independent communities because the results of the global MRPP test were not statistically significant (Table 1). Significant test results are shown in bold. n.s., not significant ($P > 0.05$).

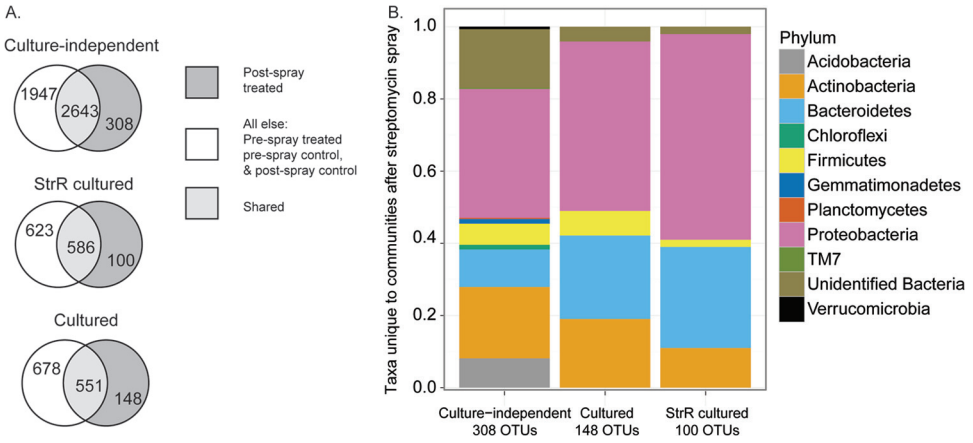

FIG 4 Taxa detected uniquely in postspray treated communities. (A) The proportion of postspray OTUs was small in each of the culture-independent, StrR-cultured, and cultured community sample groups. OTUs unique to the postspray treated samples are dark gray, OTUs from all other treatments are white, and OTUs shared among them are light gray. (B) Phylum-level affiliations of OTUs that were unique to the postspray, treated communities.

of sequencing limitations and the very low abundances of these taxa, there is no clear evidence that their occurrence patterns could be attributed to streptomycin treatment.

This work is part of a larger study to understand the impact of streptomycin on microbial communities in apple orchards. We previously reported that streptomycin had no measured effect on microbial communities associated with apple leaves, twigs (52, 53), or flowers (54). In a related study describing orchard soil microbial metagenomes, we detected *strA* and *strB*, the genes from the transposon Tn*5393* that confer streptomycin resistance (55). Of these, none were novel *strA* or *strB* genes. Furthermore, we did not detect streptomycin-resistant clones in a query of a fosmid library built from the same soil (13 Gb metagenomic DNA) and instead found 13 clones resistant to an array of antibiotics, including beta-lactams, aminoglycosides, and tetracyclines (55). The current work builds on these previous studies by providing the additional perspective of both culture-based and culture-independent analyses of orchard soil microbial communities before and after streptomycin application. Our results provide further evidence that streptomycin application has minimal immediate impact on the microbial community composition of apple trees or of the underlying soil.

Streptomycin deposition could be considered a chemical disturbance to the soil microorganisms. Because streptomycin is applied over a finite and specific time, it is a pulse disturbance. The orchard soil bacterial communities present an interesting study system for understanding how an annual pulse disturbance may select for community members adapted to survive or even thrive in an environment altered by periodic disturbance. Our data may suggest tolerance to streptomycin among some orchard soil bacteria, evidenced by growth of community members on streptomycin (15 mg/ml). A pronounced tolerance to streptomycin may increase a community's ability to resist change in the face of antibiotic disturbance (56); however, interpretation is limited by the lack of information about streptomycin resistance among as-yet-unculturable organisms. It is also unknown whether the observed communities are at an "endpoint" of selection because of the history of streptomycin application at the study site or are still changing under conditions of selection. Alternatively, the antibiotic resistance detected could represent a baseline for the organisms in the orchard soil (see, e.g., reference 8) which might enhance the pace of recovery (resilience) of the community after streptomycin application. It is also possible that the streptomycin applied at the frequency (annual) and intensity (21% streptomycin sulfate con-

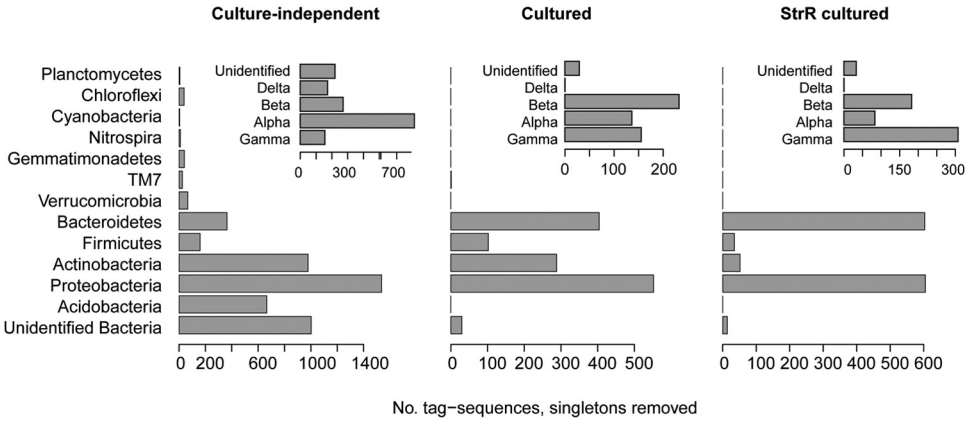

FIG 5 Community composition at the phylum level, as assessed by proportion of recovered tag sequences. Note the differences in the *x* axes. Insets show *Proteobacteria* classes.

centration) used in this study did not reach the soil in sufficient quantities to alter the community. Long-term monitoring of both community structure and antibiotic resistances in agricultural fields would be necessary to address these issues.

In conclusion, there was no evidence that spraying streptomycin for prevention of fire blight affected the nontarget soil bacterial community in the short term. This finding may represent many other situations in which an agroecosystem is buffered from change by its architecture, community robustness, or narrowly targeted interventions.

ACKNOWLEDGMENTS

USDA Microbial Observatory grant 2006-35319-17466 funded this work. A.S. is a Gordon and Betty Moore Foundation Fellow of the Life Sciences Research Foundation.

We thank L. Forest and R. Aburomia for providing orchard resources.

REFERENCES

1. **McManus PS, Stockwell VO, Sundin GW, Jones AL.** 2002. Antibiotic use in plant agriculture. Annu. Rev. Phytopathol. **40:**443–465.
2. **Tollefson L, Angulo FJ, Fedorka-Cray PJ.** 1998. National surveillance for antibiotic resistance in zoonotic enteric pathogens. Vet. Clin. North Am. Food Anim. Pract. **14:**141–150.
3. **Payot S, Dridi S, Laroche M, Federighi M, Magras C.** 2004. Prevalence and antimicrobial resistance of *Campylobacter coli* isolated from fattening pigs in France. Vet. Microbiol. **101:**91–99.
4. **Payot S, Avrain L, Magras C, Praud K, Cloeckaert A, Chaslus-Dancla E.** 2004. Relative contribution of target gene mutation and efflux to fluoroquinolone and erythromycin resistance, in French poultry and pig isolates of *Campylobacter coli*. Int. J. Antimicrob. Agents **23:**468–472.
5. **Nijsten R, London N, van den Bogaard A, Stobberingh E.** 1996. In-vitro transfer of antibiotic resistance between faecal *Escherichia coli* strains isolated from pig farmers and pigs. J. Antimicrob. Chemother. **37:**1141–1154.
6. **Nijsten R, London N, van den Bogaard A, Stobberingh E.** 1996. Antibiotic resistance among *Escherichia coli* isolated from faecal samples of pig farmers and pigs. J. Antimicrob. Chemother. **37:**1131–1140.
7. **Saida K, Ike Y, Mitsuhashi S.** 1981. Drug resistance and R plasmids of *Escherichia coli* strains isolated from pigs, slaughterers, and breeders of pigs in Japan. Antimicrob. Agents Chemother. **19:**1032–1036.
8. **Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J.** 2010. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. **8:**251–259.
9. **Gilbert J, O'Dor R, King N, Vogel T.** 2011. The importance of metagenomic surveys to microbial ecology: or why Darwin would have been a metagenomic scientist. Microb. Inform. Exp. **1:**5. doi:10.1186/2042-5783-1-5.
10. **Cho I, Blaser MJ.** 2012. The human microbiome: at the interface of health and disease. Nat. Rev. Genet. **13:**260–270.
11. **Pusey PL.** 2002. Biological control agents for fire blight of apple compared under conditions limiting natural dispersal. Plant Dis. **86:**639–644.
12. **Shwartz H, Shtienberg D, Vintal H.** 2003. The interacting effects of temperature, duration of wetness and inoculum size on the infection of pear blossoms by *Erwinia amylovora*, the causal agent of fire blight. Phytoparasitica **31:**174–187.
13. **Nuclo RL, Johnson KB, Stockwell VO, Sugar D.** 1998. Secondary colonization of pear blossoms by two bacterial antagonists of the fire blight pathogen. Plant Dis. **82:**661–668.
14. **Thomson SV.** 1986. The role of the stigma in fire blight infections. Phytopathology **76:**476–482.
15. **Bubán T, Orosz-Kovács Z, Farkas Á.** 2003. The nectary as the primary site of infection by *Erwinia amylovora* (Burr.) Winslow *et al.*: a mini review. Plant Syst. Evol. **238:**183–194.
16. **United States Department of Agriculture National Agricultural Statistics Service.** 2008. Agricultural chemical usage 2007 field crops summary. United States Department of Agriculture National Agricultural Statistics Service, Washington, DC.
17. **Selber JN, Wilson BW, McChesney MM.** 1993. Air and fog deposition residues of four organophosphate insecticides used on dormant orchards in the San-Joaquin Valley, California. Environ. Sci. Technol. **27:**2236–2243.
18. **Sundin GW, Monks DE, Bender CL.** 1995. Distribution of the streptomycin-resistance transposon TN5393 among phylloplane and soil bacteria from managed agricultural habitats. Can. J. Microbiol. **41:**792–799.
19. **van Overbeek LS, Wellington EMH, Egan S, Smalla K, Heuer H, Collard J-M, Guillaume G, Karagouni AD, Nikolakopoulou TL, van Elsas JD.** 2002. Prevalence of streptomycin-resistance genes in bacterial populations in European habitats. FEMS Microbiol. Ecol. **42:**277–288.
20. **Tolba S, Egan S, Kallifidas D, Wellington EMH.** 2002. Distribution of streptomycin resistance and biosynthesis genes in streptomycetes recovered from different soil sites. FEMS Microbiol. Ecol. **42:**269–276.
21. **Westergaard K, Muller AK, Christensen S, Bloem J, Sorensen SJ.** 2001. Effects of tylosin as a disturbance on the soil microbial community. Soil Biol. Biochem. **33:**2061–2071.
22. **Sengeløv G, Agerso Y, Halling-Sørensen B, Baloda SB, Andersen JS, Jensen LB.** 2003. Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. Environ. Int. **28:**587–595.
23. **Rodriguez-Sanchez C, Altendorf K, Smalla K, Lipski A.** 2008. Spraying of oxytetracycline and gentamicin onto field-grown coriander did not affect the abundance of resistant bacteria, resistance genes, and broad host range plasmids detected in tropical soil bacteria. Biol. Fertil. Soils **44:**589–596.
24. **Ding C, He JZ.** 2010. Effect of antibiotics in the environment on microbial populations. Appl. Microbiol. Biotechnol. **87:**925–941.
25. **Zielezny Y, Groeneweg J, Vereecken H, Tappe W.** 2006. Impact of sulfadiazine and chlorotetracycline on soil bacterial community structure and respiratory activity. Soil Biol. Biochem. **38:**2372–2380.
26. **Binh CTT, Heuer H, Gomes NCM, Kotzerke A, Fulle M, Wilke BM, Schloter M, Smalla K.** 2007. Short-term effects of amoxicillin on bacterial communities in manured soil. FEMS Microbiol. Ecol. **62:**290–302.
27. **Cermák L, Kopecky J, Novotná J, Omelka M, Parkhomenko N, Plhackova K, Sagova-Mareckova M.** 2008. Bacterial communities of two contrasting soils reacted differently to lincomycin treatment. Appl. Soil Ecol. **40:**348–358.
28. **Mohamed MAN, Ranjard L, Catroux C, Catroux G, Hartmann A.** 2005. Effect of natamycin on the enumeration, genetic structure and composition of bacterial community isolated from soils and soybean rhizosphere. J. Microbiol. Methods **60:**31–40.
29. **Liu F, Wu JS, Ying GG, Luo ZX, Feng H.** 2012. Changes in functional diversity of soil microbial community with addition of antibiotics sulfamethoxazole and chlortetracycline. Appl. Microbiol. Biotechnol. **95:**1615–1623.
30. **Buyer JS.** 1995. A soil and rhizosphere microorganism isolation and enumeration medium that inhibits *Bacillus mycoides*. Appl. Environ. Microbiol. **61:**1839–1842.
31. **Gilbert G, Clayton M, Handelsman J, Parke J.** 1996. Use of cluster and discriminant analyses to compare rhizosphere bacterial communities following biological perturbation. Microb. Ecol. **32:**123–147.
32. **Cadillo-Quiroz H, Bräuer S, Yashiro E, Sun C, Yavitt J, Zinder S.** 2006. Vertical profiles of methanogenesis and methanogens in two contrasting acidic peatlands in central New York State, USA. Environ. Microbiol. **8:**1428–1440.
33. **Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, Mcdonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R.** 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods **7:**335–336.
34. **Edgar RC.** 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics **26:**2460–2461.
35. **Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R.** 2010. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics **26:**266–267.
36. **Wang Q, Garrity GM, Tiedje JM, Cole JR.** 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. **73:**5261–5267.
37. **Kunin V, Engelbrektson A, Ochman H, Hugenholtz P.** 2010. Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. Environ. Microbiol. **12:**118–123.
38. **Shade A, Hogan CS, Klimowicz AK, Linske M, McManus PS, Handels-**

man J. 2012. Culturing captures members of the soil rare biosphere. Environ. Microbiol. **14:**2247–2252.
39. **Clarke KR, Gorley RN.** 2006. PRIMER v6: user manual/tutorial. PRIMER-E, Plymouth, United Kingdom.
40. **Development Core Team R.** 2005. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
41. **Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H.** 2011. vegan: community ecology package. http://cran.r-project.org/web/packages/vegan/index.html.
42. **Anderson MJ, Ellingsen KE, McArdle BH.** 2006. Multivariate dispersion as a measure of beta diversity. Ecol. Lett. **9:**683–693.
43. **Anderson MJ, Crist TO, Chase JM, Vellend M, Inouye BD, Freestone AL, Sanders NJ, Cornell HV, Comita LS, Davies KF.** 2011. Navigating the multiple meanings of beta diversity: a roadmap for the practicing ecologist. Ecol. Lett. **14:**19–28.
44. **Legendre P, Lengendre L.** 1998. Numerical ecology. Elsevier, Amsterdam, Netherlands.
45. **Horn HS.** 1966. Measurement of "overlap" in comparative ecological studies. Am. Nat. **100:**419–424.
46. **Palacios C, Zettler E, Amils R, Amaral-Zettler L.** 2008. Contrasting microbial community assembly hypotheses: a reconciling tale from the Rio Tinto. PLoS One **3:**e3853. doi:10.1371/journal.pone.0003853.
47. **Kuczynski J, Liu Z, Lozupone C, McDonald D, Fierer N, Knight R.** 2010. Microbial community resemblance methods differ in their ability to detect biologically relevant patterns. Nat. Methods **7:**813–819.
48. **Mielke PW, Berry KJ.** 2001. Permutation methods: a distance function approach. Springer Verlag, New York, NY.
49. **Anderson MJ.** 2005. Permutational multivariate analysis of variance. Department of Statistics, University of Auckland, Auckland, New Zealand.
50. **Anderson MJ.** 2006. Distance-based tests for homogeneity of multivariate dispersions. Biometrics **62:**245–253.
51. **Ugland KI, Gray JS.** 1982. Lognormal distributions and the concept of community equilibrium. Oikos **39:**171–178.
52. **Yashiro E, Spear RN, McManus PS.** 2011. Culture-dependent and culture-independent assessment of bacteria in the apple phyllosphere. J. Appl. Microbiol. **110:**1284–1296.
53. **Yashiro E, McManus PS.** 2012. Effect of streptomycin treatment on bacterial community structure in the apple phyllosphere. PLoS One **7:**e37131. doi:10.1371/journal.pone.0037131.
54. **Shade A, McManus PS, Handelsman J.** 2013. Unexpected diversity during community succession in the apple flower microbiome. mBio **4:**e00602–12. doi:10.1128/mBio.00602-12.
55. **Donato JJ, Moe LA, Converse BJ, Smart KD, Berklein FC, McManus PS, Handelsman J.** 2010. Metagenomic analysis of apple orchard soil reveals antibiotic resistance genes encoding predicted bifunctional proteins. Appl. Environ. Microbiol. **76:**4396–4401.
56. **Shade A, Peter H, Allison SD, Baho DL, Berga M, Bürgmann H, Huber DH, Langenheder S, Lennon JT, Martiny JB, Matulich KL, Schmidt TM, Handelsman J.** 2012. Fundamentals of microbial community resistance and resilience. Front. Microbiol. **3:**417. doi:10.3389/fmicb.2012.00417.